
Synthesis, Structural Determination, and Pharmacology of Putative Dinitroaniline Antimalarials

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Synthesis, Structural Determination, and Pharmacology of Putative Dinitroaniline Antimalarials


Abstract: A series of novel, homologous compounds possessing the general formula $N^1-N^{n}$-bis(2,6-dinitro-4-trifluoromethylphenyl)-1,n-diamino alkanes (where $n = 4, 6, 10$ or 12), were designed to probe inter- and intra- binding site dimensions in malarial parasite (Plasmodium) tubulin. Various crystal structures, including chloralin and trifluralin, an isopropyl dimer, and 2,6-dinitro-4-trifluoromethylphenylamine, were determined. Dinitroanilines, when soluble, were evaluated both in culture and in vivo. Trifluralin was up to 2-fold more active than chloralin against cultured parasites. The isopropyl dimer was water soluble (>5 mM) and revealed activity superior to that of chloralin in culture. The effects of selected dinitroanilines upon the mitotic microtubular structures of Plasmodium, the putative target of these dinitroanilines, were also determined. Electronic properties of the molecules were calculated using DFT (HF/6-31+G* level) to ascertain whether incorporation of such a pharmacophore could allow both QSAR and rational development of more selectively toxic antiparasitic agents.

Introduction

Malaria is a parasitic infection transmitted to humans (and other animals) by female Anopheles mosquitoes when taking a blood meal.[1] The increase in drug resistant forms of Plasmodium, especially of the P. falciparum species,[2] continues to threaten tropical and sub-tropical regions of the world and with climate change, infections could spread to other, currently malaria free zones.

The most recent guidelines for the treatment of uncomplicated malarial infections suggest various antimalarial combination therapies which include: arteether and lumefantrine, artesunate and (amodiaquine or mefloquine); dihydroartemisinin + piperaquine or a triple combination therapy of artesunate + sulfadoxine-pyrimethamine. [3] However, adverse side effects of certain antimalarial drugs in current use, [4] and the appearance and propagation of drug resistant strains of Plasmodium necessitate the discovery and development of new and less toxic chemotherapeutic agents with novel modes of action.[5]

A wide variety of endogenous compounds and xenobiotics produce directly (or indirectly) reactive oxygen species (ROS) that can induce oxidative stress in both the infected host and parasite. Often ROS are generated by electron transfer (ET) or other routes mediated by free radicals. Principal ET functionalities are quinones (or their precursors), conjugated imines, metal complexes and aromatic nitro compounds (ArNO2). Certain dinitroaniline herbicides, [6] (Figure 1) and their analogues [7] which are photo-labile[8] also exhibit activity against Leishmania, a disease endemic to tropical and sub-tropical regions, [9] both in culture and in vivo.[9, 10] These compounds have also exhibited activity against a range of parasites commonly infecting humans and domesticated animals, including Plasmodium,[7] Cryptosporidium,[11] Leishmania,[12, 13] Entamoeba[14] and Toxoplasma.[15] One such nitroaromatic compound, trifluralin (Figure 1), [10] a widely used commercial pre-emergent herbicide, was initially considered the active agent against leishmaniasis. [10] However, subsequent investigations implicated its industrial precursor, chloralin as the true active molecule.[11, 17] Chloralin 5 was found to be a contaminant of commercial samples of trifluralin, confounding ascription of bioactivity within the dinitroaniline class of compounds. Since 5 is one hundred times more potent than 1 against Leishmania promastigotes and targets parasite tubulin,[10] it has attracted significant attention from parasitologists. Reversible polymerisation of tubulin facilitates organelle transport, chromosome segregation, and maintains cell structure integrity. [10a, 19] Although, tubulins are well-conserved protein families, slight sequence differences between parasite and human tubulins can drastically affect the activity of certain microtubule inhibitors making them candidate drug targets.[18c, 21] In the case of benzimidazole antihelminitics, this differential toxicity has been exploited in chemotherapy. [22] The inhibitory activity of 5 has been associated with displacement of its reactive C-4 chloro moiety by cysteine residues present in Leishmania tubulin, thereby inhibiting microtubule assembly. [17b]

Dinitroanilines appear to inhibit distinct parts of the developmental cycles of protozoal parasites. For example oryzalin 3 irreversibly arrested trophozoite mitosis in E. histolytica. [13] In Toxoplasma gondii, dinitroanilines appear to block nuclear division by inhibition of intra-nuclear spindle formation, but other cytoskeletal components were also differentially affected by the drugs tested. [16] Isolation of oryzalin-resistant Toxoplasma gondii
clones shows that resistance can be induced by point mutations, in α-tubulin. \[23\] Since both structural and electronic features dictate pharmacodynamic (drug-receptor interactions), this information was sought through X-ray diffraction of selected dinitroanilines. We also report the preparation and activity of a novel trifluralin dimer active against \textit{P. falciparum} in culture.

Results and Discussion

Synthetic studies and compound selection procedure

Several compounds were chosen from our drug bank for initial, compounds 1 and 2 (Figure 1) are lethal strains of \textit{P. berghei} in mice and chloroquine-sensitive and -resistant strains of \textit{P. falciparum} in culture. Selected substances were not compromised by poor physical properties \[28\] in order to facilitate testing in culture. Experiments in vivo were not restricted by aqueous solubility, because the subcutaneous (s.c.) route was used for injections formulated in dimethyl sulfoxide (DMSO) and olive oil.

Mindful that the industrial precursor of 1 has been proposed to be the active antiparasitic agent, we included in this study an isomer of 5, isochloralin and the structurally related 8. We hypothesised that evaluation of the antimalarial activity of this potentially iso-lipophilic isomer of 5 would indicate if steric congestion, around the labile halide group, depresses the previously postulated attack by thiols presumed present within the target receptor. Structure-activity relationship analyses of 5 implicates the displaceable chloride moiety activated by a single nitro group as the pharmacophore; these contributed to selecting compound 7 for antimalarial tests. Hence, a judicious selection and evaluation of compounds depicted in Figure 1 could reveal a functional antimalarial pharmacophore for dinitroanilines.

The initial aim was to estimate the average \textit{inter- or intra-}

binding site dimensions in tubulin polymers using a homologation strategy. Consequently, a series of dimers (compounds 9 - 13, Figure 1) were designed for preliminary evaluation in culture.

Studies of the effects of dinitroaniline compounds against malarial parasites have shown that they are selectively toxic to parasite rather than host cells but lack the potency required for further development as antimalarial agents. \[10a,24\] The study of Kaidoh et al. \[24\] showed by electron microscopy that micromolar concentrations of 1 caused fragmentation and increased microtubule \textit{bifurcation} and 

dissolution of the sub-pellicular microtubule complex in \textit{P. falciparum} gametocytes. Fennell et al. \[25\] using asexual blood-staged parasites demonstrated that 1 and 3 inhibited progression through schizontogenesis, blocked mitotic division, and caused accumulation of abnormal microtubular structures. Moreover, radiolabelled 1 interacted with purified, recombinant parasite tubulins but to a much lesser extent with bovine tubulins. 3 was also an inhibitor of liver-stage schizogony. \[26\]

Identification of parasite tubulin as a novel target in \textit{Leishmania} \[17a\] suggested to us that low cost dinitroaniline herbicides, such as 1, merited further evaluation and development as potential antimalarial agents. This study consisted of the syntheses, purification and quantum mechanical study and pharmacological evaluation of selected dinitroaniline analogues against \textit{Plasmodium} in culture and \textit{in vivo}. The presence of fluorine in these compounds imparts desirable pharmacological properties and delays premature metabolism. \[27\]

X-ray Crystallography

The structures of 1, 5, 8, 9 and 14 are given in Figure 2, and structural details have been deposited with the Cambridge Crystallographic Data Centre (reference numbers CCDC 2193522 - 2193556). Crystal data and refinement details are shown in Table S1. In the structure of 1, the nitrogen atom has a trigonal environment with the three subtended angles at nitrogen adding up to 359.6°. The plane of C(4), N(7), C(71), C(81) intersects

Figure 1. Chemical structures and numbering. Trifluralin 1, pendimethalin 2, oryzalin 3, desaminomethylfurfurylazin 4, chlorin 5, 2-chloro-3, 5- dibenzoxytrifluoride (isochloralin) 6, 4-chloro-3-nitrobenzotrifluoride 7, 2,4- dinitro-5-benzotrifluoride 8, N'-bis-(2,6-dinitro-4-trifluoromethyl-phenyl)- propane-1,2-diamine, where \( n = 2 \); \( n = 9 \), where \( n = 4 \); \( n = 10 \), where \( n = 6 \); \( n = 11 \), where \( n = 10 \); \( n = 12 \), where \( n = 12 \), \( n = 11 \), 2,6-dinitro-4-trifluoromethyl-phenylamine 14, chloramphenicol 15, 2,2-nitro-1H-imidazol-1-yl-N-(2,2,3,3,3-pentafluoropropyl)- acetamide (EFS), 16, [(7-nitro-2,3-dioxo-1,2,3,4-tetrahydroquinolin-5-ylmethyl)-amino]-methylphosphonic acid (AMP397) 17.
that of the phenyl ring at an angle of 36.4(2)°. The torsion angles of the ethyl groups are somewhat surprising being C4-N7-C71-C72 126.1(4), N7-C71-C72-C73 -63.2(7) and C4-N7-C81-C82 118.9(5), N7-C81-C82-C83 -172.3(4)°. Hence, one propyl group is in a gauche conformation and the other is in the expected trans environment. The two nitro groups make angles of 47.5(2) and 54.6(2)° with the phenyl ring.

Clearly, the rotation of the two-alkyl groups out of the plane is a steric effect concomitant with an opposite rotation from the nitro groups. Thus, with reference to Figure 2, O(32) is below the plane while C(71) is above and O(52) is above the plane while C(81) is below. The O...C distances in the two cases are 2.869(6) and 2.942(5) Å.

In the structure of chloralin 5 the two molecules in the asymmetric unit show different types of disorder. Molecule A contains rotational disorder of the -CF3 group, whereas molecule B contains rotational disorder of one nitro group with the oxygen atoms alternatively above and below the ring plane as well as rotational disorder of the -CF3 group. In A, the two nitro groups intersect the ring plane at angles of 61.6(3), 34.9(5)° and in B 63.0(3) and 53.9(3) or 56.9(10)°. So it is clear that the presence of the chloride substituent intermediate between the two nitro group has had a similar effect on the orientation of the nitro groups to the -N(propyl)2 group in 1.

In the structure of 8 the nitro group is twisted out of the plane of the aromatic ring by an angle of 38.3(2)°. This is despite the fact that there is a substituent only on one side of the nitrate. In this structure the -CF3 group is ordered unlike the situation in 1 and 5, presumably this is due to the presence of the adjacent Cl(6) atom. It is noteworthy that the -CF3 group takes up a conformation in which two of the fluorine atoms are almost equidistant from the adjacent Cl(6) atom at 3.114(3), 3.126(4) Å.

The structure of the trifluralin dimer N1,N2-di-(2,6-dinitro-4-trifluoromethylphenyl)-1,3-propylenediamine 9 was obtained as a racemic crystal and is somewhat surprising because the two amine nitrogen atoms are only 2.908(9) Å apart because the linkage between them contains several torsion angles that are gauche rather than trans as might be expected;

**Figure 2.** The structures of 1, 5, 8, 9 and 14 with ellipsoids at 25 % probability, and intramolecular hydrogen bonds are shown as dotted lines. 1 has one propyl group in a gauche and the other in a trans conformation. The -CF3 group is disordered over two orientations, only the major component is shown. Molecule A is shown for 5 with one set of positions for the disordered -CF3 group. Molecule B has a similar basic geometry but rotational disorder in one nitro and a -CF3 group. The conformation of the -CF3 group in 8 ensures that fluorine atoms are staggered with respect to the adjacent Cl(6). 9 has N...N distances of 2.642(5) and 2.639(5) Å.

being for C(5)-C(4)-N(41)-C(42) -152.3(4)°, C(4)-N(41)-C(42)-C(43) 136.7(4)°, N(41)-C(42)-C(43)-N(44) 65.4(4)°, C(42)-C(43)- N(44)-C(51) -103.8(5)°, C(43)-N(44)-C(51)-C(56) 159.3(4)°. The two amine nitrogen atoms N(41) and N(44) both form intramolecular hydrogen bonds with their adjacent oxygen atom O(72), O(52), respectively, with dimensions N...O, N-H...O and H...O 2.642(5) Å, 130.6°, 2.00 Å and 2.639(5) Å, 127.6°, 2.02 Å respectively). These two nitro groups intersect the plane of the
benzene ring at angles of 9.5(11), 7.9(7)°, respectively, while the other nitro groups which are sterically hindered by the carbon atoms C(1) and C(3) are twisted by angles of 40.2(6), 53.8(5)° out of the plane of the benzene rings. The two aromatic rings intersect at an angle of 60.7(2)° though there are no intramolecular hydrogen bonds between them, nor indeed any close contacts between the two aromatic rings and their substituents.

As further confirmation of the correlation between the rotation of the nitro group relative to the phenyl ring and the substituent(s) on the amine nitrogen, the structure of 2,6-dinitro-4-trifluoromethyl-phenylamine 14 was also determined.

Here, the two-nitro groups are adjacent to an amine group and formed hydrogen bonds via O32 and O52. Dimensions were O…H 2.01, 2.01 Å, O…H-N 128, 130° and O…N 2.630(9), 2.642(9)Å, respectively. As a consequence the angles of rotation of the two groups relative to the ring were 9.8(12) and 8.6(10)° thus showing that steric effects are paramount in considering the angle of rotation of the nitro groups in the molecules reported here.

Theoretical calculations

Quantum mechanics calculations using the Gaussian03 program [32] were carried out to establish whether packing effects had any serious effects on the molecular geometry of these complexes. Calculations on trifluralin using the crystal structure as a starting model converged at the B3LYP/6-31+G* level with the N(7) having a trigonal environment. The angle between the plane of C(4), N(7), C(71), C(81) and the phenyl ring was 38.6º confirming that the conformation found in the crystal structure is not affected significantly by crystal packing, although it might be expected that both N-propyl groups would have the trans conformation though this would not feasible due to clashes between the chains.

Equivalent calculations have been carried out on this molecule with the NPr2 groups replaced with (a) NH2 and (b) NHMe. In molecule (a), the calculation resulted in a planar conformation in which both nitro groups and the NH2 group were coplanar with the phenyl ring, angles of intersection 0.4º presumably forming some weak interaction between the amine hydrogen atoms and the nitro groups. This can be compared to the crystal structure of 14 where the nitro groups are slightly twisted out of the plane of the phenyl ring by angles of 9.8(12), 8.6(10)°. In the case of molecule (b), the nitro group adjacent to the N-H group remained closely planar with the phenyl ring at an angle of 60.7(2)° though there are no intramolecular hydrogen bonds between the chains.

These experimental and theoretical results show that the bulk of the NPr2 group in 1 has a similar effect to that of the chlorine in chloralin on the twist in the adjacent nitro groups. However, with a primary amine, both adjacent nitro groups will be coplanar with the nitro group while only one nitro group in a secondary amine is twisted significantly from the plane. This difference in structure of the adjacent nitro groups may indicate that only the tertiary amine will be active and that the presence of a hydrogen atom bonded to the nitrogen and forming a weak hydrogen bond to the adjacent nitro group may affect activity.

We next investigated the electronic properties of molecules 5, 6, 7 and 8 (Figure 1) to establish whether there were any obvious correlations with activity, the first two being active and latter two inactive. Selected parameters are shown in Table 1. Clearly all but the charge on the chlorine can be correlated with biological activity but with only four test compounds, no decisive conclusions can be drawn here but may be helpful in drawing conclusions as to which substituents enhance electron transfer in future studies (see EPR section).

### Table 1. Selected physiochemical and electronic properties of compounds tested in culture and in vivo.

<table>
<thead>
<tr>
<th>Charge</th>
<th>HOMO (a.u.)</th>
<th>LUMO (a.u.)</th>
<th>C-Cl (Å)</th>
<th>μ(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>on Cl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.361</td>
<td>-0.319</td>
<td>0.136</td>
<td>1.720</td>
</tr>
<tr>
<td>6</td>
<td>0.430</td>
<td>-0.323</td>
<td>-0.147</td>
<td>1.722</td>
</tr>
<tr>
<td>7</td>
<td>0.360</td>
<td>-0.310</td>
<td>0.136</td>
<td>1.760</td>
</tr>
<tr>
<td>8</td>
<td>0.357</td>
<td>-0.302</td>
<td>0.126</td>
<td>1.741</td>
</tr>
<tr>
<td>9</td>
<td>0.327</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Pharmacological evaluations**

Six potential antimalarias were evaluated against the cloned *P. falciparum* subline FCH5.C2 [33] and the chloroquine- and pyrimethamine-resistant strain K1/Thailand (Table 2), and *in vivo* against lethal *P. berghei* (Table 3), previously utilised to construct our functional *in vivo* receptor for bisquinoines. [34] Both evaluations used commercially (Sigma-Aldrich) available chloroquine diphosphate and arteisinin as standard antimalarial drugs.

### Table 2. Antimalarial activity of dinitroanilines against two cultured strains of *P. falciparum*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 FCH5.C2 (µM)</th>
<th>IC50 K1/Thailand (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>72 h</td>
</tr>
<tr>
<td>1</td>
<td>5.0[a]</td>
<td>2.7[c]</td>
</tr>
<tr>
<td>5</td>
<td>7.1</td>
<td>5.9</td>
</tr>
<tr>
<td>6</td>
<td>7.3</td>
<td>7.8</td>
</tr>
<tr>
<td>7</td>
<td>&gt;64[c]</td>
<td>&gt;64[c]</td>
</tr>
<tr>
<td>8</td>
<td>&gt;64[c]</td>
<td>&gt;64[c]</td>
</tr>
<tr>
<td>9</td>
<td>4.8[b]</td>
<td>4.9</td>
</tr>
</tbody>
</table>

[a] Different from chloralin at p<0.05, Student’s t-test. [b] Different from chloralin at p<0.01, Student’s t-test. [c] Different from chloralin at p<0.001, Student’s t-test.

### Table 3. Antimalarial activity of dinitroaniline compounds against *P. berghei* in mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose/µmol kg⁻¹</th>
<th><em>P. berghei</em> Parasitaemia % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>89 ± 11</td>
</tr>
<tr>
<td>2</td>
<td>149</td>
<td>84 ± 16</td>
</tr>
<tr>
<td>5</td>
<td>298</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>6</td>
<td>597</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>7</td>
<td>46 T</td>
<td>71 ± 14</td>
</tr>
<tr>
<td>8</td>
<td>92 T</td>
<td>79 ± 7</td>
</tr>
<tr>
<td>9</td>
<td>185 T</td>
<td>49 ± 9[a]</td>
</tr>
<tr>
<td>10</td>
<td>46 T</td>
<td>66 (54 – 78) [a]</td>
</tr>
</tbody>
</table>

[a] Compounds were injected s.c. twice daily (total number of doses = 5) in mice (initial group sizes 5-10) inoculated with *P. berghei* N. Parasitaemias were
Antimalarial activity in vivo

Results show that 1 was not antimalarial in the in vivo three-day suppression test, against P. berghei N in mice, used in this study with doses in the range 25 - 200 mg kg\(^{-1}\) (75 – 597 µmol kg\(^{-1}\)) s.c. twice daily (Table 3). All animals in this study survived treatment with 1. In contrast, 5 was found to be toxic; a single injection of chloralin 200 mg (739 µmol) or 100 mg (370 µmol) kg\(^{-1}\) s.c. caused death or necessitated humane killing, therefore, doses of 12.5, 25 and 50 mg (46, 92 and 185 µmol respectively) kg\(^{-1}\) s.c. twice daily were used in the three day malaria suppression test. Antimalarial activity was evident with 5 at 25 (92 µmol; P < 0.1) and 50 mg (185 µmol; P < 0.05) kg\(^{-1}\) s.c.; ID\(_{50}\) 50 mg (185 µmol) kg\(^{-1}\), but toxicity was evident as hypothermia, weight loss and decreased motor activity in some animals. A readily available, theoretically isophasic isomer of 5, 2-chloro-5,5-dinitrobenzotrifluoride 6, was also toxic following a single injection s.c. of 100 mg (370 µmol) kg\(^{-1}\). Reduced doses of 25 mg (92 µmol) and 12.5 mg (46 µmol) kg\(^{-1}\) twice daily also caused toxic effects similar to 5 in mice used in the antimalarial screen. Although, 6 at 12.5 mg (46 µmol) kg\(^{-1}\) reduced P. berghei parasitaemia to 66 % of that seen in vehicle-treated mice, the low number of survivors on day 3 (2 from initial group of five mice) makes this result statistically insignificant. Compounds 4-chloro-3-nitrobenzotrifluoride 7 (50 and 100 mg kg\(^{-1}\); 228 and 443 µmol kg\(^{-1}\), respectively) and 2,4-dichloro-5-nitrobenzotrifluoride 8 (25, 50 and 100 mg kg\(^{-1}\); 96, 192 and 385 µmol kg\(^{-1}\), respectively) were neither antimalarial (Table 1) nor toxic in mice during the period of the three day suppression test.

It is relevant to note that 1 is also inactive in vivo against Cryptosporidium parvum (dosed at 100 mg kg\(^{-1}\))\(^{[23]}\) and is neither carcinogenic or tumorigenic in in male B6C3F1 mice or in Osborne-Mendel rats of either sex.\(^{[36]}\) 1 is rapidly cleared in rats by faecal and urinary excretion with only 10 % remaining unmetabolised.\(^{[36]}\) Rapid metabolism and clearance in rodents\(^{[37]}\) may account for the inactivity of 1 in rodent models of Plasmodium and Cryptosporidium in vivo. Another possibility is that the high log P value of 1 (4.81) constrains this at or around the site of injection.\(^{[11]}\)

We were unable to separate the antimalarial effects of 5 or its analogue, 6 from their toxicity in mice. In mice Componds 4-chloro-3-nitrobenzotrifluoride 7 and 2,4-dinitro-5-nitrobenzotrifluoride 8 had no antimalarial activity or toxicity and thus appear to lack the pharmacophore associated with 5 and 6.

Antimalarial activity in culture

Componds 9 – 13 were not fully soluble in DMSO at suitable concentrations (>5 mM). Limiting acquisition of a more complete QSAR profile. Although, 5 may well be the active toxic and antileishmanial component of 1 in other investigations.\(^{[174]}\) It was not a contaminant in our rigorously purified samples.

Antimalarial activity was evaluated after 48 and 72 h incubations using asynchronous cultures of chloroquine-sensitive and resistant strains of P. falciparum (Table 2). 1 was slightly but significantly more active than 5 against both strains. The observed activity of 1 is consistent with previously published data.\(^{[24-26, 38]}\)

The potency of the compound was unaffected by the resistances of the K1/Thailand strain to unrelated agents. The theoretically isophasic isomer of 5, 6, displayed similar activity to 5. Activity was abolished by the removal of one of the nitro- groups of 5, 7, and was not restored by the extra Cl in 8. The trifluoril dimer 9 was slightly more active than 1 so is possible that only one half of this dimer interacts at the receptor site, particularly as the relatively folded conformation makes it unlikely that both aromatic rings can simultaneously interact at a putative target site. However, interestingly, the activity of 9 was either superior or equal to the monomeric 5 and 6. Disappointingly, other members of the homologous series proved insoluble at the levels required for testing on cultured parasites. When strategies for improving the solubility of these putative antimalarials are discovered, it will be interesting to see whether a dimer can be found that is superior to 1 as a result of an optimised linker length.

Examination of mitotic microtubular structures of 1- or 5-treated parasites by immunofluorescence revealed a breakdown of the normal hemispindles and microtubule-organising centres and their replacement by fragmented tubulin labelling (Figure 3).

The activities of 1, 5, pendimethalin, benfluralin and 3 against P. berghei in culture and in a rat model of malaria. They reported that while moderately active against cultured parasites (IC\(_{50}\), 1.3 µM) 1 did not reach sufficient plasma levels in rats to obtain an antimalarial effect in vivo. Their results differ slightly from ours in the lower activity of 5 against cultured parasites...
parasites (IC50, 16 µM) but this could be a species difference. 5 was not tested in vivo by these investigators.

Mechanism of antimalarial action

Support for an meta-de-chloro-thiolation mechanism (compare to the aforementioned synthesis of 9, where the amine is replaced by a cysteine residue) is provided by evidence that 5 interferes with *Leishmania* tubulin[10] and microtubule assembly in culture (IC50 = 22 µM), whereas 1, which possesses the much poorer amino-bis-propyl leaving group, does not. [43] The mechanism of inhibition of thiol dependent enzymes has been reviewed. [44] However, the proposed mechanism of antitubulin action involving certain mono-nitroanilines has been questioned. [18b] Analogues tested against purified tubulin failed to inhibit microtubule assembly, but had antileishmanial activity in culture, which suggests that further analogue development may reveal non-toxic agents. 1 may bind to its receptor by non-covalent electrostatic and van der Waals interactions, whereas 5 can form a covalent adduct with nucleophilic side chains of parasite protein amino acids. Computational modelling has suggested binding sites within both dinitroanilines in kinetoplastid and apicomplexan α-tubulin. However, there are four amino acid differences between the kinetoplastid and apicomplexan binding site residues, which might contribute to the differential efficacy of specific dinitroanilines.[42]

Although, one electron reduction of the nitro group is the basis of the antiparasitic action of nitroimidazole drugs, [43] this does not appear to be the mode of action in intact cells. [44] For instance, 5-nitrofurural N-butyl semicarbazone [45] has been shown to have antitrypanosomal activities, targeting trypanothione reductase through a nitro anion radical mechanism. Nitric oxide donors have been reported to inhibit *Leishmania* cytochrome proteinase [46] and a similar mode of action could apply to *Plasmodium* [47] although nitrofurantoin radical anion and GSH cannot be detected under physiological conditions, within parasites. [43a] such experiments need to be performed for dinitroanilines against both drug-resistant and -sensitive *Plasmodium*.

EPR Studies of trifluralin-heme interactions

Previous investigations have indicated that 3 has an effect on the liver stage of the *Plasmodium* life cycle. [26] As this organ is a major site of drug deactivation and occasionally inactivation during xenobiotic metabolism especially with heme containing enzymes such as cytochrome P450’s, the interaction of reduced 1 with heme was of interest. In order to elucidate the interaction of 1 with heme, continuous wave (CW) electron paramagnetic resonance (EPR) spectra (Figure 4) were recorded in order to investigate the spin state of the heme iron. The spectrum of heme is typical of high spin ferric heme (S = 5/2) with turning points at \( g_x = 6.0 \) and \( g_y = 1.99 \). Upon addition of 4-fold molar excess of 1 under aerobic conditions a dramatic decrease in the intensity of the ferric resonance was seen with no subsequent new signals as has previously been observed with compounds related to mechanism 3. These observations suggest a change of iron spin state to an EPR silent state. The mechanism of action will need further investigation.

Mechanism of toxicity

The presence of halogen in natural products, previously considered a rare occurrence, has been identified in over 2600 compounds. [48] Non-toxic, low molecular weight drugs containing both nitro and trifluoromethyl groups have been successfully designed including EFS 16 [49] and antiepileptic AMP397 17, [50] although possessing a “structural alert” (the nitro group), [51] sufficient safety data have initiated clinical development. [52] Similar effects on mammalian tubulin have been described with small aromatic electrophiles such as 1-fluoro-2, 4-dinitrobenzene and 2,4-dichlorobenzyl thiocyanate [53] suggesting that the antimalarial activity may involve, in part, formation of a covalent adduct within parasite tubulin [54] but may also result in toxicity to the host.

The mechanism of action of several clinically useful drugs including vasodilators such as nitro-glycerine, radiosensitisers such as etanidazole (SR2508), which targets brainstem glioma, [55] and antibiotics such as metronidazole, [56] certain antileishmanials (57) and putative anticancer agents [43a] require the presence of a nitro group. Chloramphenicol 15, a halogenated nitrobenzene antibiotic produced by *Streptomyces venezuelae* [48] and found in the moon snail *Lunatia heros*, [58] lacks systemic toxicity following topical application that prevents opportunistic infections following eye surgery [59]. However, it has been noted that compounds containing a nitro group are over-represented in the Ames test whether the compounds are metabolically activated or not. [56] In the case of 5 the results of this study indicate toxicity may preclude further development of this compound. [60] 5 was found in this study to be antimalarial both in culture and in vivo, but its antimalarial activity was associated with host toxicity. This toxicity indicates a lack of selectivity by 5 for *Plasmodium* and suggests the compound is not preferentially sequestered by the parasite to an adequate degree for antimalarial drug use. 1 displayed antimalarial activity in culture, but not in vivo in mice. 1 also failed to display significant toxicity in this study. As indicated above, Dow et al. [10b] reported a similar profile; 1 inhibited *P. berghei* in culture but not in vivo in rats following oral administration. These researchers suggested 1 was inactive in vivo because plasma concentrations attained after oral dosing were inadequate for antimalarial activity, perhaps due to the high
solubility of 1 in host body fat, [56] or reduction by a first pass effect in the liver. Measurements of the uptake of radiolabelled 1 into P. falciparum suggested that accumulation in membranes may also limit the activity of 1 in cultured parasites. [57] However, since the trifluralin class of compounds undergo extensive metabolism, at least within the rat, [58] secondary xenobiotic metabolites could also be responsible for antiparasitic activity. [26] Since dinitrophenols, putative metabolites of chloralin type compounds, have significant toxicity, development of this class of compound will require extensive pre-clinical toxicity screening.

Conclusions

The long-term aims of our research on dinitroanilines as potential antiparasitic drugs are to: i) reduce the toxicity of the nitro moieties; ii) decrease the possibility of idiosyncratic drug reactions by structural modification; [59] (iii) ensure selective uptake into parasites; iv) measure intra and inter-subunit tubulin binding site dimensions and v) identify the trifluralin pharmacophore. These aims require compounds with improved physical properties compared to trifluralin and the novel dimers reported here.

5 can be considered a target for further development, because it is active against Plasmodium, however, it does display toxicity in the host. The discrepancy between the antimalarial activities of 1 in culture and in vivo cannot be readily explained. Low bioavailability due to uptake into host body fat and/or rapid metabolism and excretion may explain the lack of activity in laboratory rodents in vivo. The number of compounds examined so far is insufficient for establishing robust Q SAR. In addition, compounds with better drug formulation profiles could be generated by molecular modifications that enhance aqueous solubility. Unlike in Leishmania, 1 [and also 9] was found in this study to have activity against parasites in culture that could not be ascribed to contaminants in the test substances. Identification of a compound of similar potency to 1 against drug-resistant and sensitive strains of Plasmodium in culture 9 indicates that the presence of the halide is not an absolute requirement for antimalarial activity. This observation suggests the nitro group may be undergoing bioactivation and may act by production of ROS as evidenced by interaction with reduction of Fe(III) within heme. [46]

Both QSAR studies [54] and drugs co-crystallised with putative target receptors have enabled rational drug evolution towards more selectively toxic antiparasitic agents. [20] A computational docking study of 3, 1 and 5 to tubulin, found in Toxoplasma gondii, suggests that in this parasite, they exert their action by disrupting M-N loop contacts. [23] Studies have shown that antimitotic herbicides can bind to an unidentified site on malarial parasite tubulin and, notably, block development of liver-stage Plasmodium parasites. [49] Consequently, emphasis must now shift to understanding which of these are responsible for activity and must in turn serve as further lead compounds in the developing the next cycle in eventually developing clinically useful drugs.

Further studies are needed to develop selective accumulation of such compounds into parasites whilst sparing host mitochondrial apparatus, the original concept of selective toxicity as promoted by A. Albert [68] has indicated that it is not that a compound is not toxic but it should be selectively toxic. If we develop compounds known to selectively accumulate in mitochondria, incorporation of dinitroaniline groups into frameworks [67] may provide more effective compounds. In conclusion, until the target site of dinitroanilines in Plasmodium has been characterised, and details of the receptor site clarified, it will be difficult to identify selectively toxic compounds of this structural class that have the high safety margins required for clinical use.

Supporting Information Summary

Full experimental details and spectroscopic characterizations are given in the supporting information.

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