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Microtubule Inhibitors: Structure-Activity Analyses Suggest Rational Models To Identify Potentially Active Compounds

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Trifluralin, a dinitroaniline microtubule inhibitor currently in use as an herbicide, has been shown to inhibit the proliferation of *Plasmodium falciparum*, *Trypanosoma brucei*, and several species of *Leishmania*, in vitro. As a topical formulation, trifluralin is also effective in vivo (in BALB/c mice) against *Leishmania major* and *Leishmania mexicana*. Although trifluralin and other dinitroaniline herbicides show significant activity as antiparasitic compounds, disputed indications of potential carcinogenicity will probably limit advanced development of these substances. However, researchers have suggested that the activity of trifluralin is due to an impurity or contaminant, not to trifluralin itself. We have pursued this lead and identified the structure of the active impurity. This compound, chloralin, is 100 times more active than trifluralin. On the basis of its structure, we developed a rational structure-activity model for chloralin. Using this model, we have successfully predicted and tested active analogs in a *Leishmania* promastigote assay; thus, we have identified the putative mechanism of action of this class of drugs in *Leishmania* species. Potentially, this will allow the design of noncarcinogenic, active drugs.

Leishmaniasis, a disease endemic to 80 countries, is a major public health problem worldwide, with approximately 12 million to 40 million cases estimated and 350 million people at risk (39). The treatment of choice is pentavalent antimony in the form of sodium stibogluconate (Pentostam) or meglumine antimonate (Glucantime). Both are intravenous drugs which produce severe adverse side effects. Hospitalization of the patient during treatment (usually 30 days) is required. In addition, clinical failures are not uncommon, both because the compounds have low activity against some *Leishmania* strains (16) and because the occurrence of antimony-resistant leishmaniasis has been increasing in frequency worldwide (26, 28, 31, 37). Pentostam-resistant parasites have also been identified in vitro (3, 16, 20). Drugs that are more effective, less toxic, and easier to use are urgently needed.

Microtubule inhibitors have been exploited previously as anthelmintic drugs, in cancer therapy (22), and as herbicides (27, 35). Trifluralin, a microtubule inhibitor herbicide, has been shown to inhibit the proliferation of *Plasmodium falciparum* (25), *Trypanosoma brucei* (8, 17), and several species of *Leishmania* (6, 8, 10) in vitro. Trifluralin is also effective in vivo against *Leishmania major* and *Leishmania mexicana* in a murine model for testing topical formulations (8). Other antimicrotubule herbicides, as well as a synthetic antimicrotubule compound, have shown activity in vitro against *Leishmania* species (9, 10, 13). Thus, microtubule inhibitors have the potential to be promising lead-drug candidates for several tropical diseases, including leishmaniasis, African trypanosomiasis, and malaria.

The mechanism of action of these microtubule inhibitors in plants has been well studied (11, 19, 24, 27, 34–36). Dinitroaniline herbicides such as trifluralin and oryzalin interact directly with the major microtubule protein, tubulin, leading to disruption of mitosis. Morphologically, the plant roots are swollen because of inhibition of growth and elongation which are caused by a lack of cortical microtubules. Karyotypic examination shows a characteristic chromosome condensation like that caused by colchicine, where mitosis is arrested during prometaphase because of a lack of spindle microtubules needed to move chromosomes to the poles.

Similar features have been observed in *P. falciparum* treated with trifluralin (21) and in *L. mexicana* treated with oryzalin (9). At sublethal doses of these compounds, the shape of the *Leishmania* promastigotes changed, becoming rounded, presumably for the same reason as in plants, a lack of structural microtubules. The authors speculated that these cells were arrested during cell division. Surprisingly, flagellar multiplication either was not arrested in concert with cell division or was actually stimulated, since treated parasites had two to four flagella each. *Leishmania* species also appear to have the same biochemical mechanism of inhibition as plants, since oryzalin was found to specifically inhibit polymerization of *Leishmania* tubulin, but not rat tubulin, in vitro (9). Thus, leishmanial tubulin appears to be more similar to plant tubulin than to mammalian tubulin, with distinct structures that can be targeted by drugs.

Although the antimicrobial dinitroaniline herbicides show great potential as antiprotozoal compounds, disputed indications of potential carcinogenicity will probably keep trifluralin from being developed for human use (4, 7). However, recent work by Chan and others (10) indicates that an impurity or contaminant in trifluralin, not trifluralin itself, may be responsible for the observed in vitro activity against *Leishmania* species. The activities of trifluralin samples from two sources differed more than threefold in an in vitro *Leishmania* promastigote assay. These and other data strongly suggested that an impurity found in the samples was responsible for the differences in activity. We have pursued this lead and identified the structure of the active impurity, evaluated its in vitro activity in a *Leishmania* promastigote assay, developed a rational struc-

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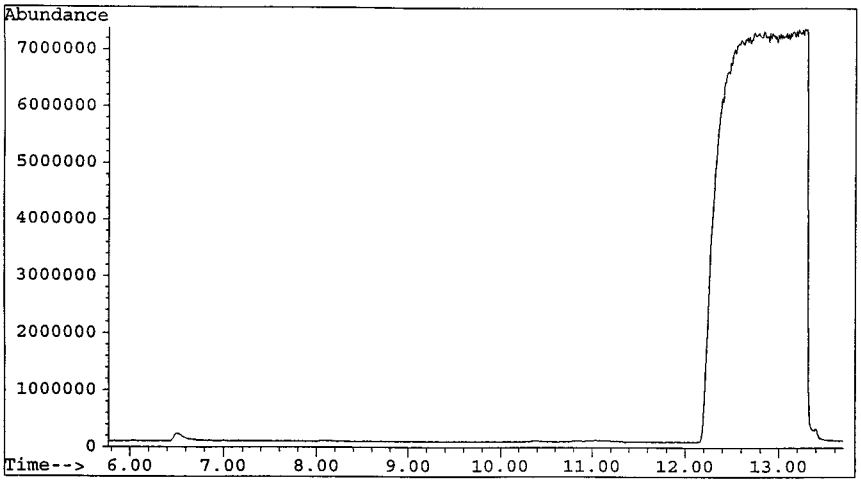

FIG. 1. Gas chromatogram of trifluralin from Reidel-de Haen, showing the impurity present in trifluralin. Time is in minutes.

ture-activity model, and, on the basis of this model, predicted and tested potentially active analogs in the *Leishmania* assay.

MATERIALS AND METHODS

Chemicals. 4-Chloro-3,5-dinitrobenzotrifluoride (chloralin), 4-chloro-3-nitrobenzotrifluoride (compound 4), 3-amino-4-chlorobenzotrifluoride (compound 5), 2-bromo-3,5-bis(trifluoromethyl)aniline (compound 6), 4-methoxy-3-nitrobenzotrifluoride (compound 7), and 2-nitro-4-(trifluoromethyl)thiophenol (compound 8) were obtained from Aldrich Chemical Company and were used without further purification. Trifluralin was obtained from Reidel-de Haen and was used without further purification. 4-Chloro-3-nitro-5-sulfonylbenzotrifluoride (WR 165317; compound 2) and 4-chloro-3-nitro-5-carboxylbenzotrifluoride (WR 191564; compound 3) were obtained from the Walter Reed Army Institute of Research inventory and were used without further purification. All drugs were initially dissolved in dimethyl sulfoxide and were diluted at least 100-fold in parasite culture medium before being tested against *Leishmania* species.

Gas-liquid chromatography–mass spectrometry. Identification of chloralin in the Reidel-de Haen trifluralin samples was conducted with a Hewlett-Packard HP 5890A gas chromatograph linked to an HP 5970 mass-selective detector and HP G1034C software for the MS ChemStation. The structures of these compounds were confirmed by a probability-based matching search with the ChemStation software using the National Institute of Standards and Testing-National Bureau of Standards (NIST-NBS) Spectral Library database. In order to detect the chloralin peak at 6.5 min, the column was overloaded with a 0.02 M solution of trifluralin in acetone. This solution was directly injected into the gas chromatograph in a volume of 5 μl. The operation conditions were as follows: injector temperature, 250°C; detector temperature, 250°C; and temperature program, 100 to 250°C at 5°C/min.

Cell culture. The *L. major* clonal derivative of LT252, CC-1, was maintained

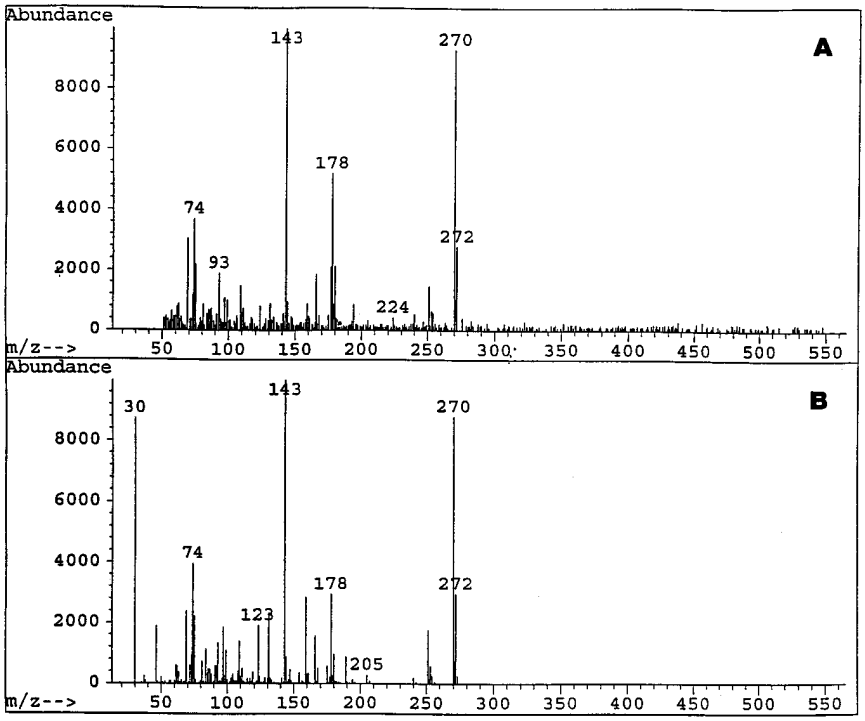

FIG. 2. Mass spectrum of the trifluralin peak in the Reidel-de Haen trifluralin sample (A) and its 99% match with trifluralin in the NIST-NBS Spectral Library database (B) following a probability-based matching search with ChemStation software. m/z, molecular mass.

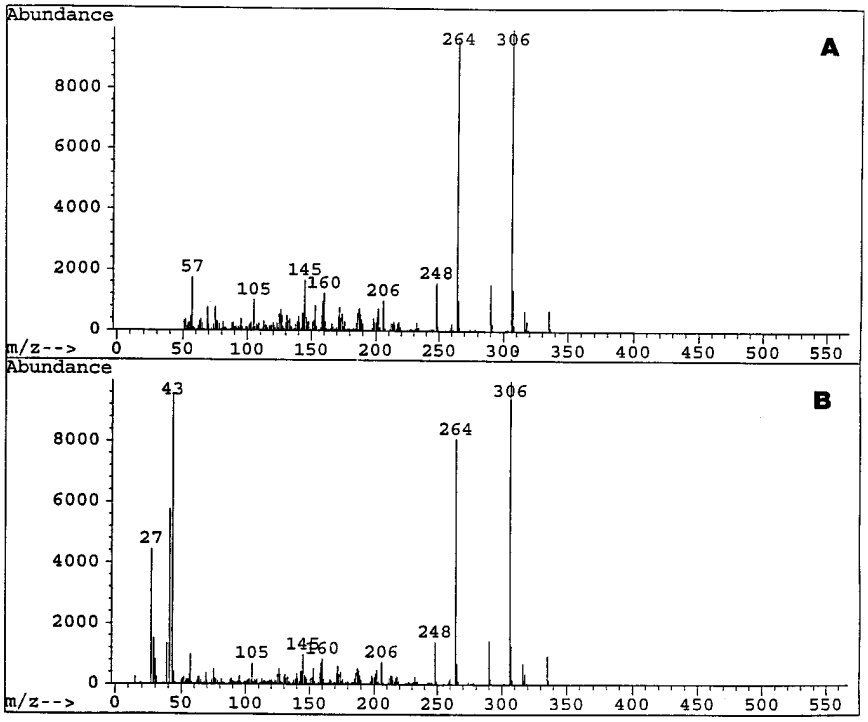

FIG. 3. Mass spectrum of the impurity peak in the Reidel-de Haen trifluralin sample (A) and its 91% match with chloralin in the NIST-NBS Spectral Library database (B) following a probability-based matching search with ChemStation software. m/z, molecular mass.

in, and all drug assays were performed in, modified M199 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Intergen) and 1% penicillin (50 U/ml)-streptomycin (50 μg/ml) (Sigma).

Parasite drug susceptibility assay. Parasite drug susceptibility determinations were made by the method described previously (5). Briefly, parasites were seeded at an initial concentration equivalent to early log phase (2×10^5 parasites per ml) and allowed to grow for 48 h in media alone or in the presence of serial dilutions of a drug until late log phase (1×10^7 parasites per ml). Drug dilutions encompassed the 10% inhibitory concentration (IC_{10}), IC_{50}, and IC_{90}. Parasite counts were performed with a Coulter Counter Multisizer II. Experiments were repeated at least three times in triplicate. The IC_{50} is the concentration of drug which decreases cell numbers by 50% compared with control cells grown in the absence of drug.

RESULTS

Characterization of trifluralin by gas-liquid chromatography–mass spectrometry. Chromatographic analysis of Reidel-de Haen samples of trifluralin by gas-liquid chromatography–mass spectrometry resulted in a chromatogram with two peaks (Fig. 1). The major peak was identified as trifluralin, and the very small peak was found to be chloralin. Both peaks displayed parent ion and loss patterns consistent with their structures; their identities were confirmed by a probability-based matching search with ChemStation software using the NIST-NBS Spectral Library database (Fig. 2 and 3). The Reidel-de Haen samples were run at a molecular weight range between 50 and 550 g/mol, while the spectrums from the library search were collected between 0 and 550 g/mol. Thus, the peaks present at 30 g/mol and at 27 and 43 g/mol in the library spectrums of chloralin and trifluralin, respectively, are not present in the sample spectrums.

Although previously the impurities in trifluralin were hypothesized to be the result of the photolability of trifluralin (10), chloralin is present because it is an intermediate product in the synthesis of trifluralin. The commercial synthesis of trifluralin is shown in Fig. 4 (29).

Structure-function analysis of chloralin and trifluralin. Since chloralin was 100 times more active than trifluralin against *Leishmania* promastigotes in vitro (IC_{50}s, 0.89 ± 0.3 μM versus 72 ± 21 μM, respectively), it became our lead compound.

Chloralin is a textbook example of a compound which should react as shown in Fig. 5 in the presence of a nucleophile. For a compound to undergo this reaction, it needs electron-withdrawing groups (in this case, two NO_2 groups) and a good leaving group (in this case, Cl). The electron-withdrawing groups are necessary both to create an electron-deficient environment in which a nucleophile can react and to stabilize the resultant anionic intermediate. A good leaving group is necessary for the reaction to proceed to completion. In the case of chloralin, the two NO_2 groups are extremely strong electron-withdrawing substituents and the Cl substituent is an excellent leaving group (Fig. 5).

This information suggested a model for the selection of active chloralin analogs. Our hypothesis was that the ability of a compound to undergo this reaction should correlate with its activity against *Leishmania* species. To test our hypothesis, analogs with suitable electron-withdrawing groups and leaving groups were chosen.

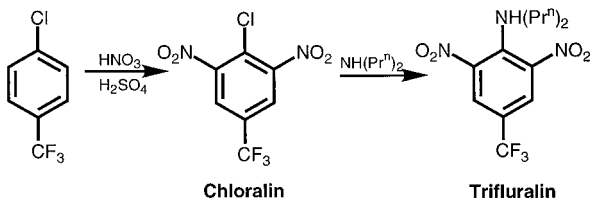

FIG. 4. Commercial synthesis of trifluralin.

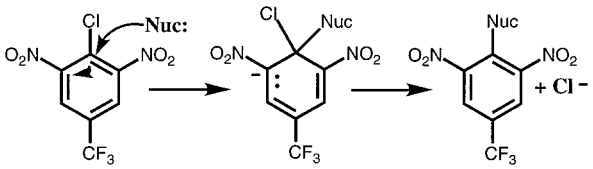

FIG. 5. Reaction of chloralin with a nucleophile (Nuc) to form a drug-nucleophile adduct.

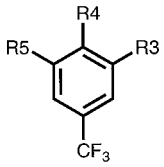

FIG. 6. General chemical structure of a simple aromatic compound. The substituents R3, R4, and R5 are given in Table 1.

Structure-function analysis of chloralin analogs. A comparison of the antileishmanial activities of analogs with electron-withdrawing groups of different strengths (but the same leaving group, Cl) showed a correlation between the strength of the electron-withdrawing group and the in vitro activity of the compound against *Leishmania* promastigotes (Fig. 6 and Table 1). The most active compound was still chloralin, which has two NO_2 groups; NO_2 groups are the strongest electron-withdrawing groups of the analogs we tested.

The other analogs generally followed the same pattern (Fig. 6 and Table 1). Replacement of a single NO_2 group with SO_2H (compound 2) resulted in almost a sevenfold loss of antileishmanial activity, which correlates with the loss of electron-withdrawing ability; the SO_2H substituent is not as strongly electron withdrawing as an NO_2 substituent. Similarly, the elimination of an electron-withdrawing group (NO_2, compound 4) resulted in an even greater loss of antileishmanial activity (nearly 40-fold less than that of chloralin). Substitution of an amino group (NH_2; compound 5) for the remaining NO_2 substituent on compound 4 resulted in an additional twofold loss of activity; NH_2 groups are weaker electron-withdrawing moieties than NO_2 substituents.

The addition of a trifluoromethyl substituent (CF_3; compound 6) (Fig. 6 and Table 1) to compound 5 resulted in a threefold increase in antileishmanial activity, despite the presence of a bromine leaving group rather than a chlorine leaving group (chlorine is a better leaving group than bromine). This is not too surprising since CF_3 is considered an electron-withdrawing substituent. However, this result is interesting, since this compound is as active as compound 4 and fivefold more active than compound 3 and compounds 3 and 4 each contain one NO_2 substituent. Our hypothesis regarding the inactivity of compound 3 is presented in the Discussion.

Compounds 4, 7, and 8 differ only in their leaving groups (Fig. 6 and Table 1). Compound 4, which contains chlorine as a leaving group, was twice as active as compound 7, which has a methoxy leaving group (OCH_3). Since chlorine is a much better leaving group than the methoxy moiety, this supports the theory that compounds with better leaving groups will be more active in vitro against *Leishmania* promastigotes. In contrast, the finding that compound 8, which has a thiol leaving group (SH), was more than twice as active as the chlorine analog (compound 4) does not appear to support the model, since thiol substituents are not considered good leaving groups; normally only the ionized form, SH_2^+, can function as a leaving group. Our hypothesis regarding the activity of compound 8 is presented in the Discussion.

DISCUSSION

We have identified and characterized the active impurity in the Reidel-de Haen sample of trifluralin as chloralin, an intermediate in the synthesis of trifluralin. Chloralin was 100 times more active than trifluralin in our in vitro promastigote drug assay, and it was 2 and 20 times more active than the reference antileishmanial compounds pentamidine and paromomycin, respectively (5a). The pentavalent antimonials Glucantime and Pentostam, which have little in vitro activity against promastigotes, have 50% inhibitory concentrations ranging from <10 to <100 mg/ml. Thus, chloralin has become one of our lead compounds.

Because chloralin is a simple aromatic compound with an easily predictable mechanism of reaction with nucleophilic substituents (e.g., the SH groups of cysteine residues on tubulin), we designed a series of analogs to test whether chloralin's antileishmanial activity was effected through this mechanism. Determining chloralin's mechanism of action would allow us to design other equally active, and potentially less toxic, antiparasitic drugs. The results of our in vitro antileishmanial tests support our hypothesis that a simple nucleophilic-substitution reaction is involved in chloralin's mechanism of action.

Although chloralin remains our most active compound, six

TABLE 1. The effects of electron-withdrawing and leaving groups on the antileishmanial activity of chloralin

Compound	Chemical name	EWG[a]		LG[b] (R4)	IC_{50} (μM)[c]
		R3	R5		
Chloralin	4-Chloro-3,5-dinitrobenzotrifluoride	NO_2	NO_2	Cl	0.89 ± 0.3
2	4-Chloro-3-nitro-5-sulfonylbenzotrifluoride	NO_2	SO_2H	Cl	6.1 ± 1.4
3	4-Chloro-3-nitro-5-carboxylbenzotrifluoride	NO_2	COOH	Cl	120 ± 30
4	4-Chloro-3-nitrobenzotrifluoride	NO_2	H	Cl	35 ± 9
5	3-Amino-4-chlorobenzotrifluoride	NH_2	H	Cl	77 ± 5
6	2-Bromo-3,5-bis(trifluoromethyl)aniline	NH_2	CF_3	Br	26 ± 3
7	4-Methoxy-3-nitrobenzotrifluoride	NO_2	H	OCH_3	68 ± 10
8	2-Nitro-4-(trifluoromethyl)thiophenol	H	NO_2	SH	15 ± 3

[a] EWG, electron-withdrawing group.
[b] LG, leaving group.
[c] The 50% inhibitory concentration (IC_{50}) is the concentration of drug which decreased cell numbers by 50% compared with control cells grown in the absence of drug. Values given are the means of at least three experiments ± standard errors.

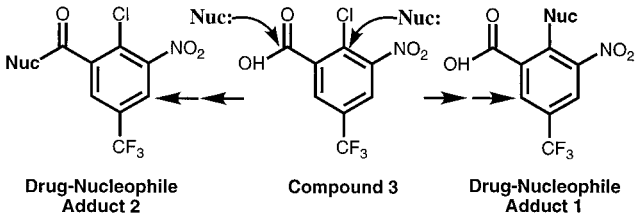

FIG. 7. Carbonyl group on compound 3 provides second site for nucleophilic attack. Nuc, nucleophile.

of the seven compounds we tested are as active as or more active than trifluralin (compounds 2 and 4 to 8). The reason for the inactivity of compound 3 (which is more than 130 times less active than chloralin) against *Leishmania* promastigotes in vitro cannot be stated unequivocally. This analog still has one excellent withdrawing group (the NO_2 substituent) and one weak withdrawing group (the carboxyl group, COOH). On the basis of this information, one would predict antileishmanial activity between the activities of compounds 2 and 4 (IC_{50} between 6 and 35 μM). One explanation is that in compound 3 there is a second site for nucleophilic attack: the carbonyl group (Fig. 7). A reaction at this second site may not be inhibitory to parasite growth and will reduce the concentration of drug available to react by the active mechanism (Fig. 5). More importantly, the inactivity of compound 3 against *Leishmania* promastigotes in vitro proves that the NO_2 groups are not simply acting in a nonspecific, toxic manner.

The NO_2 groups on trifluralin have previously been linked to questions about carcinogenicity (4, 7). Our reading of the literature (2, 12, 14, 15, 32, 38) suggests that the trifluralin used in the carcinogenicity tests was contaminated with residual *N*-nitrosodi-*n*-propylamine (38) as a result of the reaction of dipropylamine [$HN(C_3H_7)_2$] with chloralin in the last step of trifluralin synthesis (Fig. 4). Nitrosoamines are potent carcinogens and their presence could easily account for the one study out of three that linked carcinogenic potential with trifluralin (2, 12, 14, 15). Although NO_2 groups can also be carcinogenic, it would be shortsighted to rule out active compounds solely on the basis of whether they contain these substituents, since these moieties are present in similar currently prescribed drugs (for example, nifedipine).

Although the intracellular site of chloralin's action was not determined, the site of action of the parent compound, trifluralin, and another dinitroaniline, oryzalin, has been previously determined to be leishmanial tubulin (6, 9). This is also the apparent site of action in plants for dinitroanilines as well as some other herbicides (1, 11, 18, 23, 24, 30, 33). The specific binding site of some of these herbicides has been mapped on plant tubulin and frequently appears to contain a cysteine residue.

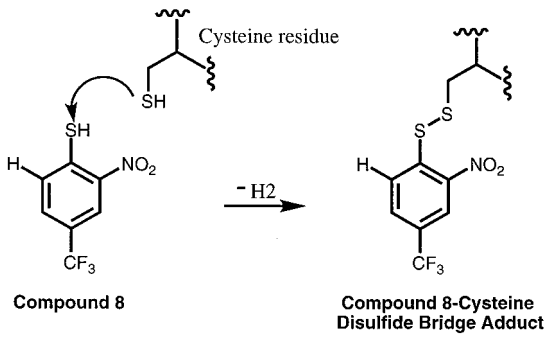

FIG. 8. Disulfide linkage formed via oxidative coupling of thiol groups on compound 8 and cysteine residue.

We expect that chloralin, like its parent dinitroaniline, trifluralin, also targets tubulin. If it does, we hypothesize that the interaction proceeds by nucleophilic substitution, with the sulfhydryl substituent on a cysteine residue(s) acting as the nucleophile (Fig. 5). This model also explains why compound 8 is unexpectedly active. In this case, instead of a nucleophilic-substitution reaction proceeding as shown in Fig. 5, there is an oxidative coupling occurring between the two thiol groups, resulting in the formation of a disulfide linkage (Fig. 8). This reaction may be either kinetically or thermodynamically favored over the nucleophilic-substitution reaction, leading to higher activity (a lower IC_{50}).

Mitotic-disrupter herbicides like trifluralin are good lead compounds for antiparasitic drugs because they have already been shown to lack activity against mammalian cells and are inexpensive to produce. In addition, preliminary work with *Leishmania* species indicates that, at least with the dinitroaniline mitotic disrupters, *Leishmania* organisms act more like plants than like animals (6, 8–10). The possibility that this is also true for other parasites exists, since both *T. brucei* (10, 17) and *P. falciparum* (25) have been shown to be sensitive to trifluralin in vitro. Further structure-function research in this potentially fruitful chemotherapeutic area is needed to identify other effective, nontoxic, antiparasitic drugs.

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