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Design, Synthesis, and Biological Activities of Closantel Analogues: Structural Promiscuity and Its Impact on *Onchocerca volvulus*

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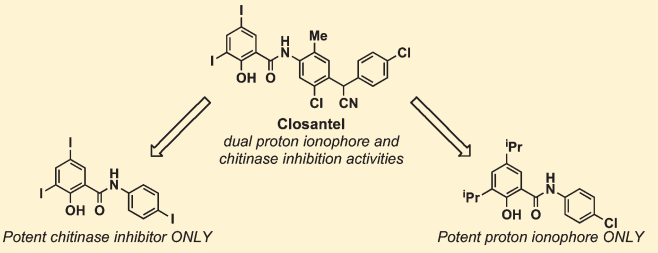
Supporting Information

ABSTRACT: Onchocerciasis, or river blindness, is a neglected tropical disease that affects more than 37 million people worldwide, primarily in Africa and Central and South America. We have disclosed evidence that the larval-stage-specific chitinase, OvCHT1, may be a potential biological target for affecting nematode development. On the basis of screening efforts, closantel, a known anthelmintic drug, was discovered as a potent and highly specific OvCHT1 inhibitor. Originally, closantel's anthelmintic mode of action was believed to rely solely on its role as a proton ionophore; thus, the impact of each of its biological activities on *O. volvulus* L3 molting was investigated. Structure−activity relationship studies on an active closantel fragment are detailed, and remarkably, by use of a simple salicylanilide scaffold, compounds acting only as protonophores or chitinase inhibitors were identified. From these data, unexpected synergistic protonophore and chitinase inhibition activities have also been found to be critical for molting in *O. volvulus* L3 larvae.



INTRODUCTION

Onchocerciasis, or river blindness, is a neglected tropical disease that affects more than 37 million people worldwide, primarily in developing nations including Yemen and many countries found in Africa and Central and South America.[1] The disease is caused by the filarial parasitic nematode *Onchocerca volvulus*, which is transmitted to humans by the blackfly (*Simulium* spp.), and its symptoms are resultant of the death of the microfilariae in the skin and eyes. Despite several efforts for control, current strategies for blocking transmission are finding limitations,[2,3] and the establishment of new drug targets is greatly needed.

Recently, our group presented data that chitin metabolism, more specifically, a larval-stage-specific chitinase, may be a potential biological target for affecting nematode development.[4] Chitin, one of the most widespread amino polysaccharides in nature, is a major structural component of arthropod exoskeletons, fungal cell walls, and the microfilarial sheath and eggshells of parasitic nematodes; however, it is entirely lacking in vertebrates.[5] The dynamic biosynthesis and degradation of chitin are crucial for the growth and development of these organisms and are regulated by two classes of enzymes, chitin synthases and chitinases. The family 18 chitinases are particularly interesting targets, as these enzymes are widely expressed in archaea, prokaryotes, and eukaryotes, and many of the members are active enzymes capable of chitin hydrolysis.[5] Currently, only one chitinase from *O. volvulus* has been identified.[6] OvCHT1 is expressed solely in the infective L3 larvae and is stored within the granules of the cells of the esophageal glands until post-infective development, after which it is secreted and found mostly in the cuticle.[6] Although its exact mechanism is not clear, it has been hypothesized to play one or more roles in parasite transmission, ecdysis, and remodeling of the L4 cuticle.[6] Because of the critical nature of these processes in the life cycle of the parasite, probing of this larval chitinase was deemed a relevant therapeutic target in *O. volvulus*.

To identify inhibitors of OvCHT1, our group examined a drug repositioning approach that involves identifying and developing new uses for existing drugs.[7] Using a previously reported fluorescence-based assay, we screened the Johns Hopkins Clinical Compound Library (JHCCL), a commercially available library of 1514 known drugs, against OvCHT1. From our screening efforts, we discovered one drug with potent inhibition against OvCHT1, the known veterinary anthelmintic drug closantel (Figure 1),[8] with an IC_{50} of 1.6 ± 0.08 μM and a competitive inhibition constant (K_i) of 468 ± 84 nM.[4] This compound was also found to be highly specific for filarial family 18 chitinases compared to those from protozoans and the human chitinase, human chitotriosidase.[4] Of

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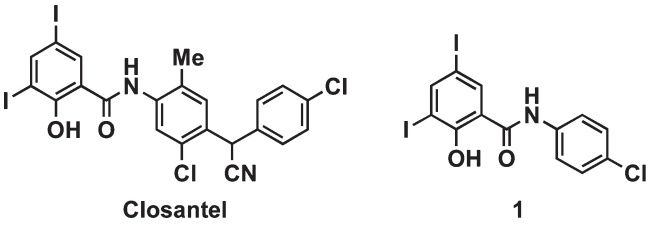


Figure 1. Structures of closantel and its most potent chitinase inhibitor fragment.

significance, closantel almost fully inhibited the L3 to L4 molt of developing *O. volvulus* larvae, and ultrastructural studies revealed an interesting closantel-induced phenotype in that the separation between the L3 cuticle and the newly formed L4 cuticle was inhibited and the cuticular material between the cuticles was not fully degraded.[4] It is important to note that a similar phenotype has also been observed when L3 larvae were cultured with cysteine protease inhibitors[9] or when the transcripts corresponding to *O. volvulus* cysteine proteases or a serine protease inhibitor were knocked down using RNA interference.[10]

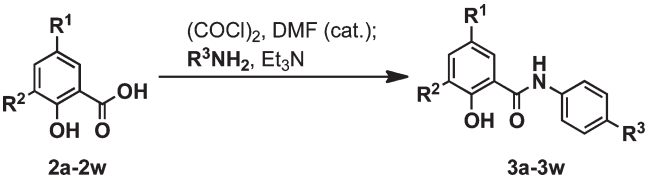
Closantel's previously documented anthelmintic mode of action was thought to solely rely on its role as a proton ionophore, or protonophore,[11] and its chitinase inhibitor activity was previously unknown, hence implicating a potential bi- or multimodal mechanism of action for its observed biochemical activity. With the newly discovered dual biochemical roles for closantel, studies were initated toward dissecting its activity determining features. Using a "retrofragment" based approach, compound **1** was identified with chitinase inhibition activity similar to closantel (IC_{50} of 5.8 ± 0.3 μM) (Figure 1).[4] From this lead fragment, investigations were initated to determine the required molecular features underlying the protonophore activity as well as the chitinase inhibition activity in an effort to map out which molecular fragments are required for individual and/or dual activities.

Herein, we report structure−activity relationship (SAR) studies on closantel's most potent fragment **1**. Remarkably, using a structurally simple salicylanilide scaffold, compounds with a range of activities including those acting only as protonophores or only as chitinase inhibitors were identified. We also present molting data in the presence of these compounds. From these data, we have identified that both protonophore and chitinase inhibition activities are necessary for affecting the molting of *O. volvulus* L3 larvae. Additionally, we report initial data on the activities of closantel and its analogues in a related parasitic species, *O. ochengi*.

RESULTS

Chemistry. The salicylanilide scaffold is known to exhibit proton ionophore activity;[12] however, prior to our work, no indication of chitinase inhibition activity was reported. Thus, SAR studies were conducted on this scaffold in the hope of obtaining protonophore- and chitinase inhibitor-selective probes in an effort to delineate the impact of each mechanism of action on *O. volvulus*. It has been well established that retention of the critical hydroxy and amide functionalities is likely required for protonophoric activity, as a dissociable proton is necessary to affect mitochondrial uncoupling.[13−16] In addition, as a chitinase inhibitor, the retention of hydrogen-bonding (H-bonding) moieties may be key to affect the catalytically required acidic residues of the active site.[17] Since the presence of available protons/

Table 1. Structures of Closantel Fragment Analogues



compd	R^1	R^2	R^3
3a	I	I	H
3b	I	I	Me
3c	I	I	OMe
3d	I	I	F
3e	I	I	Br
3f	I	I	I
3g	I	I	^{i}pr
3h	I	H	H
2i	I	H	Me
3j	I	H	OMe
3k	I	H	Cl
31	Cl	Cl	H
3m	Cl	Cl	Me
3n	Cl	Cl	OMe
3o	Cl	Cl	Cl
3p	Br	Br	H
3q	Br	Br	Me
3r	Br	Br	OMe
3s	Br	Br	Cl
3t	^{i}Pr	^{i}Pr	H
3u	^{i}Pr	^{i}Pr	Me
3v	^{i}Pr	^{i}Pr	OMe
3w	^{i}Pr	^{i}Pr	Cl

hydrogens is likely important for both modes of action, we desired to keep this salicylanilide scaffold intact. In addition to these H-bonding moieties, the presence of electron-withdrawing substituents has also been demonstrated to be necessary for potent ionophore activity because of their pK_a lowering effect.[12,15,16] Since the activity of this scaffold is largely due to its ability to form a cyclic salicylanilide anion,[15,16] modification of these moieties appeared to be the most fruitful for modulation of specificity and enhancement of potency, although it was unclear at the outset whether the protonophore activity could be dialed down in such a simple scaffold.

By use of compound **1** as a starting point, analogues capable of examining the effects of halogen-bonding (X-bonding), in both the salicylic acid and aniline portions, were designed. Analogues using various salicylic acids were synthesized to determine if the role of both iodines was due to X-bonding effects or size/lipophilicity (R^1 and R^2, Table 1). For chitinase inhibition activity, the aryl halide moieties may be critical for competitive binding because of potential interactions with the acidic active site residues, solvent-exposed tryptophan residues, and the numerous aromatic residues of the substrate- and chitin-binding domains.[17] Thus, toward the goal of identifying highly selective chitinase inhibitors, we also modified the halogen substituents of the aniline ring (R^3, Table 1). As no previous structural data exists for the use of salicylanilides in chitinase inhibition, both

electron-donating and hydrophobic substituents on the aniline ring were examined to obtain a full picture of chitinase inhibition with this scaffold. All analogues (**3a**−**w**) were synthesized in one step via standard amide bond formation from the corresponding acid chlorides of compounds **2** in good to moderate yield (39−88% yield).

Chitinase Inhibition. The closantel fragment analogues depicted in Table 1 was first examined for in vitro OvCHT1 inhibition activity using a previously reported fluorescence-based assay.[4] Compounds were initially tested at 25 μM, and positive hits were selected by observing a decrease in fluorescence signal that correlates with a decline in the hydrolytic cleavage of the fluorescently labeled chitin substrate 4-methylumbelliferyl-*N*,*N*′,*N*″-β-chitotrioside (see Experimental Section for details). Our screening results are shown in Figure 2. Structures of the most potent closantel-based fragment analogues are subsequently shown in Figure 3 with IC_{50} values ranging from 0.63 to 12.4 μM (Table 2). Interestingly, compounds **3e** and **3f** bearing larger halogen substituents on the aniline ring are over 2 times more potent than closantel itself. In addition, compounds with estimated cLogP of $\gtrsim$ 6.5 (Table 2) appear to be more active in the chitinase inhibition assay (closantel and compounds **3e**, **3f**, and **3g**), although exceptions were found (compounds **1**, **3b**, **3c**, **3d**, **3o**. and **3r**). The estimated cLogP for all analogues **3** ranged from 4.53 to 6.90 (not shown). Thus, these data indicate that this salicylanilide scaffold is indeed the required structure for chitinase inhibition. Further evidence for this stems from the fact that the arylacetonitrile portion of closantel was previously found to be over 40 times less potent than the parent compound.[4]

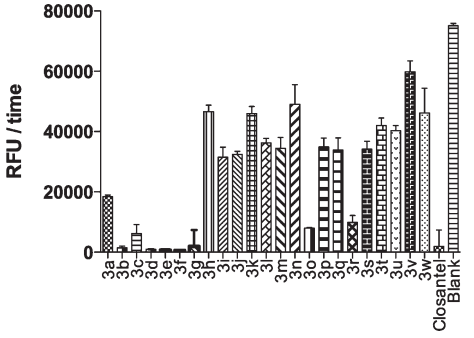


Figure 2. Evaluation of closantel fragment analogues in a fluorescence-based OvCHT1 inhibition assay. All analogues were examined at 25 μM. RFU = relative fluorescence units (λ_{ex} = 350 nm, λ_{em} = 440 nm). Blank = DMSO in the absence of compound.

Proton Ionophore Activity. The closantel fragment analogues were examined for in vitro protonophore activity using an established mitochondrial uncoupling assay. All compounds were tested using a cationic, mitochondria-selective fluorescence probe that serves as a membrane potential sensor. Briefly, HEK-293T cells were treated with analogue (50 μM) and subsequently stained with probe. Flow cytometric analysis was then used to analyze membrane polarization (see Experimental Section for details). Valinomycin, a strong potassium channel ionophore, was used as a positive depolarization control. Although many of the compounds exhibited weak protonophoric activity (see Supporting Information), compound **3w** (Figure 4F) (estimated cLogP of 6.90) showed strong uncoupling activity relative to both valinomycin and closantel (Figure 4A−D). On the other hand, the most potent chitinase inhibitor identified, compound **3f**, exhibited no protonophoric activity. Thus, using a small series of compounds based on a known proton ionophore scaffold, we were able to identify compounds with differing biological activities.

Molting Inhibition in *O. volvulus* L3 Larvae. With compounds **3f** and **3w** in hand, molting in *O. volvulus* L3 larvae was examined. In previous studies, we showed that closantel almost completely inhibited the molting of L3 to L4 larvae at 100 μM (97.6% inhibition).[4] L3 stage larvae were cultured in the presence of either 50 or 100 μM **3f** or **3w**, and the number of molting larvae was determined on day 6 (see Experimental Section for details). As Figure 5A shows, similar to closantel, each compound completely abolished molting at 100 μM, indicating that treatment with either a potent chitinase inhibitor or proton ionophore

Table 2. IC_{50} of Chitinase Inhibition and Estimated cLogP for Select Closantel Fragment Analogues

compd	IC_{50} (μM)	cLogP [a]
closantel	1.6 ± 0.08	7.64
1	5.8 ± 0.3	6.31
3b	1.7 ± 0.05	5.89
3c	8.7 ± 0.04	5.33
1d	0.88 ± 0.02	5.74
3e	0.68 ± 0.09	6.46
3f	0.63 ± 0.03	6.72
3g	1.6 ± 0.06	6.82
3o	12.4 ± 0.11	5.51
3r	5.5 ± 0.04	4.88

[a] cLogP values were calculated using ChemDraw Ultra 12.0.

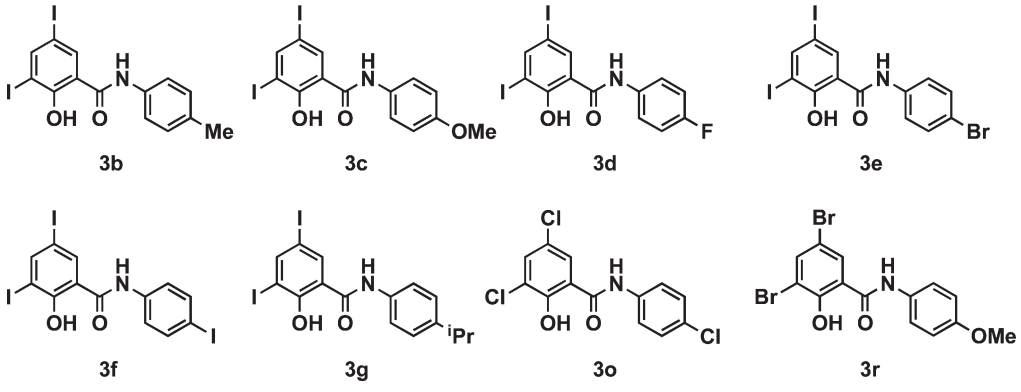


Figure 3. Structures of the most potent OvCHT1 inhibitors.

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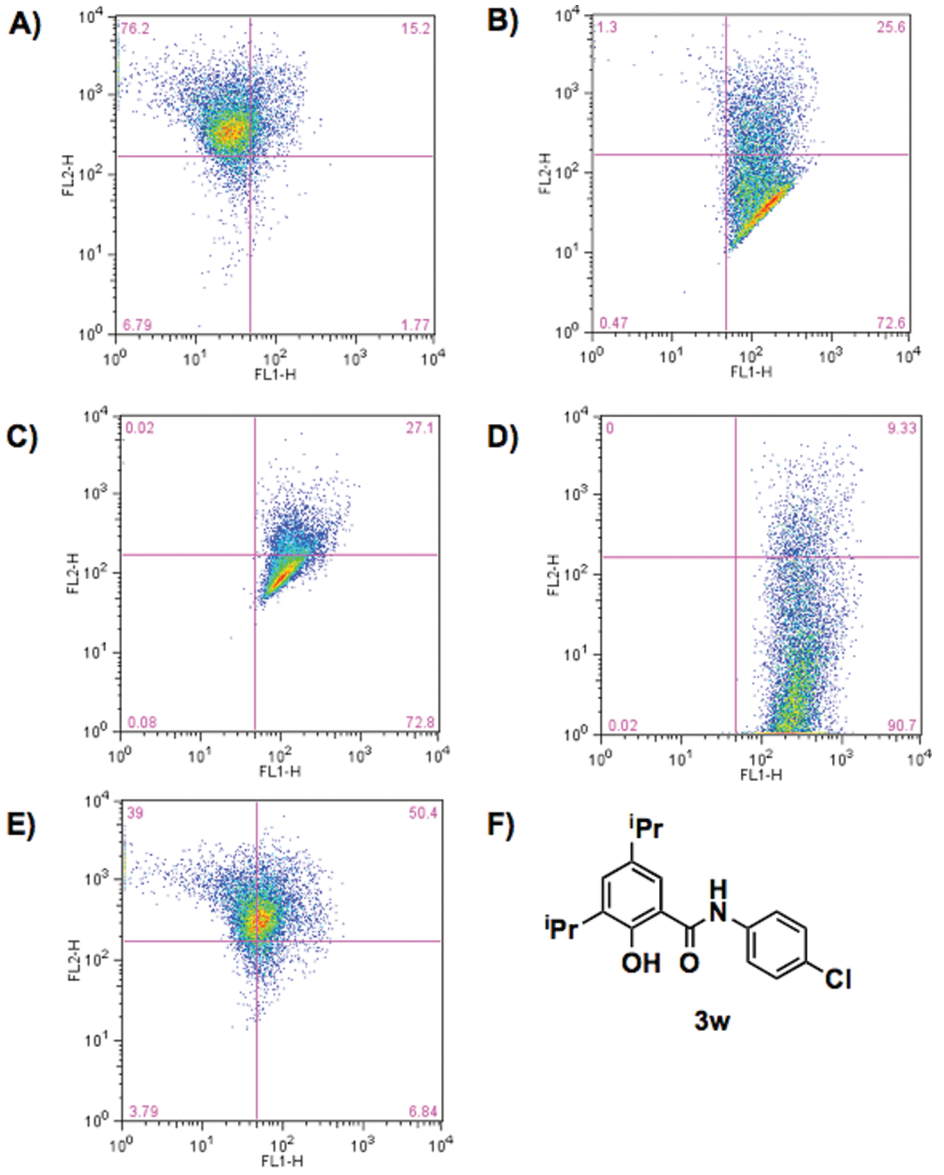

Figure 4. Proton ionophore activity of controls, closantel, and select analogues. All analogues were examined at 50 μM except valinomycin. (A) No compound (i.e., dye only). (B) Valinomycin (200 nM). (C) Closantel. (D) Compound **3w**. (E) Compound **3f**. (F) Structure of compound **3w**.

at this concentration is capable of affecting this developmental process. It is important to note here that no larval toxicity was observed with closantel or compounds **3f** and **3w**. To further our understanding of closantel's mechanism of action, the effect of treating L3 larvae with a 1:1 combination of compounds **3f** and **3w** was investigated. As Figure 5B shows, while treatment with 25 μM (not shown) or 50 μM of the individual probes had no impact on molting, 52% inhibition of molting was observed upon treatment with 25 μM each of compounds **3f** and **3w** (50 μM total for both compounds) and complete inhibition of molting was observed upon treatment with 50 μM each of compounds **3f** and **3w** (100 μM total for both compounds). These data point to the idea that closantel, in fact, does exhibit dual activities in L3 larvae, acting as both a chitinase inhibitor and a protonophore. While singular activities were sufficient to knock down molting at a higher concentration, both activities were required for more potent inhibition. Interestingly, dose-dependent inhibition was observed with the combination treatment; however, in our previous studies, a dose-dependent response was not observed with closantel. Although not shown, a similar effect was also noted with compounds **3f** and **3w**. We had originally hypothesized that this lack of dose dependence was likely due to bioavailability or tissue penetration issues;[4] however, it is also possible that closantel's dual or competing targets also impact its dosing, or a synergistic effect occurs between compounds **3f** and **3w** that affects the dose response.

Effect on *Onchocerca ochengi*. Although no animal models for *O. volvulus* exist because this parasite infects only humans and chimpanzees, a related species in cattle, *O. ochengi*, has emerged as a relevant model for chemotherapeutic research.[18,19] Because of the potential utility for closantel or one of its analogues as a promising therapy for the treatment of onchocerciasis, we were also interested in exploring the efficacy of these compounds on *O. ochengi*. All analogues were screened at 5 and 10 μg/mL for inhibition of *O. ochengi* microfilarial motility (see Experimental Section for details).[20] As Table 3 shows, only closantel and our

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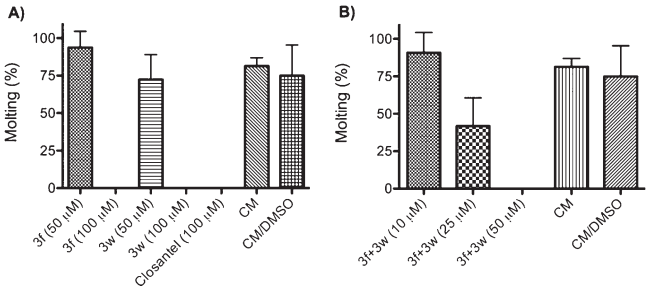

Figure 5. Inhibition of molting in the presence of closantel analogues **3f** and **3w**. The data are presented as percent molting in a total of 10 wells containing on average 5−10 larvae per well. CM = complete medium. (A) Molting inhibition in the presence of **3f** or **3w**. (B) Molting inhibition in the presence of a 1:1 combination of **3f** and **3w**. The concentrations shown in part B are the concentrations of each compound when the analogues were combined.

Table 3. Percent Motility (Viability) of *O. ochengi* Microfilariae in the Presence of Closantel and Its Fragment Analogues

compd	10 μg/mL	5 μg/mL
closantel	0	100
1	0	100
3a	100	100
3b	100	100
3c	100	100
3d	100	100
3e	100	100
3f	100	100
3g	100	100
3h	25	100
3i	21	50
3j	100	100
3k	100	100
3l	25	100
3m	50	100
3n	50	100
3o	29	100
3p	50	100
3q	100	100
3r	50	100
3s	35	100
3t	50	100
3u	25	50
3v	25	50
3w	100	100

originally identified active closantel fragment, compound **1**, fully inhibited the motility (viability) of O. *ochengi* microfilariae at 10 μg/mL (15 and 21 μM, respectively). Although exact IC_{50} values were not calculated, the data in Table 3 indicate that the values are somewhere between the concentrations tested. Of additional note, at present, no chitinase has been reported in *O. volvulus* or *O. ochengi* microfilariae; however, microfilarial chitinases have been reported in *Brugia malayi*, another filarial parasitic species.[21,22] Thus, we posit that a chitinase does exist in these species, since closantel and compound **1**, which does not display potent protonophore activity (see Supporting Information), are both active against *O. ochengi* microfilariae. Closantel and compound **1** were also examined for activity against adult *O. ochengi* worms; however, neither compound affected the viability of the worms (data not shown). This lack of activity could be due to penetrability issues or the short duration of the assay (5 days). However, it is also possible that adult *O. ochengi* worms do not produce chitinase or chitinase is present only in the embryonic stages within the female worms, neither of which would result in the killing of the adult worms.

DISCUSSION

Molting is a key developmental process for parasitic nematodes, and interference with molting is fatal to the parasite's development. As such, the identification and study of enzymes involved in molting are fundamental for furthering our understanding of the nematode life cycle and its potential manipulation as a therapeutic target. The L3 to L4 larval molt is of critical significance in the nematode lifecycle, as this represents the transitional stage between the vector and the host and is necessary for active infection of and further development within the human host. To date, four classes of enzymes have been reported to be implicated in this molting stage of *O. volvulus*: cysteine proteases,[9,10,23,24] serine proteases,[9] transglutaminases,[25] and chitinases.[6] While chemical biological probes have been identified for cysteine proteases and transglutaminases,[23−25] only recently has such a probe been discovered for the *O. volvulus* chitinase, OvCHT1. Closantel, a known veterinary anthelmintic drug, was discovered by our group as a potent inhibitor of OvCHT1.[4] Additionally, closantel was also found to inhibit the L3 to L4 larval molt in *O. volvulus* in a similar fashion as had been observed with cysteine protease and transglutaminase inhibitors cultured with this parasitic species.[4]

Because closantel's anthelmintic activity as a veterinary drug is thought to be due to its role as a proton ionophore, we were interested in discerning the impact of chitinase inhibition and mitochondrial uncoupling in the molting of *O. volvulus* L3 larvae. To answer this question, chemical synthesis was utilized and a small series of compounds based on a closantel fragment previously identified to exhibit chitinase inhibitor activity near that of the parent compound was designed and synthesized (Table 1). Various halogen substituents and a bulky alkyl substituent were examined on the salicylic acid ring, and substituents of varying electronics were examined on the aniline ring. Following synthesis, all analogues were analyzed for chitinase inhibition and protonophore activity. Interestingly, although all compounds contained the salicylanilide motif, an established protonophore scaffold, analogues with varying biological activities were discovered. Of significance, compound **3f** was found to exhibit enhanced chitinase inhibition with respect to the activity of closantel and no uncoupling activity (Table 2 and Figure 4, respectively). Additionally, compound **3w**, which exhibited no chitinase inhibition activity, was found to be a more potent ionophore than closantel (Figure 2 and Figure 4, respectively). On the basis of estimated cLogP values, lipophilicity is key for both activities (cLogP of 6.72 and 6.90 for compounds **3f** and **3w**, respectively).

In an attempt to unravel the possible mechanistic implications of closantel's dual activities, compounds **3f** and **3w** were analyzed for their impact on the molting of *O. volvulus* L3 to L4 larvae. Importantly, while 100 μM of each compound was required to

affect this molt as single compound doses, thus, similar to closantel itself, treatment with a 1:1 mixture of compounds **3f** and **3w**, each at 50 μM, was enough to completely abolish the L3 to L4 larval molt in cultured *O. volvulus*. These results support the premise that these individual closantel-based probes likely act synergistically, and both are required for potent inhibition of molting demonstrating that closantel's activity is dependent on both its chitinase inhibition and protonophore activities. Although no toxicity was observed with either probe, it appears that at higher concentrations, knockdown of only one biological process is effective.

It is interesting, however, that treatment with a combination of probes representing each of closantel's biological activities had an effect on dosing. Our experimental findings show that in the case of closantel, the individual biological modes as represented by salicylanilides **3f** and **3w** are greater than that of the composed drug. These studies mimic earlier reports concerning antibacterial agents.[26,27] Although additional experiments will need to be completed to better understand these effects, it is plausible that dual activities impact different cellular or tissue localization. In the case of closantel, these activities may compete against one another, while the target specificity of individual probes **3f** and **3w** could lend to their enhanced potency as a combination. OvCHT1 is expressed only in the glandular esophagus of the L3 larvae;[6] however, protonophores are active in mitochondria, which are found in all cell types. On the other hand, these compounds are structurally similar and may exhibit similar cellular and tissue distributions.

As an alternative hypothesis, ionophore activity and chitinase synthesis and/or activity are synergistic. Since very little is known about the *O. volvulus* chitinase, and parasitic chitinases in general, one scenario suggests a linkage between these biological functions and their role in molting. Early ultrastructural studies in *O. volvulus* demonstrated that the glandular esophagus is a prominent structure in L3 larvae making up over two-thirds of the larval body.[28] Importantly, immunoelectron microscopic studies have shown that many of the proteins essential to the development of L3 in the mammalian host are present in the granules of the glandular esophagus, indicating that this tissue has a high rate of protein synthesis. Thus, an uncoupling of the activity of the mitochondria in the glandular esophagus may impact overall protein synthesis and hence the synthesis of key secreted enzymes necessary for molting. Additional studies using compound **3w** in the presence of known inhibitors of *O. volvulus* cysteine and serine proteases and transglutaminase, enzymes also important for molting, may help to further our understanding of how mitochondrial uncoupling affects the overall biology of the glandular esophagus and its protein products.

In addition to this conjecture, we cannot rule out penetrability issues, as we had initially hypothesized in our earlier report.[4] In fact, issues of cuticle penetration have long plagued screening efforts in the nematode *Caenorhabditis elegans*,[29−31] and very few successful examples have been documented in the literature.[29,32,33] Of note, the cuticle found in *O. volvulus* L3 larvae is known to be very thick in comparison with those of other larval molt stages.[28,34] Recently, a predictive model for drug bioaccumulation and bioactivity in *C. elegans* was reported.[35] After surveying a series of commercial libraries, the researchers identified sets of key functional groups found in accumulating and nonaccumulating library members.[35] Interestingly, phenols, which are prone to metabolism, were found in a large proportion of nonaccumulating compounds. Thus, the free phenol, which is critical for proton ionophore activity, may hinder the accumulation of our closantel-based compounds. Therefore, from this study, three possibilities for enhancing nematode penetrability are taking a prodrug approach by masking the free phenol, the synthesis of other analogues incorporating the functional groups identified in this report, and the synthesis of phenol isosteric scaffolds (e.g., indazole).[36−39]

In addition, we would like to bring to light that OvCHT1 is a secreted protein found mostly in the cuticle postinfection;[6] however, the role of cuticular chitinase or secreted chitinase is still shrouded. In *Acanthocheilonema viteae*, chitinase was identified as an immunodominant filarial antigen recognized by the sera of vaccinated jirds.[40] In this report, the authors speculate that in addition to its role in degrading the nematode cuticle, chitinase may help the parasite to migrate through the host's tissues and may play a protective role when the parasite is highly vulnerable after transmission.[40] For *O. volvulus*, DNA immunization with OvCHT1 was also found to partially protect against an L3 larval challenge in mice.[41] Since protection against secreted chitinase appears to have host protective effects, it will be valuable to better define if closantel or compound **3f** is also active against cuticular chitinase.

Finally, we also tested our compounds for activity in *O. ochengi* microfilariae, a model organism for studying infection with *O. volvulus*.[18,19] *O. ochengi* and *O. volvulus* are phylogenetically the most closely related *Onchocerca* species and also share *Simulium damnosum* as a vector.[18,19] Although *O. ochengi* microfilariae are not known to produce a chitinase, both closantel and compound **1** were found to inhibit microfilarial motility (viability) in this species. Interestingly, while closantel exhibits both chitinase inhibition and proton ionophore activities, compound **1** only acts as a chitinase inhibitor. Thus, it is intriguing that this fragment analogue would emerge as an active compound. Further genome sequencing and mining studies in *O. ochengi* will be needed to determine if a chitinase does exist in this microfilarial species. Of further note, because closantel and compound **1** are active against *O. ochengi* microfilariae, regardless of their mechanism of action, we are hopeful that further animal studies using these compounds can be pursued.

■ CONCLUSIONS

We have completed SAR studies on the known veterinary anthelmintic drug, closantel, in order to more fully understand the impact of its demonstrated proton ionophore and chitinase inhibition activities on molting in *O. volvulus*, the causative agent of the neglected tropical disease onchocerciasis. Importantly, through the use of a small library of closantel-based salicylanilides, compounds that are known to exhibit proton ionophore activity, biochemical probes with differing activities were uncovered, both of which are more potent than closantel itself. Using these probes, we have discovered that the closantel-induced molting inhibition phenotype is due to both its proton ionophore and chitinase inhibition activities. Although the fragments discovered may not be druglike, they do provide the means by which to modulate molting in *O. volvulus* and potentially validate this mechanism as an antifilarial drug target not only for onchocerciasis but also for other parasite-mediated diseases. In addition, while current research strategies aimed at treating parasitic diseases in developing countries often rely on vaccination-based approaches, it has recently been shown that host immune effectors cause filarial parasites to develop more quickly and reproduce earlier.[42] Thus, the discovery and development of

small molecule-based drugs may represent a better approach for treating filarial diseases. In particular, since current drugs only target *O. volvulus* microfilariae, the addition of an antilarval drug opens up the possibility for a multidrug combination, which could synergize the reduction of worm burden by effectively targeting both the developing larvae and microfilariae, thereby decreasing the chance of transmission. We hope that our discovery of small molecule probes that target filarial species based on new mechanisms of inhibition may spark additional interest in this field.

EXPERIMENTAL SECTION

General Chemistry Methods. Reactions were carried out under a nitrogen atmosphere with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride. Yields refer to chromatographically and spectroscopically homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm EMD silica gel plates (60F-254) using ninhydrin staining or UV light (254 nm). Flash chromatography separations were performed on Silicycle silica gel (40−63 mesh). All compounds were confirmed to have ≥95% purity by HPLC (254 nm; see Supporting Information for HPLC traces). NMR spectra were recorded on a Bruker 500 MHz spectrometer and calibrated using a solvent peak as an internal reference. The following abbreviations are used to indicate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Purity was assessed using HPLC (detection at 254 nm).

General Synthetic Procedure for Salicylanilides 3a−w. A stirring solution of carboxylic acid (compounds **2a−w**) (1.0 equiv) in CH_2Cl_2 (0.25 M) was cooled to 0 °C in an ice−water bath and subsequently treated with oxalyl chloride (2.4 equiv) and DMF (∼1 drop). After the mixture was stirred for 2 h at 0 °C−25 °C, the solvent was concentrated in vacuo. The resulting residue was then dissolved in THF (0.25 M) and cooled to 0 °C. A THF solution (0.25 M with respect to carboxylic acid) of amine (R^3NH_2) (1.1 equiv) and triethylamine (3.0 equiv) was then added, and the solution was stirred overnight from 0 to 25 °C. The solvent was then concentrated in vacuo, and the resulting crude residue was extracted with ethyl acetate and washed with water and brine. After the mixture was dried with Na_2SO_4 and concentrated in vacuo, the residue was purified by flash column chromatography (EtOAc in hexanes) to yield the chromatographically pure salicylanilide (compounds **3a−w**).

Compound ***3a***[43]. 47% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.24 (br s, 1H), 10.77 (br s, 1H), 8.39 (d, *J* = 1.9 Hz, 1H), 8.21 (d, *J* = 1.9 Hz, 1H), 7.63−7.70 (m, 2H), 7.36−7.44 (m, 2H), 7.16−7.22 (m, 1H).

Compound ***3b***[44]. 49% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 12.99 (br s, 1H), 10.72 (br s, 1H), 8.25 (d, *J* = 2.0 Hz, 1H), 8.17 (d, *J* = 2.0 Hz, 1H), 7.56−7.63 (m, 2H), 7.15−7.21 (m, 2H), 2.34 (s, 3H).

Compound ***3c***[44]. 42% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.08 (br s, 1H), 10.81 (br s, 1H), 8.25 (d, *J* = 2.0 Hz, 1H), 8.18 (d, *J* = 2.0 Hz, 1H), 7.51−7.63 (m, 2H), 6.97−7.07 (m, 2H), 3.83 (s, 3H).

Compound ***3d***[43,44]. 51% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.12 (br s, 1H), 10.82 (br s, 1H), 8.27 (d, *J* = 2.0 Hz, 1H), 8.19 (d, *J* = 2.0 Hz, 1H), 7.63−7.68 (m, 2H), 7.07−7.15 (m, 2H).

Compound ***3e***[43,44]. 63% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 12.98 (br s, 1H), 10.73 (br s, 1H), 8.36 (d, *J* = 2.0 Hz, 1H), 8.23 (d, *J* = 2.0 Hz, 1H), 7.63−7.68 (m, 2H), 7.56−7.62 (m, 2H).

Compound ***3f***. 53% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.15 (br s, 1H), 10.85 (br s, 1H), 8.28 (d, *J* = 2.0 Hz, 1H), 8.20 (d, *J* = 2.0 Hz), 7.90−7.97 (m, 2H), 7.57−7.64 (m, 2H). HRMS (ESI-TOF) *m*/*z* calcd for $C_{13}H_8I_3NO_2$ $[M + H]^+$ 590.7689, found 590.9215.

Compound ***3g***. 42% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 12.99 (br s, 1H), 10.72 (br s, 1H), 8.25 (d, *J* = 2.0 Hz, 1H), 8.17 (d, *J* = 2.0 Hz, 1H), 7.56−7.63 (m, 2H), 7.15−7.21 (m, 2H), 2.82−2.87 (m, 1H), 1.20−1.24 (m, 6H); HRMS (ESI-TOF) *m*/*z* calcd for $C_{16}H_{15}I_2NO_2$ $[M + H]^+$ 506.9192, found 507.1047.

Compound ***3h***. 51% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 11.72 (br s, 1H), 10.77 (br s, 1H), 8.13 (d, *J* = 1.9 Hz, 1H), 7.63−7.70 (m, 3H), 7.36−7.44 (m, 2H), 7.16−7.22 (m, 1H), 6.81 (d, *J* = 8.4 Hz, 1H); HRMS (ESI-TOF) *m*/*z* calcd for $C_{13}H_{10}INO_2$ $[M + H]^+$ 338.9756, found 339.1285.

Compound ***3i***. 59% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 11.70 (br s, 1H), 10.62 (br s, 1H), 8.11 (d, *J* = 1.9 Hz, 1H), 7.62−7.75 (m, 3H), 7.32−7.38 (m, 2H), 6.83 (d, *J* = 8.4 Hz, 1H), 2.34 (s, 3H); HRMS (ESI-TOF) *m*/*z* calcd for $C_{14}H_{12}INO_2$ $[M + H]^+$ 352.9913, found 353.1551.

Compound ***3j***. 61% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 11.70 (br s, 1H), 10.62 (br s, 1H), 8.11 (d, *J* = 1.9 Hz, 1H), 7.60−7.70 (m, 3H), 7.30−7.39 (m, 2H), 6.80 (d, *J* = 8.4 Hz, 1H), 3.79 (s, 3H); HRMS (ESI-TOF) *m*/*z* calcd for $C_{14}H_{12}INO_3$ $[M + H]^+$ 368.9862, found 369.1544.

Compound ***3k***[43]. 68% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 11.70 (br s, 1H), 10.62 (br s, 1H), 8.15 (d, *J* = 1.9 Hz, 1H), 7.66−7.77 (m, 3H), 7.40−7.46 (m, 2H), 6.81 (d, *J* = 8.4 Hz, 1H).

Compound ***3l***[15,45]. 66% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.24 (br s, 1H), 10.77 (br s, 1H), 7.77 (d, *J* = 1.9 Hz, 1H), 7.51 (d, *J* = 1.9 Hz, 1H), 7.61−7.70 (m, 2H), 7.36−7.43 (m, 2H), 7.16−7.24 (m, 1H).

Compound ***3m***[15,45]. 80% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 12.99 (br s, 1H), 10.72 (br s, 1H), 7.80 (d, *J* = 2.0 Hz, 1H), 7.62 (d, *J* = 2.0 Hz, 1H), 7.56−7.61 (m, 2H), 7.21−7.43 (m, 2H), 2.31 (s, 3H).

Compound ***3n***[15,45]. 84% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.08 (br s, 1H), 10.81 (br s, 1H), 7.82 (d, *J* = 2.0 Hz, 1H), 7.60 (d, *J* = 2.0 Hz, 1H), 7.51−7.63 (m, 2H), 6.97−7.07 (m, 2H), 3.78 (s, 3H).

Compound ***3o***[15,45]. 73% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.02 (br s, 1H), 10.89 (br s, 1H), 7.85 (d, *J* = 2.0 Hz, 1H), 7.71 (d, *J* = 2.0 Hz, 1H), 7.68−7.73 (m, 2H), 7.43−7.49 (m, 2H).

Compound ***3p***[45]. 88% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.24 (br s, 1H), 10.77 (br s, 1H), 8.18 (d, *J* = 1.9 Hz, 1H), 8.01 (d, *J* = 1.9 Hz, 1H), 7.63−7.70 (m, 2H), 7.36−7.44 (m, 2H), 7.16−7.22 (m, 1H).

Compound ***3q***[45]. 63% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 12.99 (br s, 1H), 10.72 (br s, 1H), 8.15 (d, *J* = 2.0 Hz, 1H), 8.00 (d, *J* = 2.0 Hz, 1H), 7.56−7.63 (m, 2H), 7.15−7.21 (m, 2H), 2.32 (s, 3H).

Compound ***3r***[45]. 62% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 13.08 (br s, 1H), 10.81 (br s, 1H), 8.18 (d, *J* = 2.0 Hz, 1H), 7.99 (d, *J* = 2.0 Hz, 1H), 7.51−7.63 (m, 2H), 6.97−7.07 (m, 2H), 3.80 (s, 3H).

Compound ***3s***[45]. 50% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 12.98 (br s, 1H), 10.73 (br s, 1H), 8.20 (d, *J* = 2.0 Hz, 1H), 8.00 (d, *J* = 2.0 Hz, 1H), 7.63−7.68 (m, 2H), 7.56−7.62 (m, 2H).

Compound ***3t***. 41% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 10.81 (br s, 1H), 10.12 (br s, 1H), 7.55−7.61 (m, 2H), 7.46 (d, *J* = 1.9 Hz, 1H), 7.36−7.44 (m, 2H), 7.32 (d, *J* = 1.9 Hz, 1H), 7.25−7.31 (m, 2H), 7.16−7.22 (m, 1H), 2.95−3.02 (m, 1H), 2.85−2.90 (m, 1H), 1.11−1.20 (m, 12H); HRMS (ESI-TOF) *m*/*z* calcd for $C_{19}H_{23}NO_2$ $[M + H]^+$ 297.1729, found 297.3914.

Compound ***3u***. 39% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 10.81 (br s, 1H), 10.12 (br s, 1H), 7.55−7.61 (m, 2H), 7.47 (d, *J* = 1.9 Hz, 1H), 7.36−7.47 (m, 3H), 7.22−7.31 (m, 2H), 2.95−3.02 (m, 1H), 2.85−2.90 (m, 1H), 2.31 (s, 3H), 1.11−1.20 (m, 12H); HRMS (ESI-TOF) *m*/*z* calcd for $C_{20}H_{25}NO_2$ $[M + H]^+$ 311.1885, found 311.4180.

Compound ***3v***. 52% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 10.81 (br s, 1H), 10.12 (br s, 1H), 7.55−7.61 (m, 2H), 7.46 (d, *J* = 1.9 Hz, 1H), 7.32−7.41 (m, 2H), 7.25−7.31 (m, 2H), 3.81 (s, 3H), 2.95−3.02 (m, 1H), 2.85−2.90 (m, 1H), 1.11−1.20 (m, 12H); HRMS

(ESI-TOF) m/z calcd for $C_{20}H_{25}NO_3$ $[M + H]^+$ 327.1834, found 327.4174.

Compound ***3w***. 39% yield; ^{1}H NMR (500 MHz, DMSO, 25 °C) δ 10.81 (br s, 1H), 10.12 (br s, 1H), 7.55−7.61 (m, 2H), 7.46 (d, J = 1.9 Hz, 1H), 7.36−7.44 (m, 2H), 7.32 (d, J = 1.9 Hz, 1H), 7.25−7.31 (m, 2H), 2.95−3.02 (m, 1H), 2.85−2.90 (m, 1H), 1.11−1.20 (m, 12H); HRMS (ESI-TOF) m/z calcd for $C_{19}H_{22}ClNO_2$ $[M + H]^+$ 331.1339, found 331.8365.

Assay Materials. Closantel was purchased from Sigma-Aldrich. OvCHT1 was purchased from New England Biolabs. 4-Methylumbelliferyl-*N*,*N*′,*N*″-β-chitotrioside was purchased from Calbiochem. Valinomycin was purchased from Sigma-Aldrich. JC-1 was purchased from Invitrogen. All purchased reagents were used as received.

Chitinase Inhibition Assay. Chitinase activity was measured using a 96-well fluorescence assay using 4-methylumbelliferyl-*N*,*N*′,*N*″-β-chitotrioside as a profluorescent substrate.[46] All screening reactions were performed in 50 μL volumes at 37 °C containing 1.25 units of OvCHT1, 20 μM 4-methylumbelliferyl-*N*,*N*′,*N*″-β-chitotrioside, and 25 μM closantel fragment analogue in 200 mM NaCl with 20 mM Na_3PO_4 (pH 6.0) and 1.0 mM EDTA. As a blank, a sample was prepared as described above but without compound. In all reactions, the DMSO concentration was kept constant at 0.5%, and no influence on enzymatic turnover was observed. The reactions were monitored over 10 min for an increase in fluorescence signal using a SpectraMax M2e microplate reader (Molecular Devices) (λ_{ex} = 350 nm, λ_{em} = 440 nm). All reactions were performed in triplicate. All data were analyzed using GraphPad Prism, version 5.0a, for Mac OS X (GraphPad Software, www.graphpad.com).

Mitochondrial Uncoupling Assay. Adherent human epithelial kidney cells (HEK-293T/17) were trypsinized and resuspended at a density of 1 × 10^6 cells per 1.0 mL of sterile 1× PBS in 5 mL BD Falcon round-bottom tubes (BD Biosciences). Cells were then incubated with 200 nM valinomycin, 50 μM closantel, or 50 μM closantel fragment analogues for 10 min at 37 °C. JC-1 (1.0 μL, 2.5 mg/mL) was added, and the cells were incubated for another 10 min at 37 °C. Cells were washed and centrifuged 6× with sterile 1× PBS and resuspended in a final volume of 1.0 mL of PBS. Cells were sorted using a FACSCalibur cytometer (BD Biosciences) (FL-1 = FITC green filter; FL-2 = PE red filter). Data were analyzed in real time using Cell Quest (BD Biosciences) and later modified to create dot plots using FlowJo 9.2 (Tree Star).

***O. volvulus* L3 Molting Assay.** Cryopreserved L3 stage larvae were rapidly thawed in a 37 °C water bath and washed in wash medium (NCTC/IMDM (1:1) with 1× GPS (glutamine, penicillin, streptomycin)). The number of worms was set to 5−10 worms per 50 μL in complete medium (CM) containing 20% heat inactivated FCS. Worms were distributed to 10 wells of a 96-well plate per treatment group, and 1.5 × 10^5 normal PBMCs were added per well in 50 μL. Then 2× dilutions of compound were added to each well, 100 μL per well. Controls using DMSO in CM and CM were included. The 96-well plates were then incubated at 37 °C in a 5% CO_2 incubator until day 6 when molting was recorded under inverted microscope. Molting was assessed by the presence of L4 larvae and the empty cast of the L3.

***O. ochengi* Microfilarial Motility Assay.** *O. ochengi* microfilariae were isolated from skin snips of cattle skin.[20] The assay was conducted in 96-well plate containing fully confluent monkey kidney epithelial cells, serving as feeder layer, cocultured with at least 15 microfilariae in 200 μL of complete culture medium (CCM) (RPMI-1640 supplemented with 25 mM HEPES, 2 g/L $NaHCO_3$, 2 mM L-glutamine, 5% newborn calf serum, 150 units/mL penicillin, 150 μg/mL streptomycin, and 0.5 μg/mL amphotericin B, pH 7.4) at 37 °C in humidified air containing 5% CO_2 for 5 days without any change of medium.[20] The medium used in preparing the feeder cell layer was removed by swift decantation before fresh CCM containing compound (100 μL) and worms (100 μL) were immediately added. Amocarzine (10 μg/mL) and 2% DMSO served as positive and negative controls, respectively. Microfilarial motility scores (viability) were done on a scale of 0−100% motility: 0 (immotile), 25% motility (only tail or head shaking occasionally), 50% motility (whole body motile but sluggishly or with difficulties), and 100% motility (almost vigorous to vigorous motility). Motility was examined every 24 h, terminating at 120 h using an inverted microscope. Microfilarial viability was assessed at 120 h (day 5) after addition of compound. Each assay was done in duplicate. Interwell variations in microfilarial motility were never observed. A compound was considered active if there was a 100% reduction in microfilarial motility compared to the control. It was considered moderately active if reduction of motility was 50%−99% and inactive if the reduction was less than 50%.

■ ASSOCIATED CONTENT

Supporting Information. HPLC spectra for all compounds and characterization of protonophore activity for all analogues and compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

L3, third larval stage; L4, fourth larval stage; *O. ochengi*, *Onchocerca ochengi*; *O. volvulus*, *Onchocerca volvulus*; OvCHT1, *O. volvulus* chitinase; SAR, structure−activity relationship

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