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Antileishmanial Dinitroaniline Sulfonamides with Activity Against Parasite Tubulin

Gautam Bhattacharya, Manar M. Salem and Karl A. Werbovetz*

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210, USA

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Abstract—Novel dinitroaniline sulfonamides based on the herbicide oryzalin **3** were synthesized and evaluated for activity against the parasitic protozoan *Leishmania donovani* and against leishmanial tubulin, the putative antiparasitic target of oryzalin. A subset of these compounds possess more activity against both *Leishmania* and the target protein in vitro. Compound **20** displays improved potency against leishmanial tubulin and is 13.4-fold more active against *L. donovani* axenic amastigotes than oryzalin.

There is a general lack of effective, inexpensive chemotherapeutic agents for treating parasitic protozoal diseases that occur in the developing world. One such disease is leishmaniasis. It is estimated that approximately 1.5–2 million new cases of this disease occur each year due to infection by various *Leishmania* species.[1] Pentavalent antimonial drugs are the first line treatment for leishmaniasis in most affected areas, with amphotericin B and pentamidine being used as alternatives.[2] These agents must be administered by injection over several days to weeks, increasing the cost and inconvenience of the drugs. Resistance to antimonials has become a severe problem,[3] and treatment with amphotericin B and pentamidine is frequently complicated by the occurrence of toxic side effects.[2] Clearly, improved chemotherapeutics are needed against this disease.

Reports that the commercial herbicide trifluralin (**1**, Fig. 1), a dinitroaniline that binds to plant but not animal tubulin,[4] possessed selective antileishmanial activity were therefore cause for optimism. Trifluralin inhibited the proliferation of *Leishmania* amastigotes in macrophages, and radiolabeled trifluralin was shown to bind better to partially purified leishmanial tubulin than to rat brain tubulin.[5] Trifluralin has also been reported to possess activity in animal models of leishmaniasis.[6] Unfortunately, the story of trifluralin's antiparasitic potential has become more confusing with time. The synthetic precursor of trifluralin, chloralin (**2**, Fig. 1), is often present as an impurity in commercial trifluralin preparations, and chloralin was much more toxic to *Leishmania* in vitro than trifluralin.[7] We subsequently purified leishmanial tubulin in our own laboratory and found that chloralin, in addition to being a much more effective in vitro antileishmanial agent than trifluralin, was an inhibitor of leishmanial tubulin polymerization while trifluralin was not.[8] Other reports describing the effects of trifluralin and related compounds on protozoan parasites have appeared,[9,10] but the effects of these compounds on parasite tubulin were not examined. Trifluralin also presents serious technical problems due to its low solubility.[4] It is therefore difficult to determine whether **1** is a useful antileishmanial lead compound that targets protozoal tubulin from the studies described above.

Chan et al. have also described the antimitotic effects of the related dinitroaniline herbicide oryzalin (**3**, Fig. 1) on *Leishmania*.[11] Oryzalin, which contains a sulfonamide group in place of the trifluoromethyl functionality present in trifluralin, possesses approximately 10-fold greater aqueous solubility than trifluralin.[4] We obtained a commercial sample of oryzalin and found that it did indeed inhibit the assembly of purified leishmanial tubulin and was moderately toxic to *Leishmania* parasites in vitro (K. Werbovetz, unpublished results). On the basis of this positive result, we decided to employ oryzalin as a lead compound to explore the structure–

*Corresponding author. Tel.: +1-614-292-5499; fax: +1-614-292-2435; e-mail: werbovetz.1@osu.edu

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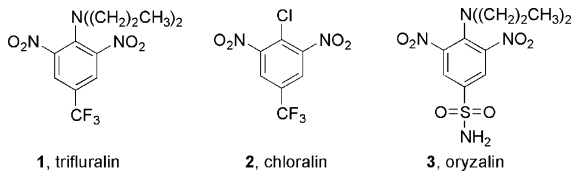


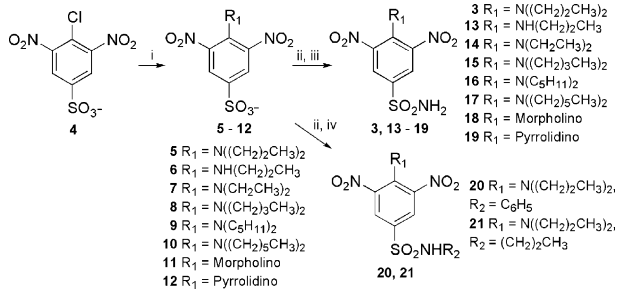
Figure 1. Structures of trifluralin, chloralin, and oryzalin.

activity relationship of dinitroanilines against leishmanial tubulin and against *Leishmania* parasites in vitro. By preparing oryzalin analogues with systematic functional group modifications, we hoped to address the following questions:

1. Do the dinitroanilines possess antileishmanial and antitubulin activity, or is the biological activity of these compounds due to the presence of electrophilic precursors?
2. Can non-electrophilic oryzalin analogues be synthesized that possess superior activity against the parasitic tubulin protein and/or cultured *Leishmania* parasites?
3. Does the in vitro antileishmanial activity of the analogues correlate with activity against parasite tubulin?

We thus synthesized oryzalin and 13 analogues that differ from **3** in one region of the molecule. Target sulfonamides **3** and **13**–**21** were prepared as shown in Scheme 1. The key intermediate 4-chloro-3,5-dinitrobenzenesulfonic acid **4**[12] was converted to amines **5**–**12** by heating **4** to reflux with the corresponding amine (for dipropyl **5**, propyl **6**, and diethylamine **7** derivatives) or by treating **4** with a methanol solution containing the desired amine (for dibutyl **8**, dipentyl (mixture of isomers) **9**, and dihexylamine **10** derivatives). The morpholino (**11**) and pyrrolidino (**12**) derivatives were prepared by stirring compound **4** in a methanolic solution containing either morpholine or pyrrolidine. Sulfonic acids **5**–**12** were then converted to the corresponding sulfonyl chloride derivatives (which were not isolated due to their unstable nature) by PCl_5/CH_2Cl_2 treatment. The sulfonyl chlorides were then transformed to sulfonamide derivatives by treatment with either ammonia in MeOH (**3** and **13**–**19**), aniline (**20**), or propylamine (**21**).

Mononitro derivative **25** was prepared as indicated in Scheme 2. 1-Chloro-2-nitrobenzene **22** was converted to **23** by heating at 120 °C in chlorosulfonic acid, which

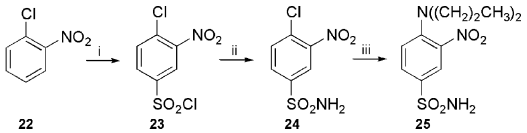


Scheme 1. Reagents and conditions: (i) R_1H/reflux, R_1H/MeOH, or R_1H/MeOH/reflux; (ii) PCl_5/CH_2Cl_2; (iii) NH_3/MeOH/50 °C; (iv) aniline or propylamine/reflux.

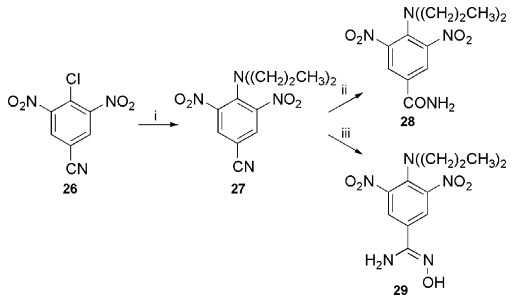
was then transformed to the sulfonamide **24** at −5 °C by reaction with ammonia in dioxane/ethyl acetate. Sulfonamide **24** was then converted to **25** by treatment with dipropylamine at reflux.

Cyano, amide and amidoxime compounds **27**–**29** were prepared according to Scheme 3. Commercially available 4-chloro-3,5-dinitrobenzonitrile **26** was converted to the corresponding dipropylamine derivative **27** by a modified Ullmann condensation using dipropylamine under reflux conditions to achieve nearly quantitative yield. Benzonitrile **27** was converted to amide **28** by hydrolysis with concd H_2SO_4 at 50 °C. The *Z*-amidoxime derivative **29** was prepared by heating **27** with $NH_2OH.HCl/Na_2CO_3$ in a solution of ethanol/water at reflux.[13] Target compounds **3**, **13**–**21**, **25**, and **27**–**29** were purified by silica gel column chromatography and crystallization when possible. All target molecules possessed satisfactory ^1H NMR and mass spectra and did not contain detectable traces of electrophilic precursors as assessed by TLC and ^1H NMR spectroscopy. A complete characterization including ^{13}C NMR and HRMS data is provided for compound **20**[14] and is also available on request for compounds **3**, **15**–**17**, and **21**.

With the desired compounds in hand, we then examined oryzalin and its analogues for their ability to block the growth of *Leishmania donovani* parasites and to inhibit the assembly of purified leishmanial tubulin by methods described previously.[15] Moderate antiparasitic and antitubulin activity was observed with **3**; mid-micromolar concentrations of this compound inhibited parasite growth and 20 μM concentrations of **3** inhibited the assembly of leishmanial tubulin by about 50% (Table 1). Compound **13**, in which the dipropyl substitution present at the N4 position of the sulfanilamide core of **3** is replaced by a monopropyl group, displays similar antiparasitic activity and potency against the target protein compared to **3**. When the alkyl chain length was shortened from three to two carbons in compound **14**, antiparasitic activity and potency against the target



Scheme 2. Reagents and conditions: (i) $ClSO_3H$/120 °C; (ii) NH_3 in dioxane/ethyl acetate/−5 °C; (iii) dipropylamine/reflux.



Scheme 3. Reagents and conditions: (i) Dipropylamine/reflux; (ii) concd H_2SO_4/50 °C; (iii) $NH_2OH.HCl/Na_2CO_3$/EtOH/reflux.

Table 1. Activity of oryzalin analogues against *L. donovani* and against leishmanial tubulin in vitro

Compd	IC_{50} versus *L. donovani* promastigotes (μM)[a]	IC_{50} versus *L. donovani* amastigotes (μM)[a]	% Inhibition of Leishmanial tubulin assembly at 20 μM compound[b]
Pentamidine	1.61±0.22	2.00±0.06	NT[c]
3	44.1±9.2	72.5±23.9	54±14
13	67.0±18.4	NT	43±19
14	69.3±23.0	NT	58±22
15	17.8±2.6	20.1±0.2	89±3
16	8.02±0.42	9.0±0.7	95±5
17	12.2±0.0	11.7±0.7	48±19
18	>100	NT	21±1
19	97.1±53.0	NT	57±18
20	14.7±1.0	5.41±0.89	108±7
21	56.0±2.3	56.9±3.4	97±1
25	89.8±15.3	NT	19±29
27	>100	NT	25±17
28	76.4±20.0	NT	34±12
29	>100	NT	35±7

[a]Mean ± standard deviation of at least two independent measurements.
[b]Mean ± standard deviation of at least one duplicate measurement. The standard deviation of the control samples in these experiments was 27.6%.
[c]NT - Not tested.

protein were again comparable with **3**. Interestingly, an increase in the number of carbons in the dialkylamine chain dramatically improved antileishmanial activity. Compounds **15**–**17** were 2.5- to 5.5-fold more active against the promastigote stage of the parasite and 3.6- to 8.1-fold more active against *L. donovani* axenic amastigotes than **3**, while **15** and **16** possess superior activity compared to **3** against the putative target protein. Restricting the conformation of the alkylamine chain, as in the morpholino analogue **18** and the pyrrolidino analogue **19**, decreased activity against *Leishmania* parasites. A difference between **18** and **19** was noted in antitubulin activity, as the pyrrolidino compound **19** was similar to oryzalin against tubulin but the morpholino compound **18** was inactive against the protein.

Another striking result was obtained with compounds **20** and **21**, both substituted at the N1 position of the sulfanilamide core, in that these two analogues are much more potent inhibitors of leishmanial tubulin assembly than **3**. Compound **20** is 3.0-fold more active against promastigotes and 13.4-fold more active against amastigotes than oryzalin **3**, approaching the in vitro antiparasitic activity of the clinical antileishmanial agent pentamidine against the axenic form of the parasite most closely related to that present in the mammalian host. Despite its improved activity against leishmanial tubulin, compound **21** is similar to **3** in antiparasitic activity. Other substitutions diminished the activity of the oryzalin analogues. Removal of a single nitro group, as in **25**, decreases the potency against cultured parasites and leishmanial tubulin. Replacement of the sulfonamide group with a cyano group, an amide functionality, or an amidoxime group as in **27**–**29** diminishes activity against *L. donovani* and leishmanial tubulin as well.

Our study confirms that the pure dinitroanilines possess antileishmanial and antitubulin activity. While other reports showed that several different dinitroaniline compounds possessed antiparasitic activity,[9,16] evidence linking dinitroanilines with tubulin inhibition in parasites was less clear. In addition to the trifluralin precursor chloralin **2**, other small aromatic electrophiles are known to inhibit tubulin assembly in general[17,18] and leishmanial tubulin assembly in particular,[15] raising the possibility that the antimicrotubule activity observed previously with dinitroanilines could have been due to electrophilic precursors. The activity observed here was clearly not due to the presence of electrophilic species.

It is also clear that several synthetic, non-electrophilic oryzalin analogues are superior to **3** in their activity against leishmanial tubulin and cultured *Leishmania* parasites. Compounds **15**–**17** and **20** are significantly more potent against parasites than lead compound **3**, with **20** being 13.4-fold more active than **3** against amastigote-stage *L. donovani*, and **15**, **16**, **20**, and **21** are more potent than **3** in blocking leishmanial tubulin assembly. Interestingly, while several of the compounds that possess activity similar to or lower than **3** are known molecules, to our knowledge **15**–**17**, **20**, and **21** have not previously been reported. These results justify the continued investigation of the dinitroanilines as lead compounds for antileishmanial drug discovery. In addition, these data are consistent with the hypothesis that tubulin is the target of the dinitroanilines in *Leishmania*. Although an exact correlation was not observed between antiparasitic activity and the inhibition of leishmanial tubulin assembly and further experiments are required to verify that tubulin is indeed the target of these new molecules, all of the compounds that are superior to oryzalin in antiparasitic activity are at least as potent as **3** in blocking parasite tubulin assembly in vitro. Thus inhibition of leishmanial tubulin polymerization appears to be a necessary but not sufficient property for members of this class of compounds to possess strong antileishmanial activity. An important factor that may help explain the lack of a direct correlation between antitubulin and antileishmanial activity of these compounds is penetration of the molecules into the parasite. Compounds **15**–**17** have more carbons in their alkyl chains than **3** and would thus be expected to be more hydrophobic than the parent compound, facilitating passage of compounds **15**–**17** across the parasite

cell membrane. The difference in activity between **20** and **21** is harder to rationalize. Although both compounds are more potent inhibitors of leishmanial tubulin assembly than oryzalin, N1-phenyl sulfanilamide **20** is much more effective than **3** at blocking parasite growth, while N1-alkyl sulfanilamide **21** and **3** possess similar antiparasitic activity. Poor accumulation of **21** within the parasite may also explain these data, but further experiments are required to adequately address this issue.

The results presented here provide motivation for further exploration of dinitroaniline sulfonamides as antileishmanial agents in particular and continued study of tubulin as an antiprotozoal drug target in general. Synthesis of new oryzalin analogues based on the structure–activity relationship outlined in this report will almost certainly yield new compounds with improved antileishmanial and antitubulin activity, and additional biological testing will hopefully provide new drug candidates against leishmaniasis and perhaps other parasitic diseases. A critical issue pertaining to the development of these agents as antiparasitic drug candidates is the selectivity of their antimicrotubule activity. Oryzalin binds more avidly to plant tubulin than to mammalian tubulin.[19] Although a single filter binding experiment suggests that the related dinitroaniline trifluralin binds more strongly to leishmanial tubulin than to mammalian tubulin,[5] the determination of dissociation constants between oryzalin and both leishmanial and mammalian tubulin would be very helpful in quantifying selectivity. It will also be vital to quantitate the selectivity for the new compounds reported here that possess superior antiparasitic and antitubulin activity compared to oryzalin. Another issue to be addressed is the potential host toxicity of these compounds due to the presence of aromatic nitro groups.[20] The main point of this work, however, is that we have significantly improved on the activity of oryzalin against *Leishmania* and the leishmanial tubulin target protein by making simple modifications to the lead compound. It is our hope that future compounds, perhaps those like compound **20** that are substituted at the sulfonamide nitrogen, will retain antitubulin and antiparasitic activity in the absence of aromatic nitro groups. By employing a wide range of drug discovery tools in the future, including synthetic medicinal chemistry, biochemical assays and computer modeling of the target protein, evaluation of new compounds against parasites and mammalian cells, and studies in animal models of parasitic disease, we are optimistic that the further study of leishmanial tubulin will lead to the development of selective antiparasitic drug candidates in the future.

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14. ***N*1-phenyl-3,5-dinitro-*N*4,*N*4-di-*n*-propylsulfonilamide (20)**. Melting point: 116 °C ^{1}H NMR (DMSO-d_6, 250 MHz) δ 0.78 (6H, t, J=7.3 Hz, CH_3), 1.47 (4H, m, CH_2), 2.91 (4H, t, J=7.2 Hz, NCH_2), 7.10 (3H, m, ArH), 7.27 (2H, m, ArH), 8.28 (2H, d, J=0.9 Hz, ArH), 10.48 (1H, s, NH). ^{13}C NMR (DMSO-d_6, 62.5 MHz) δ 11.15, 20.62, 53.59, 121.19, 125.38, 128.56, 129.80, 130.31, 137.03, 141.03, 144.61. HRMS (ES^+): m/z calcd for $C_{18}H_{22}N_4O_6SNa$ $(M+Na)^+$: 445.1158, found: 445.1164.
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