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Synthesis and evaluation of oryzalin analogs against *Toxoplasma gondii*

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ABSTRACT

The synthesis and evaluation of 20 dinitroanilines and related compounds against the obligate intracellular parasite *Toxoplasma gondii* is reported. Using in vitro cultures of parasites in human fibroblasts, we determined that most of these compounds selectively disrupted *Toxoplasma* microtubules, and several displayed sub-micromolar potency against the parasite. The most potent compound was N^1,N^1-dipropyl-2,6-dinitro-4-(trifluoromethyl)-1,3-benzenediamine (**18b**), which displayed an IC_{50} value of 36 nM against intracellular *T. gondii*. Based on these data and another recent report [Ma, C.; Tran, J.; Gu, F.; Ochoa, R.; Li, C.; Sept, D.; Werbovetz, K.; Morrissette, N. *Antimicrob. Agents Chemother.* **2010**, *54*, 1453], an antimitotic structure–activity relationship for dinitroanilines versus *Toxoplasma* is presented.

Infection by the apicomplexan parasite *Toxoplasma gondii* can result in miscarriage, birth defects in infants, vision disturbances in immunocompetent hosts, and toxoplasmic encephalitis in the immunocompromised.[1] Acute toxoplasmosis in otherwise healthy individuals is typically treated with the antifolate combination of pyrimethamine and sulfadiazine together with folinic acid.[1] Pyrimethamine has potential side effects, including bone marrow suppression[2] and teratogenicity.[3,4] Resistance to pyrimethamine in malaria chemotherapy can occur through mutations in the dihydrofolate reductase target protein,[5] although no evidence for clinical resistance to pyrimethamine in *Toxoplasma* exists to date.[6] Sulfadiazine use is also complicated by the occurrence of kidney stones,[7] allergic reactions,[8] and the development of resistance.[6] Other alternatives to pyrimethamine–sulfadiazine treatment of toxoplasmosis include clindamycin, atovaquone, and spiramycin,[1] but these drugs each possess their own limitations.[9–11] An ideal drug against human toxoplasmosis would kill the different stages of *T. gondii*, be well distributed in the main sites of infection, including the brain and eyes, and lack the potential for teratogenicity.

Tubulin, which assembles into microtubules, is an essential protein for formation of the mitotic spindle. It continues to be a prime target for established and investigational anticancer agents[12,13] and is also believed to be the molecular target of anthelmintic benzimidazoles.[14] Dinitroanilines are tubulin-binding herbicides which display microtubule selective toxicity for many different classes of protozoan parasites, including *Leishmania*, *Trypanosoma*, *Plasmodium*, and *Toxoplasma*. We have previously characterized the sensitivity of *Toxoplasma* to dinitroanilines and have found that IC_{50} values for commercially available dinitroanilines range from 45 nM to 6.7 μM.[15–17] *Toxoplasma* parasites only replicate inside of host cells, and extracellular parasites do not have dynamic microtubules. Intracellular *Toxoplasma* parasites are sensitive to disruption by dinitroanilines: dinitroaniline-treated parasites lack all microtubules and cannot carry out microtubule-dependent functions including mitosis and cytokinesis.[15,18,19] Following lysis of the original host cell, round, non-polar dinitroaniline-treated parasites are unable to invade new host cells and rapidly die. Thus, dinitroanilines could serve as excellent leads for the discovery of new drugs against toxoplasmosis since they disrupt protozoan parasite microtubules at nanomolar concentrations while showing little or no effect on host cell microtubules.[16,20–23] Computational methods have been used to identify a binding site for the dinitroanilines on parasite tubulin.[16,24] These studies predict that protofilament contacts in the microtubule lattice are disrupted when dinitroanilines selectively bind to protozoan α-tubulin beneath the H1-S2 loop. Analysis of vertebrate α-tubulin through computational methods indicates that oryzalin has non-specific, low affinity interactions with this protein and no consensus binding site, consistent with in vivo and in vitro observations that dinitroanilines do not bind to vertebrate tubulin or disrupt vertebrate microtubules.[20,25–28]

Previous work in our laboratories has shown that structural modification of the commercial dinitroaniline oryzalin leads to increased potency against kinetoplastid parasites.[23,29,30] However, a detailed structure–activity study of dinitroanilines against

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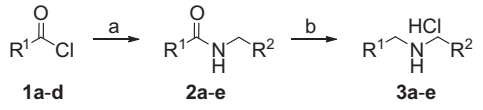
Toxoplasma has not been reported. We were therefore motivated to synthesize analogs of oryzalin (**6i**) and other dinitroaniline herbicides such as trifluralin (**14a**) and dinitramine (**18a**) for testing against *T. gondii*. In the present manuscript, we describe the synthesis and biological activity of such derivatives.

To synthesize several of the desired target compounds, unsymmetrical secondary amines were needed. The required secondary amine salts were synthesized using the method reported previously.[29,31] Hence, the standard coupling of the acid chlorides **1a**–**d** with amines gave the respective amides **2a**–**e**, followed by reduction with borane–THF solution, treatment with methanol, protection with Boc, and deprotection using HCl in dioxane to afford the secondary amine salts **3a**–**e** in good yields (see Scheme 1).



1a-d **2a-e** **3a-e**

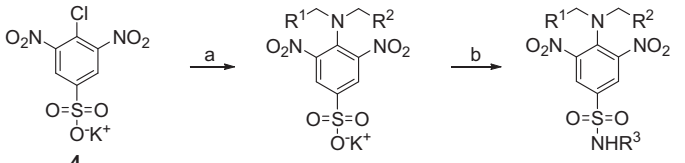
1a: R^1 = Ph
1b: R^1 = 4-Tolyl
1c: R^1 = *c*Hexane
1d: R^1 = CH_2Ph

2a, **3a**: R^1 = Ph, R^2 = CH_2CH_2OH
2b, **3b**: R^1 = Ph, R^2 = *c*Pr
2c, **3c**: R^1 = R^2 = 4-Tolyl
2d, **3d**: R^1 = R^2 = *c*Hexane
2e, **3e**: R^1 = R^2 = CH_2Ph

Scheme 1. Reagents and conditions: (a) RNH_2, Et_3N, CH_2Cl_2, 0 °C to rt, overnight; (b) (i) BH_3–THF (3 equiv), reflux, overnight; (ii) MeOH, reflux, 5 h; (iii) Boc_2O (1.5 equiv) in CH_2Cl_2, rt, overnight; (iv) 4 M HCl in 1,4-dioxane (1.3 equiv), CH_2Cl_2, rt, overnight.

Dinitroaniline sulfonamide target compounds were prepared as shown in Scheme 2. The desired amines reacted with the potassium salt of 4-chloro-3,5-dinitrobenzenesulfonate **4** in the presence of triethylamine to give N^4,N^4-disubstituted-3,5-dinitrobenzenesulfonates **5a**–**e**, **5f**–**h**,[31] and **5i**–**j**[30,31] in good yields. These sulfonates contained traces of the secondary amine salts as determined from their ^1H NMR spectra, but these salts did not interfere with further reactions. Sulfonyl chlorides were synthesized from benzenesulfonates **5a**–**j** using PCl_5 in dichloromethane. After brief work up, the resulting sulfonyl chlorides were treated with a methanolic solution of NH_3 or the desired amines in dry THF to afford the target compounds **6a**–**h**, **6i**–**j**,[29] **6k**–**l**, and **6m**–**n**[30] in good to excellent yields. Mono-N^4-alkylated and free N^4-amino sulfonamides were isolated as bi-products, especially for bulky N^4,N^4-disubstituted analogs such as **6a–g**.

The synthesis of **12**, where the nitro moieties present in **6i** were replaced with nitrile groups, was accomplished according to Scheme 3. The sulfonyl chloride corresponding to sulfonate **7**[32] was prepared in situ from **7** using PCl_5/$POCl_3$ in dry chlorobenzene; treatment of this sulfonyl chloride with methanolic NH_3 afforded **8**. An Ullmann reaction[33] between sulfonamide **8** and dipropylamine in the presence of potassium carbonate and catalytic copper gave

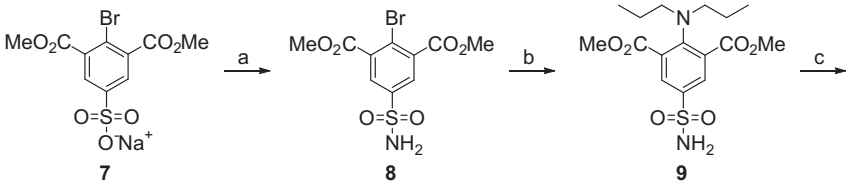


4 **5a-j** **6a-n**

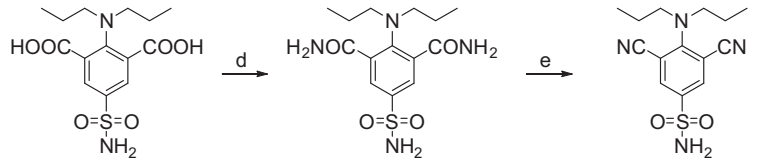
5a: R^1 = Ph, R^2 = CH_2OH
5b: R^1 = Ph, R^2 = $(CH_2)_2OH$
5c: R^1 = R^2 = 4-Tolyl
5d: R^1 = R^2 = *c*Hexane
5e: R^1 = R^2 = CH_2Ph
5f: R^1 = R^2 = Ph
5g: R^1 = Ph, R^2 = *c*Pr
5h: R^1 = R^2 = $CH(CH_3)_2$
5i: R^1 = R^2 = CH_2CH_3
5j: R^1 = R^2 = $(CH_2)_2CH_3$

6a: R^1 = Ph, R^2 = CH_2Cl, R^3 = H
6b: R^1 = Ph, R^2 = $(CH_2)_2Cl$, R^3 = H
6c: R^1 = R^2 = 4-Tolyl, R^3 = H
6d: R^1 = R^2 = *c*Hexane, R^3 = H
6e: R^1 = R^2 = CH_2Ph, R^3 = H
6f: R^1 = R^2 = Ph, R^3 = H
6g: R^1 = Ph, R^2 = *c*Pr, R^3 = H
6h: R^1 = R^2 = $CH(CH_3)_2$, R^3 = H
6i: R^1 = R^2 = CH_2CH_3, R^3 = H
6j: R^1 = R^2 = $(CH_2)_2CH_3$, R^3 = H
6k: R^1 = R^2 = CH_2CH_3, R^3 = $(CH_2)_2OH$
6l: R^1 = R^2 = CH_2CH_3, R^3 = 4-phenol
6m: R^1 = R^2 = CH_2CH_3, R^3 = Ph
6n: R^1 = R^2 = CH_2CH_3, NHR^3 = $N(CH_3)_2$

Scheme 2. Reagents and conditions: (a) secondary amine, reflux, or secondary amine hydrochloride, Et_3N, MeOH, reflux, 4 h; (b) (i) PCl_5, CH_2Cl_2, rt, 3 h; (ii) NH_3 (7 N in MeOH), or amine, Et_3N, THF, 0 °C to rt, 2 h.



7 **8** **9**



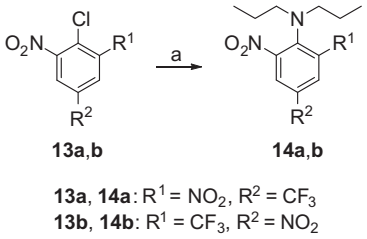
10 **11** **12**

Scheme 3. Reagents and conditions: (a) (i) PCl_5/$POCl_3$, chlorobenzene, reflux, 24; (ii) NH_3 (0.5 M in 1,4-dioxane), 0 °C, 1 h, rt, 1 h; (b) dipropylamine, K_2CO_3, Cu, dioxane, reflux, 5 h; (c) 3 N NaOH, MeOH, reflux, 2 days; (d) (i) CCl_3COCCl_3, PPh_3, THF, 0 °C, 1 h; (ii) NH_3/dioxane, rt, 1 h; (e) $PdCl_2$, CH_3CN/H_2O, 24 h.

9. Hydrolysis of this ester using aqueous NaOH in methanol afforded **10**. Treatment of dicarboxylate **10** with hexachloroacetone and PPh_3 in THF followed by the addition of NH_3 in 1,4-dioxane provided bis-amide **11**. Dehydration of **11** using $PdCl_2$ in aqueous acetonitrile[34] afforded the target compound **12** in excellent and reproducible yield.

Trifluralin (**14a**) and its regioisomer **14b**[35] were prepared according to Scheme 4. Heating **13a** and **13b** to reflux in dipropylamine gave target compounds **14a** and **14b**, respectively, in excellent yield.

Dinitramine (**18a**) and its analogs were synthesized as shown in Scheme 5. 2,4-Dichloro-3,5-dinitrobenzotrifluoride (**16**) was prepared by nitration of 2,4-dichlorobenzotrifluoride (**15**) using fuming nitric acid in the presence of fuming sulfuric acid.[36]



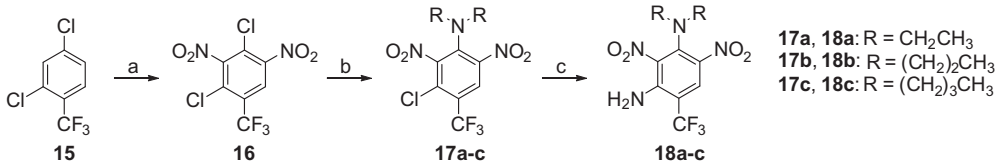
Scheme 4. Reagent and condition: (a) dipropylamine, reflux, overnight.

Dinitrobenzotrifluoride **16** was treated with 2.2 equiv of each of the desired secondary amines and heated in a sealed flask at 100 °C.[36,37] After a brief workup, the crude products **17a–c** were dissolved in 3 equiv of ammonia in dioxane and heated once again in a sealed flask at 100 °C, which gave the target compounds **18a–c** in 60–80% yield for two steps. The use of excess amine and sealed flask conditions were critical for the success of the latter two reactions.

Structures for the proposed target compounds were confirmed with the aid of ^1H and ^{13}C NMR spectroscopy and mass spectrometry analysis, while the purity of the compounds was verified by elemental analysis. Synthetic procedures and spectral data for representative target compounds **6h**, **12**, and **18b** are provided in Supplementary data.

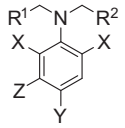
The growth inhibitory activity of compounds of interest against *T. gondii* in vitro was measured by a plaque assay,[17] with the results shown in Table 1. The IC_{50} values for **6i**, **14a**, and **18a** are consistent with those obtained previously in this assay.[17] *Toxoplasma* microtubules were examined by an in vitro immunofluorescence assay (Fig. 1). Figure 1A and B shows the effect of a selective antimitotic compound (**18b**), while Figure 1D and E illustrates the effect of a non-selective agent (**6e**). Experimental methods for both the plaque assay and the immunofluorescence assay are available in Supplementary data.

Among the compounds lacking a *meta*-amino group (**6a–n**, **12**, **14a**, and **14b**), the dinitroaniline oryzalin (**6i**) was the most effec-



Scheme 5. Reagents and conditions: (a) fuming sulfuric acid containing 30–33% SO_3/fuming 90% nitric acid; (b) secondary amine, absolute ethanol, 100 °C, 72 h; (c) NH_3 in 1,4-dioxane, 100 °C, 40 h.

Table 1
Activity of target compounds against *Toxoplasma gondii* in vitro



Compound	R_1	R_2	X	Y	Z	IC_{50} (μM) versus *Toxoplasma gondii*[a]
6a	Ph	CH_2Cl	NO_2	SO_2NH_2	H	0.85 ± 0.03
6b	Ph	$(CH_2)_2Cl$	NO_2	SO_2NH_2	H	2.6 ± 0.1
6c	4-Tolyl	4-Tolyl	NO_2	SO_2NH_2	H	Non-specific
6d	*c*Hexyl	*c*Hexyl	NO_2	SO_2NH_2	H	Non-specific
6e	CH_2Ph	CH_2Ph	NO_2	SO_2NH_2	H	Non-specific
6f	Ph	Ph	NO_2	SO_2NH_2	H	Non-specific
6g	Ph	*c*Pr	NO_2	SO_2NH_2	H	2.1 ± 0.1
6h	$CH(CH_3)_2$	$CH(CH_3)_2$	NO_2	SO_2NH_2	H	0.35 ± 0.02
6i	CH_2CH_3	CH_2CH_3	NO_2	SO_2NH_2	H	0.25 ± 0.01
6j	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	NO_2	SO_2NH_2	H	1.1 ± 0.2
6k	CH_2CH_3	CH_2CH_3	NO_2	$SO_2NH(CH_2)_2OH$	H	26 ± 2
6l	CH_2CH_3	CH_2CH_3	NO_2	SO_2NH(4-phenol)	H	2.6 ± 0.3
6m	CH_2CH_3	CH_2CH_3	NO_2	SO_2NHPh	H	6.7 ± 0.7
6n	CH_2CH_3	CH_2CH_3	NO_2	$SO_2N(CH_3)_2$	H	3.4 ± 0.2
12	CH_2CH_3	CH_2CH_3	CN	SO_2NH_2	H	4.8 ± 0.2
14a	CH_2CH_3	CH_2CH_3	NO_2	CF_3	H	0.65 ± 0.06
14b	CH_2CH_3	CH_2CH_3	NO_2, CF_3	NO_2	H	7.8 ± 1.0
18a	CH_3	CH_3	NO_2	CF_3	NH_2	0.070 ± 0.004
18b	CH_2CH_3	CH_2CH_3	NO_2	CF_3	NH_2	0.036 ± 0.002
18c	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	NO_2	CF_3	NH_2	0.51 ± 0.02

[a] Mean ± standard deviation of three independent measurements, experimental methods are given in Supplementary data.

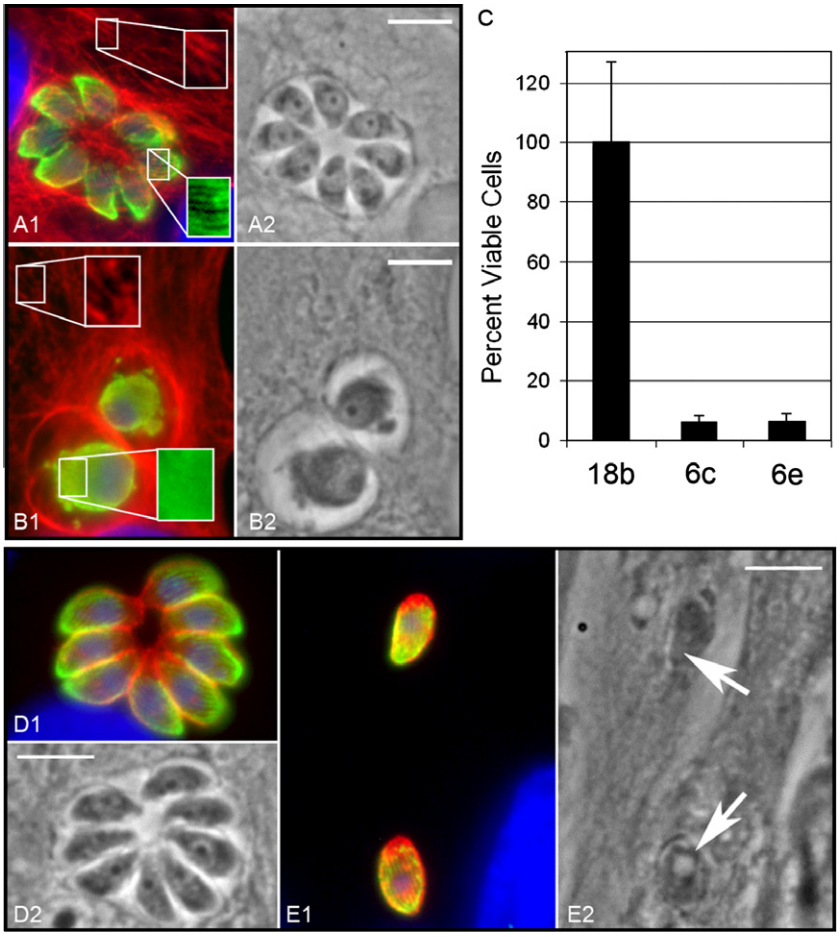


Figure 1. (A and B) Compound **18b** selectively disrupts microtubules in intracellular *Toxoplasma gondii* but not host vertebrate cells. (A1) Immunofluorescence of DMSO control treated parasite cultures illustrates that vehicle alone does not disrupt either vertebrate host cell (red) or *Toxoplasma* parasite (green) microtubules. Red: host cell microtubules retain a filamentous pattern, which can be seen more clearly in the boxed enlargement. Green: parasite subpellicular microtubules are present in a typical linear array. Blue: DAPI staining of host cell and parasite DNA. (A2) A phase contrast image of the same field; the scale bar represents 5 μm. (B1) Immunofluorescence of parasite cultures treated for 28 h with 0.5 μM **18b** in DMSO show that parasite but not host cells are disrupted. Red: host cell microtubules retain a filamentous pattern, which can be seen more clearly in the boxed enlargement. Green: parasite subpellicular microtubules are disrupted and free tubulin dimer staining is diffuse rather than organized into linear arrays. Blue: host cell and parasite DNA. (B2) A matched phase contrast image; the scale bar is 5 μm. (C) An MTS viability assay using the *CellTiter* reagent (Promega) illustrates that **6c** and **6e** are toxic to host cells at 30 μM, while 0.5 μM **18b** (~10-fold greater than the antiparasitic IC_{50} value for this compound) does not have significant toxicity. (D and E) Compound **6e** inhibits replication of intracellular *Toxoplasma gondii* but does not disrupt parasite microtubules, indicating that the compound interacts with target(s) other than tubulin. (D1) Immunofluorescence of a control culture: the rosette of eight daughter parasites indicates that the initial intracellular parasite has undergone three cycles of replication 28 h after infection. Red: A parasite-specific surface antigen, P30/SAG1, labels the entire parasite surface. Green: parasite subpellicular microtubules are present in a typical linear array. Blue: host cell and parasite DNA. (D2) A matched phase contrast image; the scale bar is 5 μm. (E1) Immunofluorescence of a culture treated with **6e** showing two single intracellular parasites which have not replicated 28 h post infection. This culture was set up at the same time as the control culture, and parasite vacuoles should contain 4–16 parasites by 28 h. Note the intact microtubules in the parasites. Fluorochromes are the same as in the control sample. (E2) A matched phase contrast image; the scale bar is 5 μm. Full experimental methods are given in Supplementary data.

tive against *Toxoplasma* in this study, possessing an IC_{50} value of 0.25 μM. In other studies examining the efficacy of commercially available dinitroanilines, **6i** was one of the most efficacious compounds to inhibit *Toxoplasma* growth as assessed by plaque assays.[17] The other potent analogs of oryzalin are compounds **6h**, **6a**, and **6j**, with IC_{50} values ranging from 0.35 to 1.1 μM. These results indicate that alkyl chains of three or four carbons in length at the aniline nitrogen of the dinitroanilines confer strong activity against intracellular *T. gondii*. Compound **6g**, which possesses one four-carbon group and one benzyl group at the aniline nitrogen atom, is 8.5-fold less active than dipropyl derivative **6i** and twofold less active than dibutyl congener **6j**. Interestingly, compounds with two large substitutions at the aniline nitrogen (**6c**–**f**) do not appear to be tubulin specific inhibitors in *Toxoplasma*. These compounds inhibit parasite invasion, replication and growth at 10–25 μM without shortening the microtubules in the organism (Fig. 1D and E, treatment with 30 μM **6e** shown) and display host HFF cell toxicity at ~30 μM. We also used a tetrazolium dye assay (Fig. 1C) to quantify viable host HFF cells after 17 h of treatment with 30 μM of two of the non-specific compounds (**6c** and **6e**) and with a parasite-specific compound (0.5 μM **18b**). Compounds **6c** and **6e** cause host cell vacuolation and dramatically decrease the number of viable host cells. Although **6c** and **6e** inhibit parasite replication, parasite microtubules are not disrupted, suggesting that they have a distinct target. In contrast, the specific compound **18b** does not compromise host cell viability at a concentration that selectively disrupts parasite microtubules.

Substitutions on the sulfonamide nitrogen decrease activity compared to **6i**. Compounds with 4-hydroxyphenyl (**6l**), phenyl (**6m**), dimethyl (**6n**), or 2-hydroxyethyl (**6k**) substituents at the sulfonamide nitrogen are 10- to 100-fold less active than **6i**. Interestingly, replacement of the nitro groups with nitrile moieties (**12**) results in a 19-fold drop in potency compared to **6i**. This is in contrast to the structure–activity relationship observed in kineto-

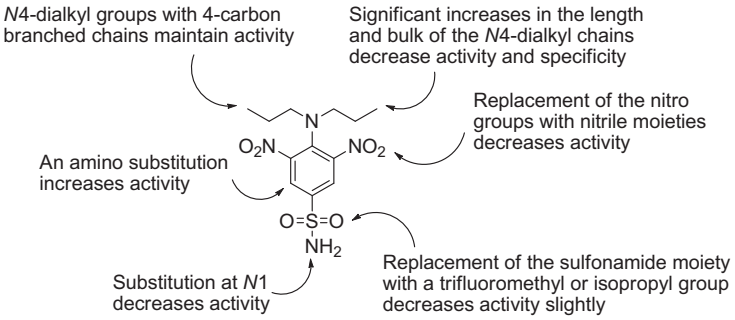


Figure 2. Activity map for dinitroaniline analogs against *Toxoplasma* microtubules.

plastid parasites, where exchanging nitrile groups for the nitro moieties present in the potent antikinetoplastid compound **6m** results in only a 1.5- to 2-fold loss of potency.[32]

Trifluralin (**14a**) is 2.6-fold less potent than **6i**, indicating that the sulfonamide group conveys greater potency against *Toxoplasma* than the trifluoromethyl group. A 12-fold decrease in potency compared to **14a** is observed for **14b**, a compound where the nitro and trifluoromethyl groups of **14a** are interchanged. The addition of the *meta*-amino group (compounds **18a–c**), however, causes a dramatic increase in activity compared to **14a**. Ma et al. recently reported that the *meta*-amino group present in **18a** participates in two critical hydrogen bonds with the α-tubulin target, significantly increasing the activity of this compound compared to both **14a** and **6i**.[17] Our present study confirms this result for **18a** and also shows that the dipropylamino group found in **18b** imparts twofold greater potency against *Toxoplasma* compared to the *N*,*N*-diethyl substitution present in **18a** and 14-fold greater potency against this parasite compared to the *N*,*N*-di-*n*-butyl substitution occurring in **18c**. The selective antimitotic action of **18b** is illustrated in Figure 1A and B. Figure 1A1 shows well-defined microtubules present in both host cells and in *Toxoplasma* in the absence of compound treatment. In the presence of 0.5 μM **18b**, host cell microtubules remain intact, while the diffuse staining of parasite tubulin reveals microtubule disruption in the parasites (Fig. 1B1). Figure 1B also indicates that parasites exposed to 0.5 μM **18b** are unable to replicate.

The results shown here, together with the data presented in Ma et al.[17] concerning the evaluation of commercial dinitroanilines against *T. gondii*, permits us to develop an antimitotic structure–activity relationship for dinitroanilines versus *Toxoplasma*. Isopropalin, a compound identical in structure to **6i** and **14a** except that it contains an isopropyl group *para* to the alkylamino position, reinforces the notion that sulfonamide-containing compounds are slightly superior to congeners with other substitutions at this position. As with compounds **6h** and **6j**, inclusion of a branched chain four carbon alkyl group in ethalfluralin leads to better activity than with benfluralin, which possesses an *n*-butyl group at the anilino nitrogen. The *meta*-amino derivatives **18a** and **18b** are 3.6- to 6.9-fold more potent than **6i** against *Toxoplasma* in vitro, and studies with **18a**–**c** suggest that the optimal chain length of the substitution on the aniline nitrogen is three carbons. These structure–activity data are summarized in Figure 2. Future efforts to optimize the anti-*Toxoplasma* potency and drug like properties of the dinitroanilines will focus on *meta*-amino containing derivatives **18a** and **18b** as lead compounds.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.003.

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