0026-895X/03/6406-1325–1333
MOLECULAR PHARMACOLOGY
Vol. 64, No. 6
2690/1111568
Mol Pharmacol 64:1325–1333, 2003
Printed in U.S.A.

Selective Antimicrotubule Activity of *N*1-Phenyl-3,5-dinitro-*N*4,*N*4-di-*n*-propylsulfanilamide (GB-II-5) against Kinetoplastid Parasites

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Received May 30, 2003; accepted September 3, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Analogs of the antimitotic herbicide oryzalin (3,5-dinitro-*N*4,*N*4-di-*n*-propylsulfanilamide) were recently prepared that were more potent in vitro than the parent compound against the kinetoplastid parasite *Leishmania donovani* (*Bioorg Med Chem Lett* **12:**2395–2398, 2002). In the present work, we show that the most active molecule in the group, *N*1-phenyl-3,5-dinitro-*N*4,*N*4-di-*n*-propylsulfanilamide (GB-II-5), is a potent, selective antimitotic agent against kinetoplastid parasites. GB-II-5 possesses IC_{50} values of 0.41 and 0.73 μM in vitro against two strains of the related parasite *Trypanosoma brucei* but is much less toxic to J774 murine macrophages and PC3 prostate cancer cells, exhibiting IC_{50} values of 29 and 35 μM against these lines, respectively. Selectivity is also observed for GB-II-5 with purified leishmanial and mammalian tubulin. The assembly of 15 μM leishmanial tubulin is completely inhibited by 10 μM GB-II-5, whereas 40 μM GB-II-5 inhibits the assembly of 15 μM porcine brain tubulin by only 17%. In cultured *L. donovani* and *T. brucei*, treatment with 5 and 0.5 μM GB-II-5, respectively, causes a striking increase in the fraction of G_2M cells compared with control. Given the potency and selectivity of this agent against kinetoplastid tubulin, GB-II-5 emerges as an exciting new antitrypanosomal and antileishmanial lead compound.

Tubulin is a highly conserved protein in eukaryotic cells. Examination of tubulin sequences from pig, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* using the ExPASy proteomics server (http://us.expasy.org/tools/sim-prot.html) reveals that the yeast and nematode α-tubulins possess 76% and 93% identity with porcine α-tubulin, respectively, whereas the β-tubulins from these organisms are 77% and 93% identical to porcine β-tubulin, respectively. Despite such high levels of identity, differences in susceptibility to antimitotic agents are known to exist between tubulins from different organisms. For example, the antifungal compound methyl *N*-(benzimidazol-2-yl)carbamate is selective for yeast tubulin. Past studies comparing yeast tubulin with the corresponding protein from bovine brain indicated that methyl *N*-(benzimidazol-2-yl)carbamate was at least 300-fold more potent as an inhibitor of yeast tubulin assembly (Kilmartin, 1981). Certain anthelmintic benzimidazoles, such as oxfendazole and thiabendazole are also more effective in vitro against nematode tubulin than mammalian tubulin (Dawson et al., 1984). Thus, differences in tubulin drug susceptibility can be the basis for selective chemotherapeutic intervention against certain infectious organisms.

Another class of molecules known to possess phylogenetic selectivity for tubulin is the antimitotic herbicides (see Morejohn and Fosket, 1991, for review). Treatment of susceptible plants with dinitroanilines such as trifluralin and oryzalin causes morphological abnormalities in the root tip, a region of the plant exhibiting high levels of cell division. The dinitroaniline herbicide oryzalin inhibits the rapid phase of paclitaxel (Taxol)-induced polymerization of rose tubulin at low micromolar concentrations but does not inhibit paclitaxel-induced bovine brain tubulin assembly at similar concentrations (Morejohn et al., 1987). In addition, the binding of 10 μM oryzalin to rose tubulin was 17-fold greater than binding to bovine brain tubulin as assessed by a DEAE cellulose filter binding assay (Morejohn et al., 1987). The phosphoric thioamide herbicide amiprophos-methyl (APM) also inhibits the paclitaxel-induced assembly of rose tubulin in vitro at low micromolar concentrations, whereas 100 μM concentrations of APM have no effect on the paclitaxel-promoted assembly of

ABBREVIATIONS: APM, amiprophos-methyl; GB-II-5, *N*1-phenyl-3,5-dinitro-*N*4,*N*4-di-*n*-propylsulfonilamide; DMEM, Dulbecco's modified Eagle's medium; PME, PIPES/$MgCl_2$/EGTA; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PIPES, piperazine-*N*-*N*'-bis(ethanesulfonic acid); DAPI, 4′,6-diamidino-2-phenylindole.

bovine brain tubulin (Morejohn and Fosket, 1984). APM competitively inhibits the binding of oryzalin to tobacco tubulin (Murthy et al., 1994). Whereas tobacco cells treated with 50 nM oryzalin or 1 μM APM display disruptions in microtubules, these structures are unaffected in mouse 3T3 fibroblasts exposed to 100 μM APM (Murthy et al., 1994).

Dinitroaniline herbicides also possess activity against protozoan parasites (Nath and Schneider, 1992; Chan et al., 1993; Stokkermans et al., 1996; Benbow et al., 1998), raising hopes that protozoal tubulin could be a target for selective chemotherapeutic agents. Surprisingly, few reports have appeared that describe the effects of such ligands on purified parasite tubulin. These studies have employed tubulin from the kinetoplastid parasite *Leishmania* sp. as a substrate, most likely because the insect form of this organism can be grown on the large scale required to purify reasonable quantities of parasite tubulin. Radiolabeled trifluralin was shown to bind better to partially purified *Leishmania* sp. tubulin than to rat brain tubulin (Chan and Fong, 1990). The related dinitroaniline herbicide oryzalin inhibited the polymerization of partially purified leishmanial tubulin at a concentration of 5 μM and caused the formation of abnormal leishmanial microtubules at a concentration of 10 μM, whereas the rat brain microtubules formed at 50 μM concentrations of this compound seemed normal (Chan et al., 1991). In initial studies from our laboratory, we were unable to demonstrate the activity of trifluralin against purified leishmanial tubulin in a spectrophotometric assembly assay (Werbovetz et al., 1999). However, the more soluble dinitroaniline sulfonamide oryzalin did show moderate inhibitory activity in our hands against leishmanial tubulin, and a series of oryzalin analogs synthesized in our laboratory possessed activity superior to oryzalin against parasite tubulin assembly and *Leishmania* sp. proliferation in vitro (Bhattacharya et al., 2002).

In the present study, we investigate the selectivity of these novel dinitroaniline sulfonamides for leishmanial tubulin compared with porcine brain tubulin. We also investigate the cellular mechanism of action of these compounds in *Leishmania* sp. and the related kinetoplastid parasite *Trypanosoma brucei*, subspecies of which cause African trypanosomiasis. Our results identify *N*1-phenyl-3,5-dinitro-*N*4,*N*4-di-*n*-propylsulfanilamide (GB-II-5) as a molecule with potent, selective antimitotic effects against leishmanial tubulin and kinetoplastid parasites in vitro.

Materials and Methods

Chemicals and Biochemicals. Where not otherwise indicated, commercial materials were obtained from Sigma (St. Louis, MO). Dinitroaniline sulfonamides were synthesized as outlined previously (Bhattacharya et al., 2002). Ansamitocin P3 was obtained from the drug inventory of the National Cancer Institute.

Determination of the Drug Susceptibility of Parasites and Mammalian Cells. The susceptibility of *Leishmania donovani* amastigote-like parasites (WHO designation MHOM/SD/62/1S-CL2$_D$) to growth inhibition by compounds of interest was measured in a 3-day assay using the tetrazolium dye-based CellTiter reagent (Promega, Madison, WI) (Werbovetz et al., 1999; Havens et al., 2000). The amastigote medium used in this assay is based on the medium mentioned by Joshi et al. (1993). Before addition of fetal bovine serum to a final concentration of 20%, this amastigote medium contains 15 mM KCl, 115 mM KH_2PO_4, 10 mM K_2HPO_4, 0.5 mM $MgSO_4$, 24 mM $NaHCO_3$, a 1$\times$ concentration of RPMI-1640 vitamins and amino acids, 2.0 mM L-glutamine, 22 mM D-glucose, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM adenosine, 1 μg/ml folate, and 25 mM MES, pH 5.5. Compounds were tested for their activity against bloodstream-form *Trypanosoma brucei brucei* (MITat 1.2, variant 221) axenically-cultured in HMI-9 medium (Hirumi and Hirumi, 1989) following the procedure of Ellis et al. (1993) with some modifications. Briefly, 100 μl of late log phase parasites were incubated in 96-well plates (Costar, Cambridge, MA) at an initial concentration of 10^5 cells/ml with or without test compounds at 37°C in a humidified 5% CO_2 atmosphere for 72 h. Twenty-five microliters of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (prepared in phosphate-buffered saline and filter-sterilized) was then added to each well, and plates were re-incubated at 37°C as before for 2 h. One hundred microliters of 20% SDS lysis buffer (prepared in 50% aqueous dimethyl formamide) was added to each well and plates were incubated as before for an additional 3 to 4 h. Optical densities were then measured at 570 nm using a SpectraMax Plus microplate reader (Amersham Biosciences, Piscataway, NJ). IC_{50} values (concentration of the compound that inhibited cell growth by 50% compared with untreated control) were determined with the aid of the software program SoftMax Pro (Amersham Biosciences). This program uses the dose-response equation $y = [(a - d)/(1 + (x/c)^b)] + d$, where x = the drug concentration, y = absorbance at 490 nm, a = upper asymptote, b = slope, c = IC_{50}, and d = lower asymptote. Assessment of the in vitro sensitivity of *T. brucei brucei* Lab 110 EATRO was performed by methods described by Donkor et al. (2001).

The toxicity of compounds to J774 murine macrophages and PC3 prostate cancer cells was measured in a 3-day assay using the CellTiter reagent. J774 macrophages in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2.0 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin were added to individual wells of a 96-well plate at a concentration of 10^4 cells/ml and a total volume of 100 μl. Macrophages were allowed to adhere for 24 h, then the medium was removed and replaced with serial dilutions of the test compounds in the DMEM mentioned above without phenol red. After 72-h incubation with the test compounds at 37°C in a humidified 5% CO_2 incubator, cell viability was determined using the CellTiter reagent by adding 20 μl of assay solution to each well. After a 6- to 7-h incubation at 37°C to allow for color development, the absorbance of each well at 490 nm was measured in a SpectraMax Pro microplate reader. The toxicity of compounds to PC3 cells was assessed in the same manner as for the macrophages, except that the medium used was RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2.0 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. IC_{50} values for the J774 and PC3 assays were determined with the software program SoftMax Pro as described above.

Tubulin. Rat brain tubulin, used for fluorescence quenching assays, was isolated from rat brain microtubule protein (Sackett et al., 1991) by differential assembly as described previously (Wolff et al., 1996). Porcine brain tubulin, employed for assembly assays, was isolated using DEAE-Sepharose ion exchange chromatography. Briefly, fresh porcine brains were obtained from a local slaughterhouse, sliced into ~1-cm sections, frozen on dry ice, and stored at −80°C until use. Approximately 100 g of frozen brain was broken into small pieces and thawed in 100 ml of PME + DTT buffer (0.1 M PIPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT). The thawed brain pieces were homogenized in a blender at 4°C using three 5-s bursts at high power. A 150-ml portion of this homogenate was centrifuged in a Ti-70 rotor at 100,000*g* for 40 min at 4°C, then the supernatant was loaded at 4°C on a column containing 11 ml DEAE-Sepharose Fast Flow matrix (Amersham Biosciences) that had previously been equilibrated with PME + DTT. This column was washed with 2 column-volumes of PME + DTT, 4 volumes of PME containing 0.3 M glutamate, and 4 volumes of PME + DTT containing 1 M glutamate. Tubulin was eluted from the column using PME + DTT containing 0.75 M glutamate and 0.3 M KCl. Fractions rich in tubulin (consisting of approximately 10 ml total) were pooled, then

GTP and DMSO were added slowly with gentle vortexing to bring the final concentrations of these components to 1 mM and 8% (v/v), respectively. This solution was incubated at 37°C for 40 min, then the cloudy solution containing microtubules was centrifuged at 100,000*g* at 35°C for 30 min. The pellet was resuspended in 2.3 ml of PME on ice and sonicated once using a Microson XL2000 sonicator (Misonix, Farmingdale, NY) at an output power of 10 W to completely redissolve the pellet. This solution was incubated on ice for an additional 30 min, then spun at 80,000*g* at 4°C for 20 min. The supernatant containing porcine tubulin was stored at −80°C in 80-μl aliqouts. Leishmanial tubulin was purified by the method of Werbovetz et al. (1999), except that parasite tubulin was eluted from the DEAE-Sepharose Fast Flow column with PME containing 0.75 M glutamate and 0.3 M KCl as above for mammalian tubulin rather than with PME containing 0.7 M KCl as reported earlier.

Tubulin Assembly Assays. Assembly reactions were performed in 96-well half-area microplates (Costar) in a final volume of 50 μl. For both leishmanial and brain tubulin, reactions contained final concentrations of 1.5 mg/ml (15 μM) tubulin, 0.1 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 10% (v/v) DMSO, and 1 mM GTP with and without compounds of interest. Components of the reaction mixtures were added to the microplate on ice, then assembly was initiated by the simultaneous addition of a GTP solution to all sample wells with a multichannel pipet. The change in turbidity was measured at 351 nm using the SpectraMax Plus microplate reader immediately after GTP addition at 30°C (for leishmanial tubulin) or 37°C (for brain tubulin). To ensure maximal solubility of GB-II-5 at a concentration of 40 μM, compounds were added to reactions containing brain tubulin in 5-μl volumes containing 100% DMSO, and assembly was initiated by adding 10 μl of 5 mM GTP in water. The assembly of leishmanial tubulin occurred too rapidly for accurate measurement under the conditions described for brain tubulin. Because lower concentrations of GB-II-5 affect leishmanial tubulin and solubility was thus not as much of a concern, compounds were added to reactions containing parasite tubulin in 5-μl volumes containing 50% DMSO, then assembly was initiated by adding 10 μl of 5 mM GTP in 25% DMSO.

Measurement of Dissociation Constants by Fluorescence Quenching. Binding of dinitroanilines to tubulin was measured by the resulting quenching of intrinsic tubulin tryptophan fluorescence, a method that has proved useful in protein binding studies of many compounds (Dufour and Haertle, 1991). The strong 300 to 350 nm absorbance of these compounds (1 mM oryzalin has absorbance of ~4.5 cm^{-1} at 325 nm) makes them good acceptors of resonance energy transfer from the photoexcited tryptophan residues, resulting in reduction of tryptophan fluorescence. Samples were studied using short pathlength (3 mm) cells (Hellma Cells, Plainview, NY). Tubulin samples with increasing concentrations of test compound were excited at 290 nm, and tryptophan fluorescence was measured between 310 and 340 nm. Spectra were recorded using an ISS PC1 photon counting spectrofluorometer (ISS, Champaign, IL) with excitation and emission slits of 2 and 1 mm, respectively. Emission intensity at 325 nm was taken for calculation of quenching. Sample fluorescence was corrected for the small photobleaching by measuring a tubulin sample with additions of DMSO. Inner filter effects were corrected using a sample of *N*-acetyltryptophanamide and additions of the test compound. To minimize inner filter corrections, absorbance at 325 nm because of test compounds was typically kept <0.15. The corrected fluorescence decrements as a function of test compound concentration were fitted to a single-site binding model using the nonlinear fitting routines of Prism software (GraphPad Software, San Diego, CA). K_d values and S.E. of the fit were also calculated by this software.

Flow Cytometry and Fluorescence Cell Sorting. Parasites were incubated in T25 flasks (3–5-ml final volume) in the appropriate media described earlier for 24 to 48 h. Cultures contained 1% (v/v) DMSO in the presence or absence of test compounds. Cells were counted using a hemocytometer, then were centrifuged at 1000*g* at 4°C for 10 min. Cells were resuspended in 150 μl of PBS (0.01 M phosphate, 0.137 M NaCl, and 2.7 mM KCl); then, 350 μl of ice-cold methanol was added and the samples were fixed at −20°C for 2 to 3 h. Cells were centrifuged as before and resuspended in PBS containing 0.1% TX-100, 5 μg/ml RNase A, and 10 μg/ml propidium iodide for 20 to 30 min. Centrifugation was repeated, then cells were resuspended in PBS to a final concentration of 5×10^5 to 10^6/ml and stored at 4°C until analysis. Fluorescence cell sorting was conducted on an Elite flow cytometer (Beckman Coulter, Fullerton, CA). In each case, gating was performed to exclude doublets and aggregates.

Fluorescence Microscopy. *L. donovani* promastigotes were incubated with or without test compounds for 2 days and centrifuged as described above. Parasites were resuspended in PBS at a concentration of 2×10^7 cells/ml; then, 10^6 parasites were applied to a microscope slide previously coated with 0.1 mg/ml poly-L-lysine. Parasites were allowed to adhere for 10 min; the cells were fixed in methanol for 5 min, rinsed with PBS, then placed in 0.1% TX-100 in PBS for 5 min. Slides were then washed with PBS, and were incubated with a 1:100 to 1:1000 dilution of a FITC-conjugated anti–α-tubulin antibody (clone DM 1A) for 1 h at room temperature. After washing the slides three times with PBS for 5 min each, 4′,6-diamidino-2-phenylindole (DAPI) was applied to slides at a concentration of 10 μg/ml. Slides were washed again in PBS, then coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA) in preparation for fluorescence microscopy using a Nikon Labophot-2 microscope equipped with a Nikon FX-35DX camera (Nikon, Tokyo, Japan).

Results

Activity of Dinitroaniline Sulfonamides against Kinetoplastid Parasites and Mammalian Cells. Earlier, we described a new series of oryzalin analogs that showed improved activity, compared with the parent compound, against axenic amastigote-like *L. donovani* parasites in culture (Bhattacharya et al., 2002). In this study, we examined oryzalin and two of its more potent analogs, GB-II-5 and GB-II-46, in more detail against *Leishmania* sp., African trypanosomes, and mammalian cells. GB-II-5 differs from oryzalin only in that the N1 position of the sulfonamide is substituted with a phenyl ring, whereas GB-II-46 is distinct from oryzalin by virtue of a dibutyl rather than a dipropyl substitution at N4. In vitro assays against *L. donovani* and *T. brucei brucei* revealed that GB-II-5 was also the most active compound against kinetoplastid parasites (Table 1). GB-II-5 was more active against African trypanosomes than *Leishmania* sp., displaying an IC_{50} of 0.41 μM against *T. brucei brucei* MITat 1.2, variant 221 and an IC_{50} of 0.73 μM against *T. brucei brucei* Lab 110 EATRO. GB-II-5 thus approaches the in vitro activity of the clinical antitrypanosomal agent suramin, which possessed an IC_{50} value of 0.23 μM against the trypanosomes. Pentamidine, another agent used clinically to treat the early stage of African trypanosomiasis, is more than an order of magnitude more active in vitro than either GB-II-5 or suramin. Assays conducted against J774 murine macrophages and PC3 prostate cancer cells revealed that GB-II-5 was 5.8- and 7.0-fold more active against *L. donovani* axenic amastigotes and 71- and 85-fold more potent against *T. brucei* MITat 1.2 bloodstream forms than against J774.G8 macrophages and PC3 prostate cancer cells, respectively. Oryzalin and GB-II-46 displayed similar toxicity to *Leishmania* sp. and mammalian cell lines and were 6- to 11-fold more effective against African trypanosomes.

Effect of Dinitroaniline Sulfonamides on the Assembly of Leishmanial and Porcine Brain Tubulin. We showed earlier that increasing the number of carbons in the N4 alkyl chain of oryzalin, as in GB-II-46, and placing a

substituent at the N1 nitrogen of oryzalin, as in GB-II-5, increases the potency of the compounds against parasite tubulin assembly compared with oryzalin (Bhattacharya et al., 2002). To provide a more quantitative measure of the effect of these compounds on leishmanial tubulin assembly, different concentrations of the dinitroaniline sulfonamides were included in purified parasite tubulin assembly reactions, and the polymerization of leishmanial tubulin into microtubules was monitored spectrophotometrically (Fig. 1). GB-II-5 at concentrations of 10 and 20 μM completely inhibited the assembly of 15 μM leishmanial tubulin, whereas 5 μM GB-II-5 slowed the kinetics and inhibited the final extent of leishmanial tubulin assembly compared with the control (Fig. 1A). Oryzalin was much less potent than GB-II-5, with 20 and 40 μM concentrations of the compound slowing the initial rate but not the final extent of leishmanial tubulin assembly after 20 min (Fig. 1B). GB-II-46 inhibited the assembly of leishmanial tubulin more effectively than oryzalin but was clearly less potent than GB-II-5 against parasite tubulin assembly (Fig. 1C). We also examined the effects of these compounds on the polymerization of purified porcine brain tubulin (Fig. 2). Inclusion of 40 μM GB-II-5 (Fig. 2A) or 40 μM GB-II-46 (Fig. 2C) slowed the kinetics and inhibited the final extent of the assembly of 1.5 mg/ml (15 μM) porcine brain tubulin after 20 min, whereas almost no effect was observed with 40 μM oryzalin (Fig. 2B). When examined in four separate assembly experiments, 40 μM GB-II-5 inhibited the assembly of 15 μM porcine brain tubulin by 17 $\pm$ 6% (mean $\pm$ S.E.M.) compared with control after 20 min at 37°C.

Binding Affinity of Dinitroaniline Sulfonamides for Leishmanial and Porcine Brain Tubulin as Determined by Tryptophan Fluorescence Quenching. As another assessment of the binding of the dinitroaniline sulfonamides to parasite and mammalian tubulin, dissociation constants were determined by measuring the concentration dependence of quenching of tubulin tryptophan fluorescence by these ligands (Table 2). GB-II-5 displayed the lowest dissociation constant for leishmanial tubulin (K_d = 1.7 μM). Although oryzalin bound more tightly to leishmanial tubulin than brain tubulin, the selectivity measured in this assay was slightly less than that seen with GB-II-5. GB-II-46 displayed comparable affinity for leishmanial and porcine brain tubulin.

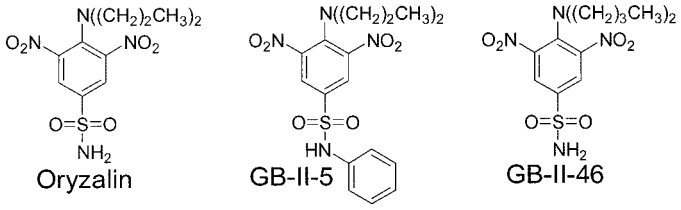
Effect of Dinitroaniline Sulfonamides on the Cell Cycle of *L. donovani* Promastigotes. To examine whether the oryzalin analogs had any influence on microtubule-mediated processes in *Leishmania* sp., we first performed a cell-cycle analysis on dinitroaniline-treated parasites. Promastigotes were cultured for 2 days with concentrations of dinitroaniline sulfonamides that were close to their in vitro IC_{50} values, then samples were stained with propidium iodide and analyzed by flow cytometry (Fig. 3). Compared with parasites grown for 2 days in the absence of compound (Fig. 3A), the fraction of promastigotes present in G_2M was greatly increased after a 2-day incubation with 5 μM GB-II-5. An increase in G_2M parasites was also apparent in promastigotes treated for 1 day with GB-II-5 but was less pronounced after this shorter incubation (data not shown). Promastigotes treated with 2 μM concentrations of the nonselective mammalian antimicrotubule agent ansamitocin P3 also gave an increase in the percentage of G_2M cell types and caused the appearance of parasites containing four times the amount of DNA as control cells in G_1 (Havens et al., 2000). There were no clear cell cycle changes with either 50 μM oryzalin (Fig. 3C) or 20 μM GB-II-46 (Fig. 3D) in this experiment. When cell cycle analysis was performed on promastigotes treated for 2 days with 10 μM GB-II-5, even more striking results were obtained (Fig. 4). During the time in which these experiments were conducted, control parasites doubled approximately three times, as assessed by hemocytometer counts. Histograms corresponding to parasites treated with 10 μM GB-II-5 show that the majority of cells contain four times the amount of DNA present in G_1-phase control parasites (note the log scale in Fig. 4).

Promastigote Cell Types Present in Control and Dinitroaniline-Treated Cultures. *L. donovani* promastig-

TABLE 1

IC_{50} values (micromolar) for oryzalin analogs against kinetoplastid parasites and mammalian cell lines

Values represent the mean $\pm$ S.E. of the measurement of at least three independent experiments.



Compound	*L. donovani* axenic amastigotes[a]	*T. brucei brucei* variant 221[b]	*T. brucei brucei* Lab 110 EATRO[c]	J774 macrophages[d]	PC3 prostate[d]
			μM		
Oryzalin	72 $\pm$ 10	11 $\pm$ 0	6.6 $\pm$ 1.0	41 $\pm$ 5	57 $\pm$ 4
GB-II-5	5.0 $\pm$ 0.6	0.41 $\pm$ 0.02	0.73 $\pm$ 0.09	29 $\pm$ 1	35 $\pm$ 1
GB-II-46	20 $\pm$ 2	2.6 $\pm$ 0.3	1.9 $\pm$ 0.7	9.4 $\pm$ 2.0	23 $\pm$ 4
Pentamidine	2.2 $\pm$ 0.2	0.019 $\pm$ 0.003	0.002 $\pm$ 0.001	NT	NT
Suramin	NT	0.23 $\pm$ 0.03	NT	NT	NT
Ansamitocin P3	NT	NT	NT	0.00087 $\pm$ 0.00019	0.000060 $\pm$ 0.000024

NT, not tested.

[a] Determined by tetrazolium dye assay after 3-day incubation with test compounds. The starting concentration of cells was 10^6 parasites/ml.

[b] Determined by tetrazolium dye assay after 3-day incubation with test compounds. The starting concentration of cells was 10^5 parasites/ml.

[c] Determined by Coulter counter after 2-day incubation with test compounds as described in Donkor et al. (2001). The starting concentration of cells was 10^5 parasites/ml.

[d] Determined by tetrazolium dye assay after 3-day incubation with test compounds. The starting concentration of cells was 10^4 cells/ml.

otes were exposed to dinitroaniline compounds for 2 days, then parasites were fixed, stained with DAPI and a FITC-conjugated anti–α-tubulin antibody, and scored microscopically to quantitate the cell types present (Fig. 5). More than 200 parasites were scored according to cell type in each sample, with K representing kinetoplast and N representing nucleus. Log phase promastigote control cultures contain a predominance of cells exhibiting one flagellum, one nucleus, and one kinetoplast and are designated as 1K1N (Havens et al., 2000). Micrographs of several typical 1K1N parasites, where nuclei and kinetoplasts are revealed by DAPI staining, are shown in Fig. 6, A to C. Approximately 5% of promastigotes in these control cultures are in a late stage of mitosis and are designated as 2K2N or 1K2N cells, depending on whether there is a discernible gap between the replicated kDNA discs

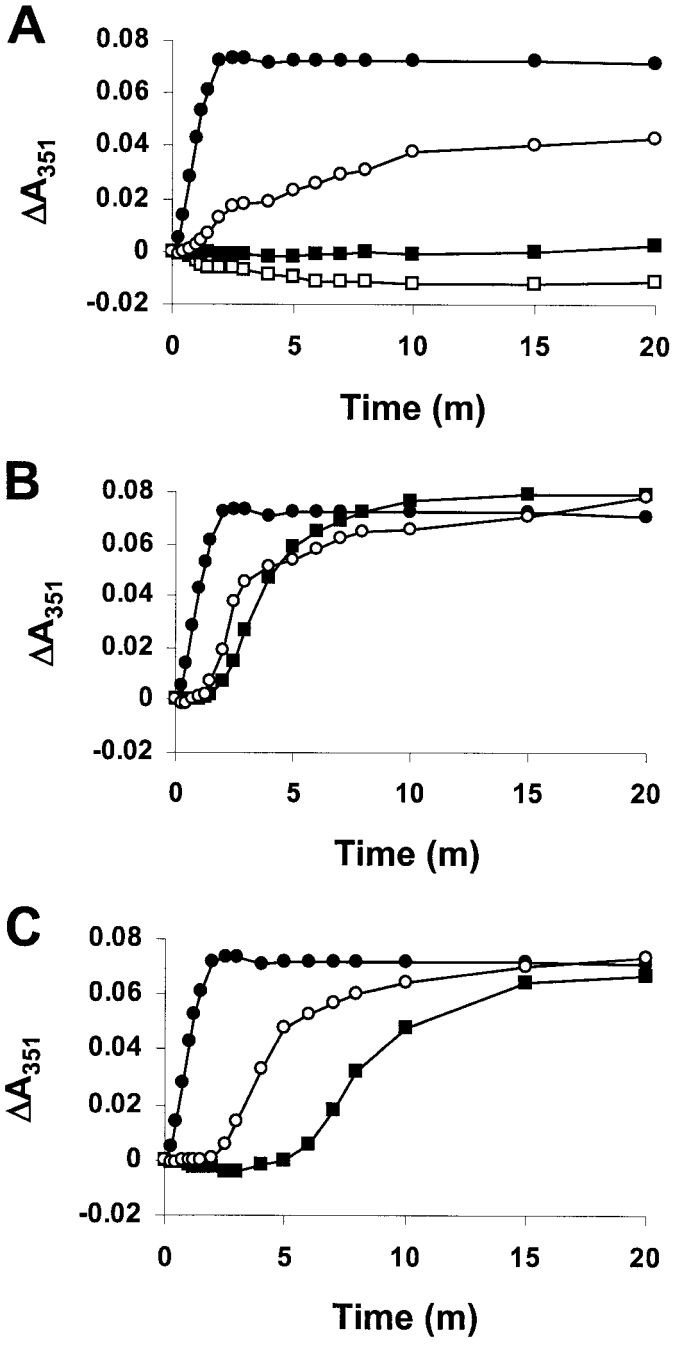


Fig. 1. Effect of dinitroaniline sulfonamides on the assembly of purified tubulin from *Leishmania* sp. The assembly of 1.5 mg/ml (15 μM) purified leishmanial tubulin was assessed at 30°C at 351 nm in the presence or absence of different concentrations of dinitroaniline sulfonamides as described under *Materials and Methods*. In each, the assembly curve for the control sample is indicated by ●. A, effect of the inclusion of GB-II-5 at 5 (○), 10 (■), and 20 μM (□). B and C, oryzalin and GB-II-46, respectively, are included at 20 (○) and 40 μM (■). Unless otherwise indicated, experiments pictured in the figures were performed in triplicate, with the results of a representative experiment shown.

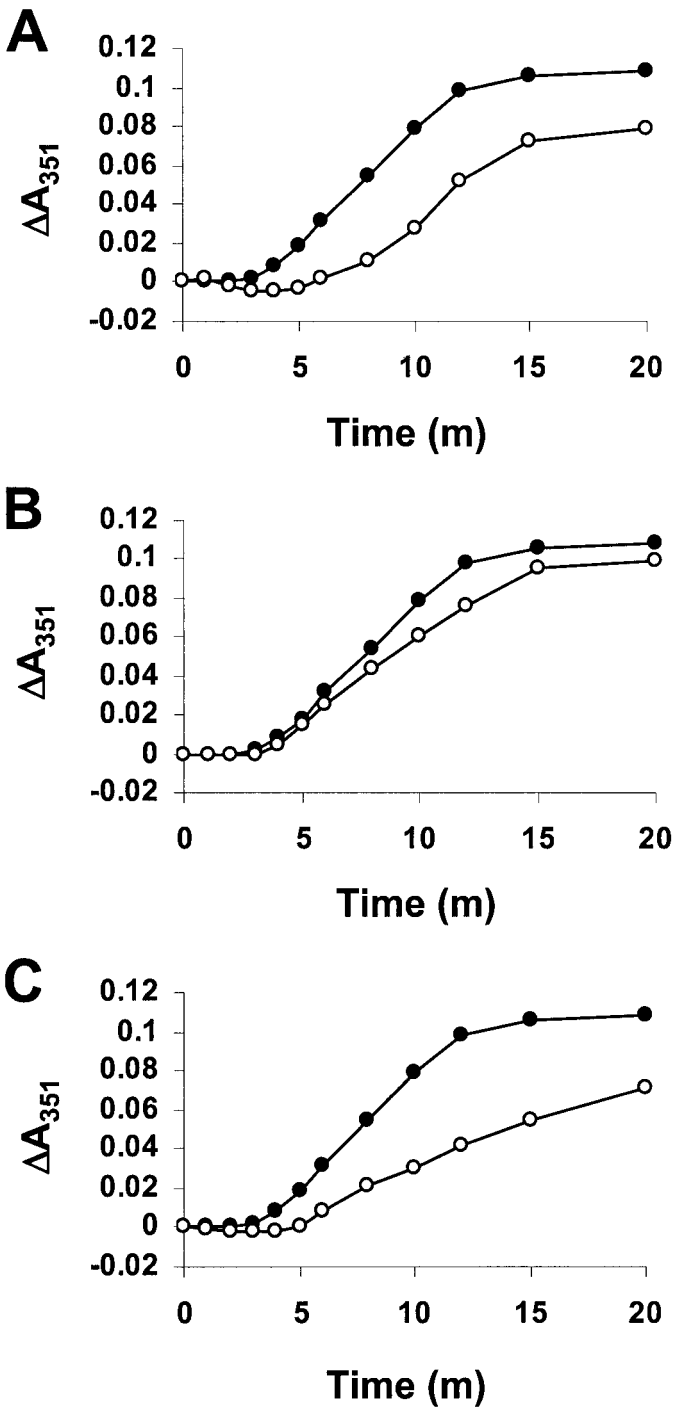


Fig. 2. Assembly of porcine brain tubulin in the presence of dinitroaniline sulfonamides. Porcine brain tubulin assembly in the presence (○) or absence (●) of 40 μM concentrations of dinitroaniline sulfonamides was assessed at 37°C at 351 nm using 1.5 mg/ml (15 μM) purified protein. A, GB-II-5; B, oryzalin; C, GB-II-46.

in the kinetoplasts. Note that the smaller kinetoplast DNA discs stain more intensely with DAPI than the nuclear DNA. A 1K2N cell is also pictured in Fig. 6, A to C. The kinetoplasts have replicated in this cell, but they remain closely associated until the daughter cells divide. A more complete discussion of the morphology of *Leishmania* sp. promastigotes undergoing mitosis has been presented previously (Havens et al., 2000).

Parasites treated with 50 μM oryzalin and 20 μM GB-II-46 showed little difference from controls in the percentage of cell types observed, but parasites exposed to 5 μM GB-II-5 displayed a dramatic increase in the fractions of 2K1N cell types, multi K,N cell types, and kinetoplastid cytoplasts with one kinetoplast and no nucleus (zoids) (Fig. 5). The same cell types were observed upon treatment of promastigotes with 2 μM ansamitocin P3. Shown in Fig. 6, D to F, are examples of two 2K1N cells and a 3K1N cell produced upon treatment of promastigotes with 5 μM GB-II-5 along with a 1K1N cell that is also present in this field. Note that the multiple kinetoplasts are distinct from one another spatially, as seen previously in *Leishmania* sp. promastigotes treated with nonselective antimicrotubule agents (Havens et al., 2000), and that the single nuclei of the aberrant cells pictured are enlarged compared with controls. Zoids (Robinson et al., 1995; Havens et al., 2000) were also observed in parasite cultures treated with 5 μM GB-II-5 and 2 μM ansamitocin P3, but a 10 μM dose of GB-II-5 decreased the percentage of zoids and increased the fraction of 2K1N and multi K,N cell types.

Cell Cycle Effects of Dinitroaniline Sulfonamides on *T. brucei*. Axenic cultures of *T. brucei* MITat 1.2 bloodstream forms were incubated for 24 h in the presence of 0.5 or 1 μM GB-II-5 and 1 nM ansamitocin P3, a compound that was previously shown to block microtubule-mediated processes in African trypanosomes (Robinson and Gull, 1991). In this experiment, 0.5 μM GB-II-5 inhibited parasite growth by approximately 50% compared with controls as assessed by hemacytometer counts, whereas 1 μM GB-II-5 and 1 nM ansamitocin P3 inhibited *T. brucei* growth by about 80%. GB-II-5 at a concentration of 0.5 μM increases the fraction of cell types in G_2M and also causes the appearance of cell types containing roughly four times the amount of DNA present in G_1 phase parasites (Fig. 7, compare control A to GB-II-5 treated sample in B). At a concentration of 1 μM, GB-II-5 caused a dramatic increase in the number of cell types possessing four times the DNA of the G_1 peak, whereas the G_1 peak itself nearly vanished (Fig. 7C). Exposure of *T. brucei* to 1 nM ansamitocin P3 (Fig. 7D) gave results similar to those observed after treatment of parasites with 1 μM GB-II-5.

TABLE 2

Dissociation constants for oryzalin analogs against leishmanial and rat brain tubulin as determined by tryptophan fluorescence quenching

Values are derived from fits to data from four to nine different concentrations of the compound. The values given are the fitted K_d ± S.E. of the fit (see *Materials and Methods*).

Compound	K_d vs. Leishmanial Tubulin	K_d vs. Brain Tubulin
	μM	
Oryzalin	19 ± 3	115 ± 15
GB-II-5	1.7 ± 0.4	13 ± 4
GB-II-46	8.3 ± 3.0	13 ± 6

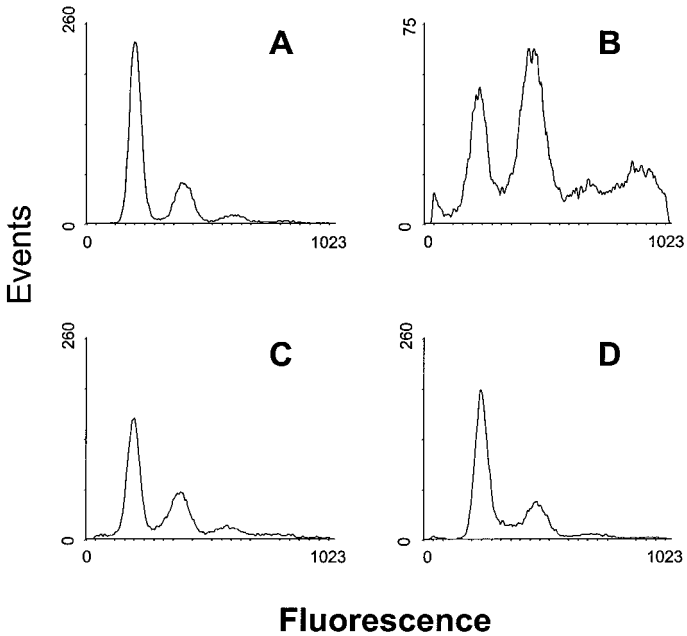


Fig. 3. Cell cycle analysis of *L. donovani* promastigotes treated with dinitroaniline sulfonamides. After 48-h treatments in the presence of 1% DMSO (A), 5.0 μM GB-II-5 (B), 50 μM oryzalin (C), or 20 μM GB-II-46 (D), parasites were fixed, stained with propidium iodide, and analyzed by flow cytometry as described under *Materials and Methods*.

Discussion

Compounds with selective activity against both protozoan parasites and protozoal tubulin are of interest as chemotherapeutic candidates and as probes for tubulin ligand binding domains. In this study, we compared the antitubulin and antiproliferative activities of oryzalin and two of its more active derivatives, GB-II-5 and GB-II-46, on kinetoplastid parasites and mammalian cells. GB-II-5 is the most potent agent in this group in vitro against *Leishmania* sp. and trypanosomes, possessing better activity than oryzalin, by at

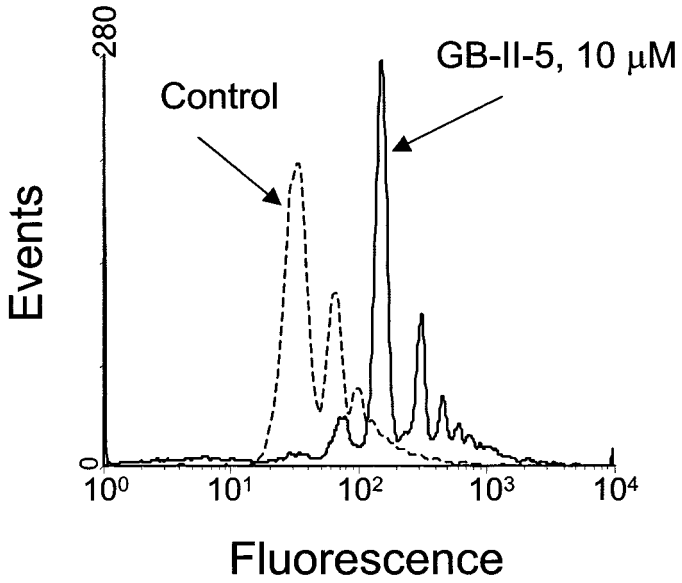


Fig. 4. Effect of 10 μM GB-II-5 treatment on the DNA content of *L. donovani*. Promastigotes treated for 48 h at 26°C with 1% DMSO or 10 μM GB-II-5 were processed as described in Fig. 3 and under *Materials and Methods*. Note the log scale of the *x*-axis in this experiment.

least an order of magnitude, against both parasites (Table 1). The selectivity of GB-II-5 for trypanosomes versus mammalian cells is particularly striking, because this compound is roughly 2 orders of magnitude more potent against *T. brucei* MITat 1.2 than against two cancer cell lines. Oryzalin possesses moderate activity against parasites and the mammalian cell lines, whereas GB-II-46 displays the highest potency against J774 macrophages and PC3 cells, exhibiting more activity against the J774 cell line than against *L. donovani*. Subsequent experiments sought to better define the molecular basis for the potent and selective activity of GB-II-5 against kinetoplastid parasites.

Our earlier report indicated that both GB-II-5 and GB-II-46 were better inhibitors of leishmanial tubulin assembly than oryzalin (Bhattacharya et al., 2002). Our current study employed a range of dinitroaniline sulfonamide concentrations and higher concentrations of leishmanial tubulin, revealing the superiority of the N1-phenyl substituted GB-II-5 over GB-II-46, a compound with longer alkyl chains at N4 (Fig. 1, compare A and C). Assembly experiments with purified porcine brain tubulin (Fig. 2) show that brain tubulin assembles more slowly than the corresponding leishmanial protein under the conditions employed here. Nonetheless, it is clear that porcine brain tubulin is much less sensitive to assembly inhibition by GB-II-5 than leishmanial tubulin (compare Figs. 1A and 2A). Oryzalin has negligible effects on porcine brain tubulin assembly at a concentration of 40 μM (Fig. 2B), whereas the action of 40 μM GB-II-46 on mammalian tubulin polymerization (Fig. 2C) is similar to 40 μM GB-II-5.

Dissociation constants obtained by fluorescence quenching experiments, shown in Table 2, are consistent with the assembly assays presented in Figs. 1 and 2. The selectivity of both oryzalin and GB-II-5 for leishmanial tubulin is clearly shown in Table 2, as is the enhanced affinity of the latter compound for parasite tubulin. GB-II-46, on the other hand, displays similar affinity for parasite and mammalian tubulin. These data indicate that substitution at the N1 position of these molecules maintains or slightly improves selectivity for parasite tubulin and improves the affinity of the molecules for the target. Increasing the alkyl chain length at N4 from three to four carbons seems to eliminate selectivity for kinetoplastid tubulin in the absence of an N1 substitution.

Figures 3 to 6 provide evidence that GB-II-5 acts through a tubulin mechanism in *Leishmania* sp. and *T. brucei*. Whereas treatment of *L. donovani* promastigotes with concentrations of oryzalin and GB-II-46 that are close to the measured in vitro IC_{50} values of these compounds have little or no effect on the parasite cell cycle, exposure of *L. donovani* to 5 μM GB-II-5 causes a dramatic increase in the fraction of parasites in G_2M (Fig. 3). At 10 μM GB-II-5, cells containing approximately four times the amount of DNA present in G_1

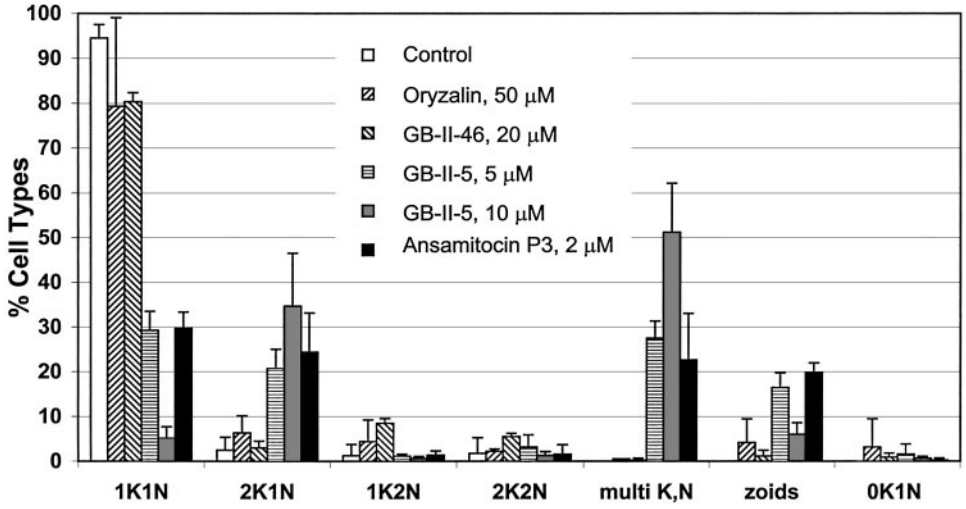


Fig. 5. Cell types of *L. donovani* promastigotes treated with dinitroaniline sulfonamides. Parasites were incubated with or without compounds for 48 h, stained with DAPI and a FITC-conjugated anti-α-tubulin antibody, and evaluated by fluorescence microscopy to quantify the cell types present. More than 200 cell types were counted for the control, and each sample was treated with a given concentration of compound. Results shown represent the mean plus the range (difference between the values) obtained in two independent experiments.

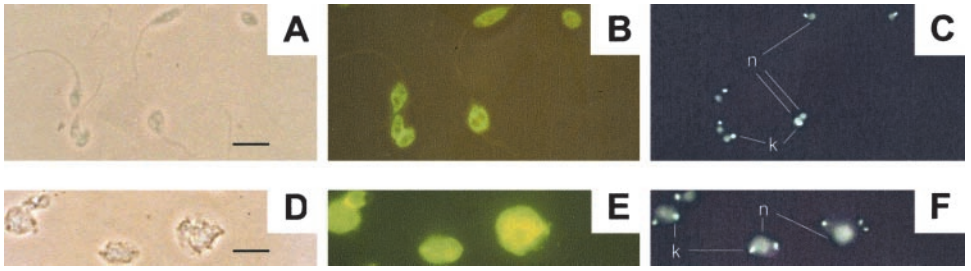


Fig. 6. Micrographs of *L. donovani* in the absence and presence of 5 μM GB-II-5. Promastigotes were incubated for 48 h at 26°C with 1% DMSO or 5 μM GB-II-5, stained with a FITC-conjugated anti–α-tubulin antibody and DAPI, then viewed. Parasites cultured in the presence of 1% DMSO are shown in A to C, whereas GB-II-5 treated parasites are pictured in D to F. Images were obtained using bright-field microscopy (A and D) and fluorescence microscopy, where staining from a FITC-conjugated anti–α-tubulin antibody (B and E) and DAPI (C and F) was observed. A to C, five parasites containing one kinetoplast (k) and one nucleus (n) which would be scored as 1K1N cells. The other parasite would be scored as 1K2N and contains nuclear DNA that has recently partitioned in preparation for cytokinesis. Kinetoplasts of parasites in mitosis, though replicated, remain closely associated until the daughter cells divide. D to F, parasite cell types that would be scored as (left to right) 2K1N, 1K1N, 2K1N, and 3K1N. Note the spatially distinct kinetoplasts and the higher apparent nuclear DNA contents of the cell types containing multiple kinetoplasts. The 3K1N cell shown in these is larger than most of the other cells observed in the field. Scale bars in A and D, 10 μm.

parasites are the most prominent species, while other cells containing greater amounts of DNA are also observed (Fig. 4). The effects of GB-II-5 on the cell cycle in *L. donovani* promastigotes are thus similar to those observed when *Leishmania* sp. are treated with the nonselective vinca domain agents ansamitocin P3 and hemiasterlin and the microtubule stabilizer paclitaxel (Havens et al., 2000). These flow cytometry data are consistent with our analysis of promastigote cell types observed after 48-h treatment with dinitroaniline sulfonamide. Exposure of parasites to 5 and 10 μM GB-II-5 dramatically decreases the number of cells containing one kinetoplast and one nucleus and gives rise to aberrant cells containing multiple kinetoplasts, whereas oryzalin and GB-II-46 have comparatively little effect on the cell types produced (Fig. 5). Although oryzalin and GB-II-46 are moderate inhibitors of leishmanial tubulin assembly, the results presented in Figs. 3 and 5 suggest that other mechanisms also contribute to the in vitro antileishmanial effects of these molecules. For GB-II-5, however, the striking effects of this compound on parasite mitosis at concentrations near this compound's in vitro IC_{50} value are strong evidence that GB-II-5 acts through a tubulin mechanism in *Leishmania* sp.

Cell-cycle analysis of trypanosomes treated with GB-II-5 suggests that this compound also works through a tubulin mechanism in *T. brucei* (Fig. 7). The clear increase in G_2M parasites observed when trypanosomes were treated with 0.5 μM GB-II-5 and the similarity between histograms for samples treated with 1 μM GB-II-5 and those exposed to 1 nM ansamitocin P3 indicate an antimitotic effect for this agent in African trypanosomes. Despite the high identity between leishmanial and trypanosomal tubulin, it is possible that single amino acid substitutions or differences in post-translational modifications lead to the higher activity of GB-II-5 in trypanosomes. Efficient entry into the organism, whether by passive diffusion or a specific transporter, could also influence the in vitro potency of the compounds. Treatment with antimitotic agents frequently triggers apoptosis in mammalian cells (Jordan et al., 1996; Casenghi et al., 1999), and markers of apoptosis have been observed in kinetoplastid parasites (Welburn et al., 1996; Arnoult et al., 2002). Different cellular responses to microtubule disruption, such as those involved in apoptotic cascades, may occur in trypanosomes compared with *Leishmania* sp. It is also possible that bloodform *T. brucei* are simply more sensitive to antimitotic agents than *Leishmania* sp. because of the rapid proliferation rate of trypanosomes. Further work is required to test these hypotheses.

Oryzalin and GB-II-46 show moderate inhibition of *Leishmania* sp. proliferation in vitro. However, clear antimitotic effects are absent in *Leishmania* sp. parasites treated with these two compounds, suggesting that the dinitroanilines act on cells by more than one mechanism. Oryzalin inhibits intracellular calcium signaling in mammalian cells (Powis et al., 1997), and the concentrations of oryzalin required to affect calcium-mediated processes in Swiss 3T3 cells are comparable with those that inhibit J774 and PC3 cell growth in our study. By extension, the other dinitroaniline sulfonamides examined here may cause moderate toxicity to mam-

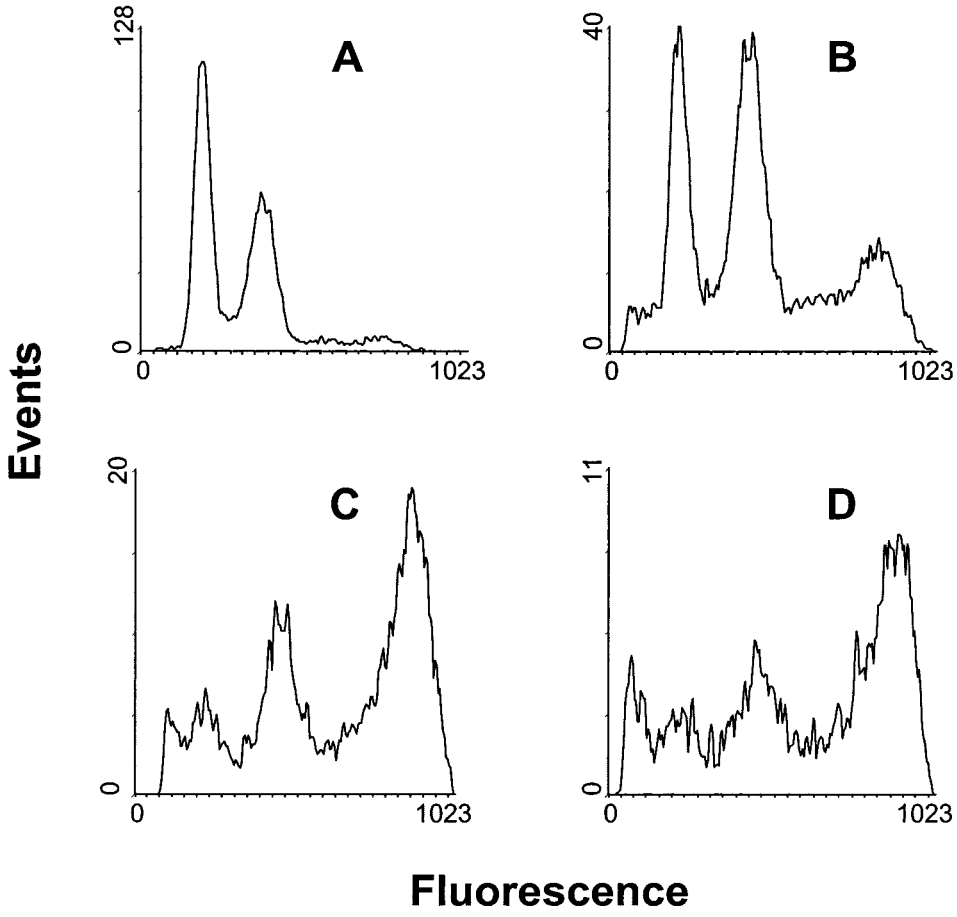


Fig. 7. Flow cytometry analysis of African trypanosomes treated with GB-II-5 and ansamitocin P3. Bloodstream-form *T. brucei* parasites (MITat 1.2, variant 221) were incubated for 24 h at 37°C in HMI-9 medium in the presence of 1% DMSO (A), 0.5 μM GB-II-5 (B), 1 μM GB-II-5 (C), or 1 nM ansamitocin P3 (D). Trypanosomes were fixed, stained with propidium iodide, and analyzed by flow cytometry as described under *Materials and Methods*.

malian cancer cell lines by a similar mechanism. Although oryzalin affects calcium-mediated processes in mammalian cells, this compound displays an LD_{50} of 10 g/kg when given i.v. to rodents (Decker and Johnson, 1976). Molecules based on GB-II-5 may thus have great potential as nontoxic antiparasitic antimicrotubule drug candidates, particularly if other biological effects can be dissociated from microtubule inhibition in parasites.

Based on its activity against kinetoplastid parasites in vitro and its potent and selective effects on leishmanial tubulin, GB-II-5 emerges as an exciting lead compound against these parasites. Our laboratory is currently evaluating a series of N1-substituted derivatives of GB-II-5 for their effects on leishmanial tubulin and kinetoplastid parasites. Given the selective antimitotic activity of GB-II-5 against trypanosomes and *Leishmania* sp., the facile preparation of these molecules, and the drug-like properties of these compounds, we are optimistic that new agents based on GB-II-5 will serve as antitrypanosomal and/or antileishmanial drug candidates in the future.

Acknowledgments

We thank Andy Oberyzyn and Mark Kotur for assistance with flow cytometry and fluorescence microscopy experiments, respectively, and Simon Croft for helpful discussions.

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