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Exploiting the Polypharmacology of ß-Carbolines to Disrupt *O. volvulus* Molting

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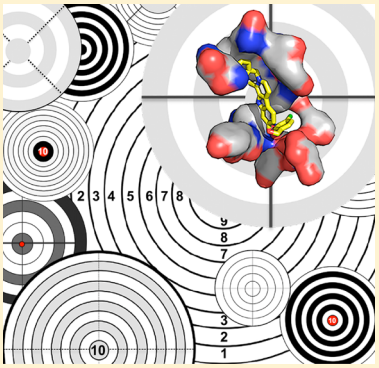
Supporting Information

ABSTRACT: Onchocerciasis is an infection caused by the filarial worm *Onchocerca volvulus*, which can eventually result in blindness. The lack of an effective macrofilaricide and the possible development of ivermectin-resistant strains of *O. volvulus* necessitate the need for alternative treatment strategies. We have shown that targeting the L3-stage-specific chitinase OvCHT1 impairs the shedding of the filarial cuticle. In our continued efforts to discover OvCHT1 inhibitors, we identified the *β*-carboline alkaloid scaffolding as a chitinase inhibitor that is capable of penetrating the worm cuticle. Herein, we disclose the rich polypharmacology of the *β*-carboline class of compounds as an approach to abrogate the molting of the parasite and thus the initiation of infection in the human host.



KEYWORDS: *Onchocerciasis, β-carbolines, chitinase inhibitor, polypharmacology*

Onchocerciasis, or river blindness, is caused by the parasitic nematode *Onchocerca volvulus* and is the second leading infectious cause of blindness (affecting over 37 million people worldwide).[1] Of crucial significance to the survival and development of *O. volvulus* in the human host is the shedding of the L3 cuticle.[2] The molting of L3 to L4 larvae is particularly important for active infection of the human host, and thus, targeting this transitional stage may help reduce parasite infection and transmission.

OvCHT1, a chitinase expressed predominantly in the infective L3 larvae, has been implicated in the development of *O. volvulus*.[2] We have previously demonstrated that inhibition of OvCHT1 activity impedes the L3-to-L4 molt[3,4] and that a dual-targeting strategy (involving mitochondrial uncoupling and chitinase inhibition) results in improved efficacy.[4] Multitarget treatments offer several advantages including increased therapeutic effects and prevention of drug resistance. As such, the polypharmacological strategy has emerged as a new paradigm in the discovery of anticancer[5,6] and anti-infective[7] medications, among others. The dearth of an efficacious macrofilaricide and the possible emergence of drug-resistant *O. volvulus*,[8] call for a need to identify alternative therapeutics for onchocerciasis.

In our continuing search for anti-onchocerciasis agents, we screened a commercial library of over 500 natural products toward OvCHT1 inhibition, as described previously.[3] Our screening initiative led to the identification of norharmane (**1**), harmane (**2**), harmine (**3**), and harmol (**4**), which all showed complete inhibition of OvCHT1 at 25 *μ*M. Compounds **1**-**4** are *β*-carboline alkaloids that are known to display a wide range of biological and pharmacological properties, including antitumor, antiviral, and antimicrobial activities.[9] In addition, *β*-carbolines have been reported to display antiparasitic activities. Harmine inhibited the growth of *Leishmania infantum* promastigotes as well as the intracellular amastigote forms of the parasite.[10] *β*-carboline derivatives were shown to significantly reduce the growth of *Trypanosoma cruzi* epimastigotes[11] and were also active against the parasites *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*.[12] In rodents infected with *Acanthoeilonema viteae*, *Brugia malayi*, and *Litomosoides carinii*, treatment with *β*-carbolines led to death of the filariae or sterilization of female worms.[13] The antiparasitic activity of *β*-carbolines was thought to be due to respiratory chain inhibition or inverse agonism of the benzodiazepine receptor; however, it was not previously linked to chitinase inhibition. Herein, we present yet another biological activity of the *β*-carboline class of compounds and further exploit its polypharmacology as a means to effectively inhibit *O. volvulus* L3 molting. We also show that the *β*-carboline derivatives are able to penetrate the highly resistant cuticle of nematodes (using *C. elegans* as a model organism of bioaccumulation), which predetermines their efficacy *ex vivo*.

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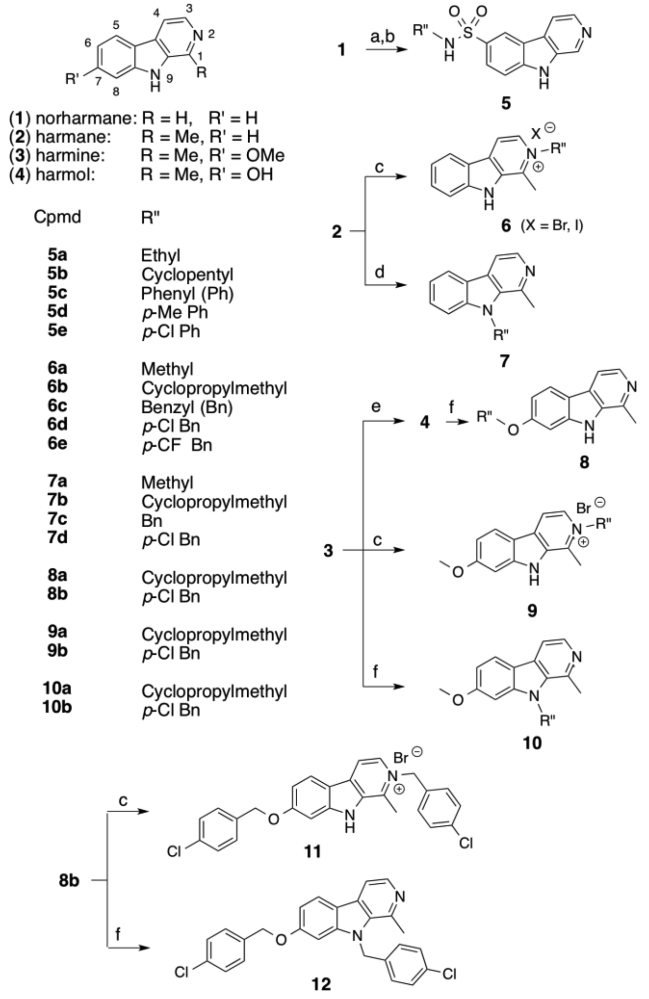
On the basis of the initial hits **1**–**4**, a small library of β-carboline analogues was prepared to identify key features that could impart potency toward OvCHT1 inhibition. An initial structure–activity relationship was gleaned from the library screening as methyl and hydroxy/methoxy substitutions are tolerated at positions 1 and 7, respectively. As derivatization of β-carbolines is already well established in the literature, we focused on simple elaborations of the β-carboline framework by diversification at positions 2, 6, 7, and 9. The synthetic routes for the preparation of β-carboline derivatives **5**–**12** are outlined in Scheme 1. Reaction of **1** with chlorosulfonic acid furnished

Scheme 1. Synthesis of β-Carboline Derivatives[a]



[a]Reagents and conditions: (a) $ClSO_3H$, 0 °C, 1 h; (b) RNH_2, DMF, rt, 1 h; (c) RBr or RI, EtOAc/*i*PrOH, reflux, 16 h; (d) NaH, RBr, DMF, 0 °C to rt, 1 h; (e) HBr/HOAc, reflux, 16 h; (f) Cs_2CO_3, RBr, DMF, rt, 12 h.

the 6-sulfonyl chloride intermediate,[14] which was subsequently reacted with alkyl/aryl amines to produce 6-sulfonamides **5a**–**e**. N^2-, N^9-, and 7-*O*-alkylated derivatives **6**–**12** were prepared according to published methods.[15,16] Next, we tested compounds **1**–**12** for OvCHT1 inhibition at 10 and 5 μM (Figure 1). Functionalization with a sulfonamide moiety at position 6 (derivatives **5a**–**e**) did not cause a significant effect on potency, relative to **1** (Figure 1A). The same could be said for compounds **6**–**8**; N^2-, N^9-, or 7-*O*-alkylation showed no significant improvement on inhibitory activity as compared to **2**. However, disubstitution of **2** at positions 2 and 7 (analogues **9** and **11**) led to increased potency against OvCHT1 (Figure 1B). N^9-Alkylation of **3** (compounds **10a**–**b**) diminished its activity, whereas simultaneous N^9- and 7-*O*-benzylation (**12**) displayed complete inhibition of OvCHT1 at 5 μM. The IC_{50} values of the initial hits **1**–**4** and the more potent derivatives are listed in Table 1. Compound **3** appeared to exhibit a competitive mode of inhibition as determined using Dixon plot, with an inhibition constant (K_i) of 3.78 ± 0.79 μM. Our straightforward derivatization of the β-carboline scaffold resulted in up to 5-fold increase in potency; analogue **11** had an IC_{50} of 1.43 ± 0.09 μM and a competitive inhibitory constant K_i of 0.98 ± 0.15 μM.

With regard to chitinase selectivity, representative compounds (**3**, **4**, **9a**, **11**, and **12**) were tested for inhibition of chitinases from other species including *Brugia malayi* (BmCHT1) and *Entamoeba histolytica* (EhCHT1). All compounds preferentially inhibited OvCHT1 over BmCHT1 and EhCHT1 except for compound **11**, which showed complete inhibition of all three chitinases at 10 μM (Supporting Information Figure S1).

Next, we conducted docking of compound **11** into the active site of OvCHT1 using the homology model described by Segura-Cabrera et al.[17] Analysis of the docked poses showed that the tricyclic indole ring fills the binding pocket by interacting with residues Tyr268, Trp361, and Thr362, while the N^9-(*p*-chloro)benzyl system is involved in π-stacking interaction with Phe365 (Figure 2). The binding mode is consistent with that of closantel,[17] where the terminal chlorobenzene and the central aromatic system occupy the same respective binding pockets. In the case of **11**, the more extended structure predisposes its 7-*O*-(*p*-chloro)benzyl moiety to interaction via van der Waals contacts with residues Tyr27, Phe58, Asp145, Ala184, and Trp361 (Figure 2), exploiting thus an additional binding pocket that is not accessible with the closantel ligand (Figure S2 in the Supporting Information).

N^2-Methylated β-carbolines were previously reported to inhibit mitochondrial respiration.[18] This activity was rationalized on their ability to form the neutral anhydronium base (through indole N^9-deprotonation), which could passively diffuse across the mitochondrial membrane.[18] To evaluate the mitochondrial uncoupling activity of the β-carboline derivatives, we performed a microplate fluorescence assay using the mitochondrion-selective probe TMRE to detect membrane depolarization.[4] Quarternary alkaloids **9a**, **9b**, and **11** proved to be good uncouplers of oxidative phosphorylation relative to CCCP, a known protonophore (Figure 3). It would seem that the uncoupling behavior is dependent on the availability of the indole N–H proton as N^9-alkylated **12** was completely devoid of protonophoric activity. Compounds **1** and **2** were found to be inactive, whereas the 7-OH/OMe-substituted counterparts (compounds **3** and **4**) displayed comparable uncoupling activity as **9a**/**9b**.

The foregoing studies reveal compounds **3**, **4**, **9a**, **9b**, and **11** to possess both protonophoric and chitinase inhibitory activities, whereas analogues **1**, **2**, and **12** act as chitinase inhibitors only. With these data in hand, we went ahead and tested the compounds for their ability to inhibit *O. volvulus* molting. At 10 μM, the dual protonophore-chitinase inhibitors **9a**, **9b**, and **11** eradicated L3 molting, while compounds **1**, **2**, and **12** (OvCHT1 inhibitors only) had no effect on the parasite's developmental process (Figure 4A). When dosed at 100 μM, compounds **1**, **2**, and **12** prevented the shedding of

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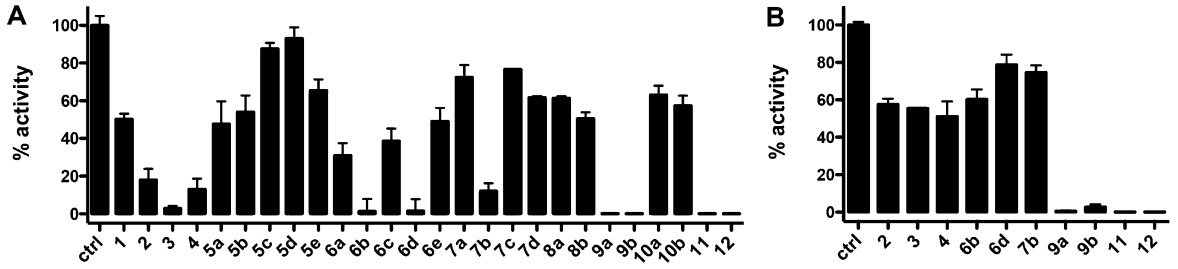

Figure 1. Evaluation of β-carboline derivatives in a fluorescence-based OvCHT1 inhibition assay. Analogues were examined at (A) 10 and (B) 5 μM. Data shown as % OvCHT1 activity, relative to control (0.5% DMSO).

Table 1. IC_{50} of *O. volvulus* Chitinase Inhibition

cmpd	IC_{50} (μM)	cmpd	IC_{50} (μM)
1	8.46 ± 1.36	**9a**	3.57 ± 0.79
2	7.33 ± 0.06	**9b**	3.49 ± 0.17
3	7.06 ± 0.42	**11**	1.43 ± 0.09
4	7.08 ± 0.20	**12**	2.08 ± 0.03

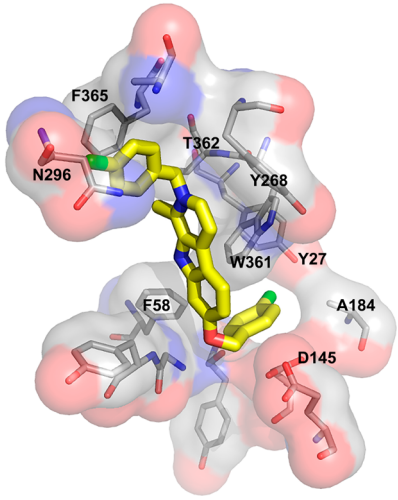

Figure 2. Lowest energy pose of compound **11** docked into *O. volvulus* chitinase (OvCHT1) using AutoDock Vina. Color scheme: oxygens are in red, nitrogens in blue, chlorines in green, and carbons in yellow (compounds **11**) or gray (OvCHT1).

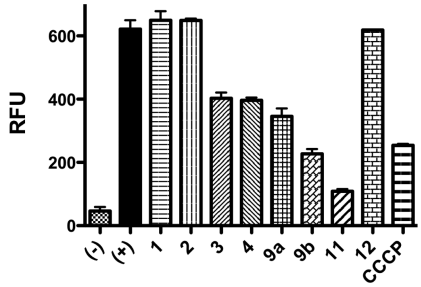

Figure 3. Evaluation of the mitochondrial-uncoupling activity of β-carboline derivatives. HEK 293*T*/17 cells were incubated with compound (50 μM) and subsequently stained with TMRE. Data shown as mean fluorescence intensity ± SD (n = 3). Unstained cells (no TMRE) and 0.5% DMSO were used as negative (−) and positive (+) control, respectively. RFU = relative fluorescence units (λ_{ex} = 488 nm, λ_{em} = 575 nm).

the L3 cuticle. Compared to **9a**/**9b**, the 2-fold less active chitinase inhibitors **3** and **4** showed 57% and 61% inhibition of

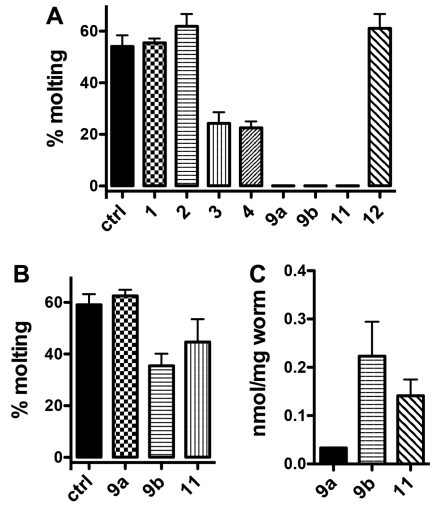

Figure 4. Molting of *O. volvulus* L3 larvae in the presence of β-carboline derivatives. Percent molting at (A) 10 and (B) 1 μM inhibitor concentration. Data presented as percent molting in a total of 2−3 wells containing on average 5−10 larvae per well. (C) Bioaccumulation in *C. elegans*. Late-stage L4 worms were incubated with 10 μM inhibitor (equivalent to 2 nmol/mg worm) for 6 h. Data shown as mean concentration ± SD (n = 3), expressed in nmol/mg worm.

molting, respectively (Figure 4A). These results demonstrate that the presence of both mitochondrial uncoupling and chitinase inhibitory activities in a single molecule more effectively inhibits the L3-to-L4 molt, as we have earlier shown for the closantel analogues.[4]

Derivatives **9a**, **9b**, and **11** were further evaluated for their effect on L3 molting at 1 μM (Figure 4B). Treatment with **9b** and **11** led to 45% and 27% inhibition, respectively, whereas **9a** had no impact on molting at 1 μM. Considering that compounds **9a**, **9b**, and **11** have similar inhibitory profiles, we reasoned that the difference in efficacy might be due to bioaccumulation issues. *O. volvulus*, as with other nematodes, is equipped with a thick cuticle composed primarily of cross-linked collagen, giving rise to a highly compact structure resistant to exogenous perturbation.[19] Thus, cuticle penetration is a pharmacological determinant of the drug sensitivity of *O. volvulus* and other filarial worms. To shed light on this matter, we used *C. elegans* as a predictive model of nematode penetrability.[20] We have previously employed this model system to determine the accumulation of closantel derivatives within the worm.[4] Late-stage L4 *C. elegans* were incubated with analogues **9a**, **9b**, or **11** at 2 nmol/mg worm for 6 h, and worm homogenates were then analyzed by LC−MS for quantitation. As depicted in Figure 4C, all three compounds penetrate the

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cuticle, albeit, at low concentrations. Levels of **9b** were 0.22 ± 0.10 nmol/mg worm, 6-fold higher than those of **9a** (0.033 ± 0.001 nmol/mg worm). Compound **9a** was also found to undergo N^2-dealkylation to **3**, which in turn had worm concentrations of 0.052 ± 0.006 nmol/mg. Note that other possible metabolism pathways, including 7-*O*-dealkylation, aromatic ring, and N^2-oxidation, were also expressly looked for but were not identified in the worm homogenates. We hasten to add that the bioaccumulation of **9a**, **9b**, and **11** correlated well with the bioactivity observed in *O. volvulus*.

It is intriguing that despite the low accumulation levels (up to 11% of the initial dose for **9b**) and modest chitinase inhibitory activities (IC_{50} values in the low micromolar concentration) of the β-carbolines, the casting of the L3 cuticle was still significantly inhibited. The efficacy observed in *O. volvulus* is likely the result of the dynamic modulation of a pharmacological network of relevant targets. Early electron microscopic studies of *O. volvulus* revealed that the infective L3 has a compact morphology, with the glandular esophagus making up over two-thirds of the larval body.[21] The glandular tissue is packed with secretory structures and rough endoplasmic reticulum,[21] signifying an enhanced rate of protein synthesis crucial for the development of the L3 larvae. Enzymes such as cysteine proteases,[22] serine proteases,[23] transglutaminases,[24] and chitinases[2] have earlier been implicated in the L3-to-L4 molt of *O. volvulus*. Thus, the uncoupling of mitochondrial activity (as demonstrated by compounds **9a**, **9b**, and **11**, Figure 3) in the esophageal glands may impact the production of essential proteins (including OvCHT1) that are necessary for molting. In addition, β-carbolines can interact with DNA through groove binding or intercalation,[25] thereby interfering with DNA replication/transcription and hence the overall protein biosynthesis in the granules of the glandular esophagus. Interestingly, no significant effects on the viability of human HEK 293*T*/17 cells were observed upon incubation with **9a**, **9b**, or **11** at a concentration (5 μM) above their IC_{50} values (Figure S3 in the Supporting Information), thus indicating a level of confidence about their safety at the effective concentration used.

β-carboline alkaloids were also reported as inverse agonists at the benzodiazepine allosteric site of the $GABA_A$ receptor,[26] a biochemical target for filaricidal agents. Incidentally, expressed sequence tag (EST) analysis of cDNA libraries from *O. volvulus* showed upregulation of an ionotropic GABA receptor in the molting L3 larvae of *O. volvulus*.[27] Compounds **9a**, **9b**, and **11** could possibly interact with this uncharacterized GABA receptor to modulate the neurotransmitter-mediated signaling pathways. A next logical step would be to isolate and characterize the molting L3 GABA receptor (or the homologue in *C. elegans* unc-49)[27,28] and investigate the inverse agonistic effects of the β-carboline analogues.

Most drug discovery efforts are directed toward selective regulation of a therapeutic target in an attempt to maximize efficacy and minimize harmful side effects. However, single-targeted compounds are often ineffective in treating complex diseases involving a wide array of cellular signaling networks. The use of polypharmacologicals renders the benefits of superior efficacy (via modulation of multiple disease pathways) while reducing the risk of drug resistance. Although it raises safety concerns, multitarget therapy does not necessarily equate to toxicity, as was shown for the analgesic tapentadol[29] and antidepressants.[30] The possible occurrence of ivermectin-resistant strains of *O. volvulus*[8] warrants the need for effective treatments of onchocerchiasis. From our screening efforts, we have identified the β-carbolines as novel inhibitors of the *O. volvulus* chitinase OvCHT1. We took advantage of the rich polypharmacology of the β-carboline class of compounds and investigated its ability to abrogate the shedding of the L3 cuticle. The multitarget derivatives (**9a**, **9b**, and **11**) with protonophoric and chitinase inhibitory activities displayed more potent inhibition of molting. As penetration of the filarial cuticle is a requisite for an effective anthelmintic drug, we also demonstrated that the β-carboline analogues accumulate within the worm using *C. elegans* as the model nematode. Future efforts will focus on the optimization of our multitarget leads and exploitation of their beneficial polypharmacology as an alternative approach to combat onchocerchiasis.

■ ASSOCIATED CONTENT

Supporting Information

General syntheses and characterization of compounds, assays on chitinase inhibition, molecular docking, bioaccumulation in *C. elegans*, L3 molting assay, mitochondrial uncoupling, and cell viability. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

O. volvulus, *Onchocerca volvulus*; OvCHT1, *Onchocerca volvulus* chitinase; L3, third larval stage; L4, fourth larval stage; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; *C. elegans*, *Caenorhabditis elegans*; LC, liquid chromatography; MS, mass spectrometry

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