Inhibitors of Tubulin Assembly Identified through Screening a Compound Library

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Abstract

Tubulin is the proposed target for drugs against cancer and helminths and is also a validated target in kinetoplastid parasites. With the aim of identifying new lead compounds against *Leishmania* sp., tubulin isolated from *L. tarentolae* was used to screen a 10 000 compound library. One compound, Chembridge No. 7992831 (5), displayed an IC$_{50}$ of 13 μM against *Leishmania* tubulin in an *in vitro* assembly assay and showed a greater than threefold selectivity over mammalian tubulin. Another compound, Chembridge No. 9067250 (8), exhibited good activity against mammalian tubulin (IC$_{50}$ = 5.0 μM). This compound was also toxic to several cancer cell lines with IC$_{50}$ values in the region of 1 μM. Subsequent testing of analogues of 8 contained within the library identified two compounds with greater potency against mammalian tubulin (IC$_{50}$ values of 1.1 and 2.8 μM). The more potent antitubulin agent also showed promising activity against cancer cell lines *in vitro*, with IC$_{50}$ values ranging from 0.18 to 0.73 μM.

Keywords

Tubulin; screen; library; *Leishmania*

Tubulin is an accepted target for treatments against cancer and helminths (1–3). This protein exists as a heterodimer consisting of α and β subunits which polymerize to form microtubules. These microtubules have a number of functions within eukaryotic organisms including chromosome segregation, motility and the maintenance of cellular morphology (4–6). Given these vital roles, tubulin is essential to all eukaryotes. The assembly–disassembly process is critical for the proper functioning of microtubules within the cell. Taxol, one of the more well-known compounds which act against tubulin, does so by stabilizing the protofilaments and thereby prevents disassembly (7). Other compounds such as colchicine and vinblastine inhibit the assembly of tubulin (8–10). Therefore, compounds which affect this assembly–disassembly process could serve as good lead compounds in drug discovery efforts targeting pathogenic eukaryotic cells.

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Tubulin is a validated target in kinetoplastid parasites (11,12) and therefore offers an excellent target against which to develop drug treatments against these organisms. *Leishmania* sp. is responsible for the disease leishmaniasis, which in the case of visceral leishmaniasis can be fatal. An estimated 12 million people currently suffer from leishmaniasis[a]. The current treatments are far from ideal and there is a clear need to identify new lead compounds.

Previous work from our laboratory has attempted to optimize the lead compound oryzalin (**1**) (13) for inhibition of *Leishmania* tubulin assembly. A series of oryzalin analogues have been synthesized with the most promising leads, GB-II-5 (**2**), GB-II-150 (**3**) and **4**, showing low micromolar activity against *Leishmania donovani* axenic amastigotes and mid-nanomolar activity against *Trypanosoma brucei* bloodstream forms (14–17) (Figure 1). However, these compounds suffer from metabolic instability, which limits their activity *in vivo* (16,18). While we continue to optimize these compounds for antikinetoplastid activity and improved metabolic stability, we are also seeking to identify new lead compounds that selectively interfere with parasite microtubules. Given the selectivity observed with these compounds *in vitro* and the selectivity observed with tubulin targeting anthelminths *in vivo* (19,20) there is clearly the potential to develop antikinetoplastid compounds with selectivity *in vivo*. This potential is perhaps greater for antikinetoplastids in comparison with anthelminths as the homology of *Leishmania* sp. and helminth α- and β-tubulin with porcine tubulin are in the regions of >80 and >90% respectively (21,22).

We have isolated tubulin from *Leishmania tarentolae* in our laboratory and have shown that this protein is a suitable alternative to tubulin purified from *Leishmania amazonensis* for use in antiparasitic drug discovery efforts (22). *Leishmania tarentolae* tubulin is nearly identical in amino acid sequence to tubulins from other *Leishmania* sp. (>98%), and dinitroaniline compounds display indistinguishable activity against and binding affinity for *L. tarentolae* and *L. amazonensis* tubulin. In addition to the obvious safety advantages of purifying tubulin from a non-pathogenic species, *L. tarentolae* is also more readily and inexpensively cultured than *Leishmania* species that infect humans. This means that the large scale production of protein required for a high throughput screen becomes more feasible using *L. tarentolae* tubulin. The use of high throughput screens to identify novel lead compounds has increased because of the greater accessibility of the required technology. This is reflected in the number of screens against protozoan parasites reported over the last few years (23–28). Given the need for new lead compounds against kinetoplastid parasites and the greater accessibility of *Leishmania* tubulin, a 10 000 compound library was screened against this protein purified from *L. tarentolae*. Secondary assays were used to determine the selectivity of these compounds for leishmanial tubulin over mammalian tubulin. The cellular effects on *Leishmania* of the most potent hit were examined. In addition, three compounds were identified that displayed activity against mammalian tubulin and cancer cell lines.

Experimental

Compounds and other reagents

The CNS-Set™ of 10 000 drug-like compounds was purchased from ChemBridge Corporation (Suite G, San Diego, CA, USA). These compounds were supplied as 10 m$_M$ stock solutions in dimethyl sulphoxide (DMSO) in 96-well plates. Hit compounds were repurchased from ChemBridge as 5 mg of solid. Unless otherwise noted, all other reagents were from Sigma-Aldrich (St Louis, MO, USA).

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[a]**Notes** http://www.who.int/tdr/diseases/leish/diseaseinfo.htm

Tubulin purification

Tubulin from *L. tarentolae* was isolated using a larger scale version of the previously reported protocol (22). *Leishmania tarentolae* were grown in brain heart infusion medium supplemented with Hemin (10 mg/mL). The parasites were cultured in 2 L flasks to a maximum cell density of approximately 2×10^8 cells/mL. In a typical purification, 20×10^{11} cells were used as the starting material and an average of 50 mg of protein were isolated at concentrations ranging from 10 to 30 mg/mL. This quantity of tubulin was sufficient to screen approximately 1600 compounds in the primary assay under the conditions described below. Tubulin from pig brain was isolated as outlined earlier (15).

Compound screening

Primary assay—Compounds were diluted in DMSO to 2 mM, then diluted to 1 mM with H_2O, giving a 50:50 DMSO:H_2O solution. Dimethyl sulphoxide and **3** (at 40 μM) were used as controls. The compounds were screened using a modification of the previously reported assay (22,29). Primary assays were carried out in 384-well plates at *Leishmania* tubulin concentrations of 1.2 mg/mL. Compounds were preincubated with tubulin for 10 min at 4 °C followed by 5 min at RT. Addition of 4 μL of assembly solution (25% DMSO, 5 mM GTP) followed, giving a final assay volume of 20 μL. The plates were read using a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 30 °C for 15 min with intervals of 15 seconds between readings. Successful assembly was considered an increase in optical density of >0.02 absorbance units at 351 nm.

IC_{50} determinations—IC_{50} values against *Leishmania* and mammalian tubulin were determined in half area 96-well plates at tubulin concentrations of 1.5 mg/mL using previously published assay conditions (15,22).

***Susceptibility testing of* L. donovani, *Vero cells and* T. b. brucei—**The susceptibility of *L. donovani* axenic amastigote-like parasites and Vero cells (African green monkey kidney epithelial cells) to growth inhibition by compounds of interest was assayed as described previously (14,30). The susceptibility of *T. b. brucei* to **5** was assayed as described previously (14).

Growth inhibition assay against cancer cell lines—Viable cells were quantified using the sulphorhodamine B (SRB) assay in several solid tumor cell lines, including four prostate cancer cell lines (LNCaP, PC-3, DU145, PPC-1), a bladder cancer cell line (TSU-Pr1), a colon cancer cell line (HT-29) and a breast cancer cell line (MCF-7). Cells were plated in 96-well plates at a density of 800–5000 cells/well (depending on the cell line) for 1 day prior to the addition of different compounds at a range of concentrations (0–100 μM). After 96 h incubation at 37 °C, cells were fixed by the addition of 50 μL of 50% cold trichloroacetic acid and incubated at 4 °C for 3 h. The plate was washed three times with tap water and was allowed to air dry. The cells were then stained with 0.4% SRB dissolved in 1% acetic acid for 30 min at room temperature. Unbound SRB was washed away with five washes of 1% acetic acid. The plate was again allowed to air dry and the bound SRB stain, representing surviving cells, was dissolved in 200 μL of Tris base (10 mM). The optical density was determined at 540 nm using a microplate reader (Dynex Technologies, Chantilly, VA, USA). Mean values were obtained from at least four wells per treatment condition. Plots of per cent inhibition of cell growth versus drug concentration were constructed, and the concentration that inhibited cell growth by 50% relative to the untreated control (IC_{50}) was determined by nonlinear least squares regression using WINNONLIN software (version 5.2, Pharsight Corp., Mountain View, CA, USA). WINNONLIN was provided by a Pharsight Academic License to The Ohio State University.

Flow cytometry—_Leishmania donovani_ promastigotes were incubated with either 1% DMSO, **3** (2 μM), or **5** (10 or 20 μM) for 48 h at 37 °C and were processed for cell cycle analysis as described previously (14).

Binding site reaction—Interaction of **8** and **5** with the colchicine site was measured by competition with a known colchicine site agent which becomes fluorescent when bound (31). Binding reaction consisted of 5 μM mammalian tubulin and 5 μM 2-methoxy-5-(2′,3′, 4′-trimethoxyphenyl)tropone (MTPT) in tubulin buffer (0.1 M Pipes, 0.5 mM MgCl$_2$, pH 7.0). 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone is a ****wellestablished probe of the colchicine site on tubulin. Fluorescence was excited at 350 nm and emission measured from 380 to 480 nm, using an ISS PC1 spectrofluoromter (ISS Inc., Champaign, IL, USA). The decrease in peak emission intensity was measured following addition of the known competitor dihydrocombretastatin A4 or the test compounds **8** and **5**. Decrease in fluorescence was taken as a direct measure of displacement of MTPT.

LCMS analysis of compounds—The DMSO stock solutions of **5, 8, 23** and **25** used in the assays were analysed by LCMS to confirm the identity and purity of the compounds. Compounds were evaluated for purity by separation and detection via LC/UV/MS on an Agilent 1100 liquid chromatography (LC) system linked to a ThermoFinnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (MS). Initial MS tuning was completed to identify parent and primary fragment ion masses for each compound. All four compounds were detectable with negative mode electrospray (ESI, compounds **8, 23** and **25**) or atmospheric pressure chemical ionization (compound **5**). Each compound yielded a parent ion peak with a mass corresponding to the loss of a hydrogen atom, [M–H]$^-$. For evaluation of compound purity, a gradient from 95% water to 95% methanol over 20 min was used to separate injected samples through a Thermo Aquasil C18 column (3 μm, 100 × 2.1 mm). The LC variable wavelength detector was set to 260 nm and MS filters were established for both full scan (100–1500 m/z) and single reaction monitoring (SRM) modes (SRM filters were established to monitor parent >fragment transitions). The DMSO stock compound solutions were diluted to 10 and 50 μM in 50% methanol for LC/MS injection (50 μL). For all sample separations, overlapping primary peaks were observed in the UV and MS filters. All observed peaks were integrated and the area of the primary analyte peak was divided by the total area of all peaks in each chromatogram. The analyte signal represented greater than 99% of the total peak area in each chromatogram. Compound purity was therefore determined to be greater than 99% for each compound evaluated.

Results and Discussion

Tubulin purified from _L. tarentolae_ was used to screen a 10 000 member library of drug-like molecules. The library selected was the CNS set. While leishmaniasis is not a disease of the central nervous system, this library was selected because of the physiochemical properties of its compounds. This library contains compounds that have a high probability of being orally bioavailable, a property that is especially important when developing drugs against neglected diseases where alternative methods of drug administration are often not viable. The assay used was a modification of the established assembly assay which monitors increases in absorbance at 351 nm as an indication of tubulin polymerization (22). The established assay involves testing in a half-area 96-well plate (final assay volume 50 μL) with tubulin at a concentration of 1.5 mg/mL. The use of _L. tarentolae_, which grows to higher cell density in a less expensive growth medium compared with other _Leishmania_ species, as a source of tubulin means lower collection volumes are required for tubulin isolation (22). We have previously shown that the use of diethylaminoethyl–Sepharose chromatography together with one cycle of assembly–disassembly allows for the ready

isolation of assembly competent *L. tarentolae* tubulin of excellent purity as assessed by SDS-PAGE (22). However, isolating sufficient tubulin to screen 10 000 compounds at the volumes and concentrations reported in the previous 96-well plate assay was undesirable. Selection of a 384-well plate to carry out the primary screen facilitated the use of lower assay volumes, and preliminary experiments showed that the volume could reasonably be reduced to 20 μL (data not shown). In addition, tubulin concentrations of 1.2 mg/mL produced reproducible results (data not shown). However, the rate of *Leishmania* tubulin assembly still presented an obstacle to screening. *Leishmania* tubulin assembles rapidly, with complete assembly observed within 3 min (see Figure 2). This makes the simultaneous measurement of tubulin-containing samples in a full 384-well plate difficult, given the time required to both add the assembly solution across the entire plate (see Figure 2) and read the first time-point. Attempts to slow the rate of assembly by modifying the assay conditions proved unsuccessful (data not shown). The final assay conditions used were the result of a series of investigations into optimizing throughput and reproducibility. These included the variation of the selected buffer, magnesium concentrations, pH, DMSO concentration, and the concentration and use of chelating agents. We determined that three columns of the 384-well plate could be tested successfully in each experiment, enough to evaluate 16 compounds with appropriate controls per assay. The use of only three columns not only decreased the time required to add the assembly solution, thereby allowing for an earlier reading of the initial time-point, but also allowed smaller time intervals between data points.

To validate the assay, plates with known assembly inhibitors and DMSO controls were tested (see Figure 2B). The identification of these known inhibitors of *Leishmania* tubulin assembly indicated that false negatives would not be a concern. While a few false positives were observed in the validation assays, the protocol was not changed because such false positives would be eliminated in a secondary screen where all hits would be retested.

The 10 000 member compound library was then screened at concentrations of 100 μM with one determination per compound. To eliminate false positives, hits were retested at least twice to verify the results. Through this process, 14 compounds were identified as inhibitors of *Leishmania* tubulin polymerization.

Having identified compounds which were active at 100 μM, the next step was to quantify the activity of the compounds by generating IC_{50} values against *Leishmania* tubulin assembly. To provide a direct comparison with antileishmanial tubulin compounds already in the literature, the IC_{50} determinations were made using the original assay format carried out in half-area 96-well plates and employing tubulin concentrations of 1.5 mg/mL. Most of the hits possess IC_{50} values in the region 50–90 μM. However, one compound, Chembridge No. 7992831 (**5**), has an IC_{50} of 13 μM, making **5** the most interesting hit identified from the antileishmanial screen. Some hits belong to similar structural classes, indicating that modifications to these scaffolds could generate compounds with increased potency. One compound was insoluble at the concentrations required for testing. Given the potential problems generated by these solubility issues, this compound was not studied further. The structures of the 13 hits are shown in Figure 3 and their biological activities are given in Table 1.

Compound selectivity

The selectivity of these hit compounds for *Leishmania* tubulin compared with the corresponding mammalian protein is important if the hits are to be considered further for antiparasitic drug discovery. Therefore, the IC_{50} values of the 13 compounds against porcine brain tubulin polymerization were also determined. Compound **5**, the most promising compound from the *Leishmania* tubulin screen, inhibited porcine brain tubulin assembly with an IC_{50} of 40 μM. Although **5** displays only a threefold selectivity for *Leishmania*

tubulin over mammalian tubulin, greater selectivity may be obtainable following synthetic optimization. Aside from **5**, only Chembridge No. 9044486 (**6**) and Chembridge No. 9056942 (**7**) displayed any selectivity for *Leishmania* tubulin (less than twofold). One compound Chembridge No. 9067250 (**8**) showed significant activity against mammalian tubulin, displaying an IC_{50} of 5.2 μ_M. This compound is 10-fold more active against mammalian tubulin than the parasite protein. Although compound **8** is not suitable as an antileishmanial candidate, it may be of interest as an anticancer lead (see below).

The activity of the 13 compounds against *L. donovani* axenic amastigotes and the cytotoxicity of these agents against Vero cells were also determined. Compound **5** is the most active of the hits against the parasite, exhibiting an IC_{50} value of 11 μ_M, similar to its IC_{50} against *Leishmania* tubulin. The remaining compounds possessed IC_{50} values against axenic amastigotes >50 μ_M. While the majority of the compounds exhibited no toxicity to Vero cells at concentrations up to 100 μ_M, a few were toxic at lower concentrations. Interestingly, compound **8** was not toxic to Vero cells up to 50 μ_M concentrations, which is surprising given the effect of this molecule on mammalian tubulin and its activity against mammalian cancer cell lines (see later sections). Compound **5** displayed an IC_{50} value against Vero cells of 29 μ_M. Two compounds, Chembridge No. 7946770 (**9**) and Chembridge No. 7960631 (**10**), also gave IC_{50} values in the region of 30 μ_M. Given that both compounds **9** and **10** have IC_{50} values in the region of 70 μ_M against mammalian tubulin, their activity against Vero cells may be due to another target besides tubulin. However, other factors, for example cell permeability, may be the cause of the observed differences in IC_{50} values between isolated tubulin and Vero cells.

Structural classes within the hits

Among the hits, there are clearly four sets of compounds that are structurally related: (i) **8, 9, 10** and Chembridge No. 9063043 (**17**); (ii) Chembridge No. 9041709 (**11**), Chembridge No. 9035650 (**12**), Chembridge No. 9035165 (**13**) and Chembridge No. 7944936 (**16**); (iii) Chembridge No. 9026860 (**14**) and **7**; (iv) Chembridge No. 9049411 (**15**) and **6**. Class (i) contains *N*-substituted benzamides. Two of the compounds in this class, **9** and **10**, show IC_{50} values in the region of 60 μ_M against both *Leishmania* and mammalian tubulin. One definite trend is that these two compounds both have IC_{50} values against Vero cells in the region of 30 μ_M, suggesting that these two compounds may possesses activity against mammalian cells through mechanism(s) other than or in addition to inhibiting tubulin assembly. However, it should also be considered that other effects, such as drug accumulation, could be the cause of these differences in the IC_{50} values. Class (ii) contains substituted aryl 4-piperazin-1-yl compounds. Three of these compounds, **11, 12** and **13**, contain a 2-phenyl-1,3-oxazole-4-carbonitrile functional group, all these agents have IC_{50} values against both *Leishmania* and mammamlian tubulin of approximately 60 μ_M. This is in contrast to **16** which gives much lower IC_{50} values, indicating the significance of the 2-phenyl-1,3-oxazole-4-carbonitrile group. Compound **12** showed slightly increased activity against *L. donovani* axenic amastigotes, displaying toxicity to the parasites at 50 μ_M. Class (iii) compounds contain a 1,2,4-oxadiazol-5-yl ring system. These compounds have lower activity against *Leishmania* tubulin with IC_{50}s around 80 μ_M. They are some of the least active against mammalian tubulin with IC_{50}s of over 100 μ_M. Interestingly, compound **7** shows slightly improved activity against *L. donovani* axenic amastigotes, displaying toxicity to the parasites at 50 μ_M, whereas compound **14** has slightly better activity against Vero cells. The agents in class (iv) contain a thioacetamide unit but differ in the ring system attached to the sulphur atom. These compounds possess low activity in both the tubulin and cell-based assays.

Compound 5, the most potent against Leishmania tubulin assembly

Compound **5** is the most promising antileishmanial compound of the hits identified, with its IC$_{50}$ of 13 μM against *Leishmania* tubulin and threefold selectivity compared with mammalian tubulin (see Figure 4 for concentration-dependent inhibition of *Leishmania* tubulin assembly by **5**).

We suspected that the observed antileishmanial properties of this compound were related to its ability to inhibit tubulin polymerization. To test this hypothesis, compound **5** was incubated with *L. donovani* promastigotes at 10 and 20 μM for 48 h, then cell cycle analysis was performed to determine if **5** arrested cells in mitosis (see Figure 5). Compound **5** appears to have little effect on the cell cycle of *L. donovani* when compared with the DMSO control. This is even more evident when compared with **3**, a known *Leishmania* tubulin inhibitor, tested at 2 μM under the same experimental conditions, which clearly shows a dramatic increase in the percentage of cells in the G$_2$/M phase compared with G$_1$ and a number of cells containing four times the amount of DNA observed in G$_1$. The results observed for compound **3** in this experiment were consistent with data previously observed for this class of compounds (15). Thus, although compound **5** clearly inhibits *Leishmania* tubulin polymerization *in vitro*, its inhibition of parasite growth could be due to an off target effect. Additionally, compound **5** was assayed against *T. b. brucei*, a related parasite, and surprisingly showed no activity at 100 μM.

SAR information around compound 5 from other compounds in the library

The compound library that was screened contains more than one compound per structural class. Given the knowledge that any compound not identified as a hit is inactive at 100 μM, some preliminary SAR information can be obtained by searching the library for compounds with structures related to the hits. To acquire some information regarding the SAR for *Leishmania* tubulin assembly inhibition around compound **5**, the library was initially searched for compounds containing substructure X shown in Figure 6. There are 40 compounds in the library with this substructure, including 19 compounds with a halogen at the 5th position. The most noticeable trend is that only one compound other than the hit contains the propyn-1-yloxy group, Chembridge No. 7905398 (**18**). Given that the structure of this compound differs significantly in two of the other functional groups, it is difficult to make any conclusions from this compound with regards the requirement of the propyn-1-yloxy group. However, two compounds Chembridge No. 7983651 (**19**) and Chembridge No. 7961265 (**20**) are also present in the library; the inactivity of these compounds where the only structural difference is in the propyn-1-yloxy group implies that this moiety may be required for activity. There are seven compounds where only the functional group of the 4th position oxygen is different and none of these displayed any activity. This supports the hypothesis that the propyn-1-yloxy group is required for activity. The inactivity of **18** would suggest that the propyn-1-yloxy group is not the only requirement for activity and that the halogen and/or the cyano group are also required for activity. Further investigations into the SAR of compound **5** should examine the activity of compounds where individual groups are held constant and the other groups are systematically modified.

Compound 8 has potent activity against mammalian tubulin

Compound **8** was identified as a potent inhibitor of mammalian tubulin assembly. While identifying inhibitors of mammalian tubulin was not the primary goal of this work, the potency of compound **8** against mammalian tubulin assembly (see Figure 7) prompted us to investigate the activity of this compound against a panel of cancer cell lines. The IC$_{50}$ values against most of these cell lines were approximately 1 μM (see Table 2). These results indicate that compound **8** is worthy of further consideration as an anticancer lead compound and

encouraged us to examine other related compounds from the library for their effects on mammalian tubulin.

SAR information around compound 8 from other compounds in the library

The library was searched for analogues of compound **8** which could also be tested against mammalian tubulin to gain some information regarding the SAR. Aromatic sulphonamides are a common structural motif in the library as evident by the identification of 179 compounds with the substructure Y (see Figure 8). This search was refined to identify compounds with substituents and substitution patterns on the aromatic ring systems similar to those of compound **8**.

Compounds **21–27**, shown in Figure 8, were identified as being closely structurally related to compound **8** and were tested against mammalian tubulin at concentrations of 100 and 50 μ_M. Two compounds Chembridge No. 7987157 (**25**) and Chembridge No. 9048759 (**23**) showed activity at both concentrations, while Chembridge No. 7973517 (**27**) displayed activity at 100 μ_M. All other analogues of compound **8** exhibited little or no effect on the assembly of mammalian tubulin. Determination of the IC_{50} values for compounds **25** and **23** revealed that they have greater potency than compound **8**, with values of 2.8 and 1.1 μ_M, respectively. These results suggest that the methyl ester is important for activity. The potent activity of compound **23** shows that the 3′ position fluorine is not required for activity. Given that both compounds **23** and **25** are more active than compound **8**, having the methyl ester in the 3rd position may increase activity. However, the inactivity of Chembridge No. 7951602 (**24**), which is a regioisomer of compound **25**, suggests that the presence of the methyl ester is not the only requirement for activity and there could be conformational and/ or steric demands. The inactivity of Chembridge No. 7973060 (**26**) could further indicate the importance of the position of the ester group or could demonstrate the importance of the methoxy group at the 4′ position. Further investigations should involve the synthesis and evaluations of derivatives with the methyl ester in the *ortho, para* and *meta* positions to directly determine the effect of the position of this group on activity.

Other sulphonamides have been shown to possess activity against mammalian tubulin. In particular, the compounds E7010 (ABT-751) (**28**), T-138067 (**29**) and T-900607 (**30**) are in clinical trials (32–39). Recent publications have reported the results of phase I and phase II clinical trials with ABT-751 (**28**), which is orally bioavailable (37,39). Reports on T-138067 (**29**) in phase II clinical trials have shown that this compound displays low levels of toxicity (35,38). Structural similarities between these compounds and other sulphonamides with antitubulin activity and compound **8** are evident (see Figure 9). Interestingly, compound **29** has a methoxy group in the 4th position and a fluorine in the 3rd (compared with 4′ and 3′ for compound **8**). Compound **29** has an IC_{50} value in the region of 2 μ_M against mammalian tubulin and IC_{50} values from 11 to 165 n_M against a panel of cancer cell lines (40). The activity against mammalian tubulin is similar to that of compound 8 (5 μ_M); however, it shows greater potency against cancer cell lines (1 μ_M in the case of compound **8**). Likewise, compound **28** has an IC_{50} against mammalian tubulin of 2.2 μ_M and IC_{50}s against tumour cell lines of 0.06–0.8 μg/mL (0.16–2.2 μ_M) (41), similar to the values obtained for compound **23** in this study.

One of the other striking similarities with regard to the structures is the presence of a methoxy group in the *para* position. The importance of this functional group is particularly illustrated when comparing the activities of compounds **30–32** (42,43). Compound **32**, while active against cancer cell lines, has an IC_{50} of >100 μ_M against mammalian tubulin and is proposed to interfere with the G1 phase of the cell cycle rather than G2/M. However, compound **31**, the methoxy analogue of **32**, has an IC_{50} of 9.5 μ_M against mammalian

tubulin. Additionally, compound **30** has an IC$_{50}$ of 2.1 μ_M against mammalian tubulin, again highlighting the importance of the methoxy group. The difference in activity between compounds **30** and **31** illustrates the importance of the other functional groups. These observations are consistent with the data obtained for compound **8** and its analogues.

Investigating the mammalian tubulin binding sites of compounds 8 and 5

Compounds **28** and **29** are known to bind to the colchicine site of mammalian tubulin (41,44). If compound **8** binds to the same site as these compounds as expected, the observed increased activity of these analogues against mammamlian tubulin compared with *Leishmania* tubulin would be consistent with the inactivity of classical colchicine site agents against *Leishmania* tubulin (41,44).

Competition studies were performed with mammalian tubulin and MTPT, an established probe for the colchicine site on tubulin which becomes fluorescent upon binding (31) (see Table 3). Incubation of MTPT with mammalian tubulin in the presence of 2, 20 or 50 μ_M compound **8** caused a dose-dependent inhibition in MTPT binding as measured by a decrease in observed fluorescence. Inhibition by compound **8** was less than, but comparable with, that observed with dihydrocombretastatin A4, a known colchicine site ligand. This lends support to the argument that compound **8** and its derivatives bind tubulin at the colchicine binding site, thus explaining the increased activity of compound **8** against mammalian tubulin over *Leishmania* tubulin. Compound **5** had no effect on the binding of MTPT indicating that the observed inhibition of polymerization (Table 1) is caused by a binding site on mammalian tubulin different from the colchicine site. Given its increased potency against *Leishmanial* tubulin over mammalian tubulin, this was expected.

Although the number of antileishmanial hits identified from this screen was rather disappointing and the level of activity observed from the majority of these hits was also less than desired, the screen successfully identified the interesting antimicrotubule compounds **5, 8, 23** and **25**, the identity and purity of which were confirmed by LCMS. In addition, the structural similarity of some of the hits may indicate new classes of molecules which have the potential to be optimized to produce improved lead compounds.

Conclusion

Our screening efforts have identified one compound with good activity against *Leishmania* tubulin assembly, **5**. Compound **5** shows threefold selectivity for Leishmania tubulin over mammalian tubulin. This selectivity could improve through the systematic synthesis of analogues. While this compound also showed activity against *L. donovani* axenic amastigotes, cell cycle analysis revealed that inhibition of tubulin may not be the primary cause of activity. Despite the questions over the mechanism of action of compound **5** against amastigotes, this is still a promising compound with potential as an antileishmanial lead.

Compound **8** was identified as an inhibitor of mammalian tubulin assembly. Further investigations revealed its promising activity against a number of cancer cell lines. Two analogues of compounds **8, 23** and **25** were later identified from the library as compounds with increased potency against mammalian tubulin, and compound **23** also showed excellent activity against several cancer cell lines. These compounds are structurally similar to E7010 (**28**) and T-138067 (**29**), two compounds currently in clinical trials. Compounds **28** and **29** bind at the colchicine binding site, suggesting the same is true of compound **8** and its analogues. This is supported by the observed inhibition of MTPT binding by compound **8**. The SAR information surrounding compound **8** and the identification of the more potent analogues provides a good framework around which to design SAR studies. There is still some potential for improvement in the tolerance to cycles of treatment for ABT-751 (39)

and clinical effectiveness for T-138067 (35,38). Despite the structural similarities between ABT-751 and T-138067, the former compound appears to be more effective in cancer trials. Compounds **8, 23** and **25** may thus provide structural clues for the discovery of tubulin-targeted anticancer agents that might possess subtle differences in pharmacokinetic properties, tumor cell potency and host cell toxicity compared with ABT-751 and T-138067. Such differences could translate into improved efficacy against some forms of cancer.

Acknowledgments

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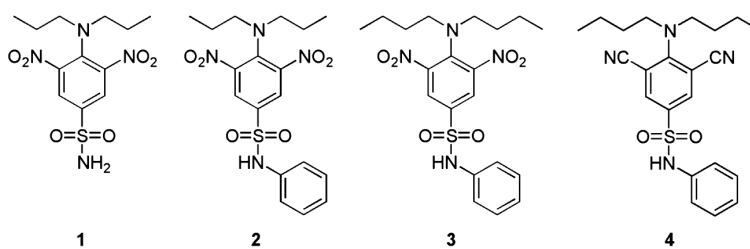
Figure 1.
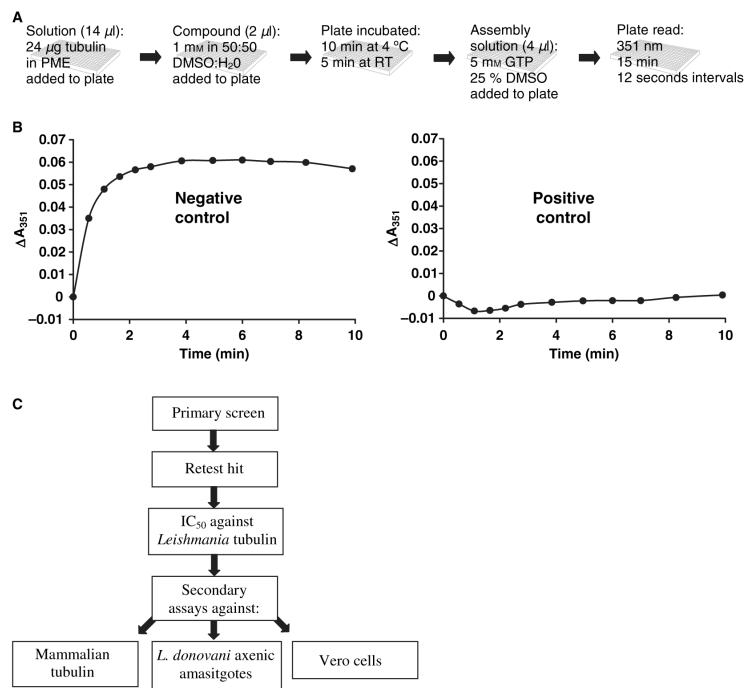Structures of oryzalin (**1**) and analogues with known antitubulin activity.

A

Solution (14 μl):
24 μg tubulin
in PME
added to plate

→

Compound (2 μl):
1 mM in 50:50
DMSO:H$_2$0
added to plate

→

Plate incubated:
10 min at 4 °C
5 min at RT

→

Assembly
solution (4 μl):
5 mM GTP
25 % DMSO
added to plate

→

Plate read:
351 nm
15 min
12 seconds intervals

B

Negative control

Positive control

C

Primary screen

↓

Retest hit

↓

IC$_{50}$ against
Leishmania tubulin

↓

Secondary
assays against:

Mammalian
tubulin

L. donovani axenic
amasitgotes

Vero cells

Figure 2.
Assays used to evaluate the 10 000 compound library for activity against *Leishmania*
tubulin. (A) Schematic of the primary assay used for screening. (B) Examples of positive
and negative control data obtained in the primary assay. The data shown are taken from
validation studies using DMSO as the negative control and GB-II-150 as the positive
control. (C) Schematic for the screening process, including secondary assays.
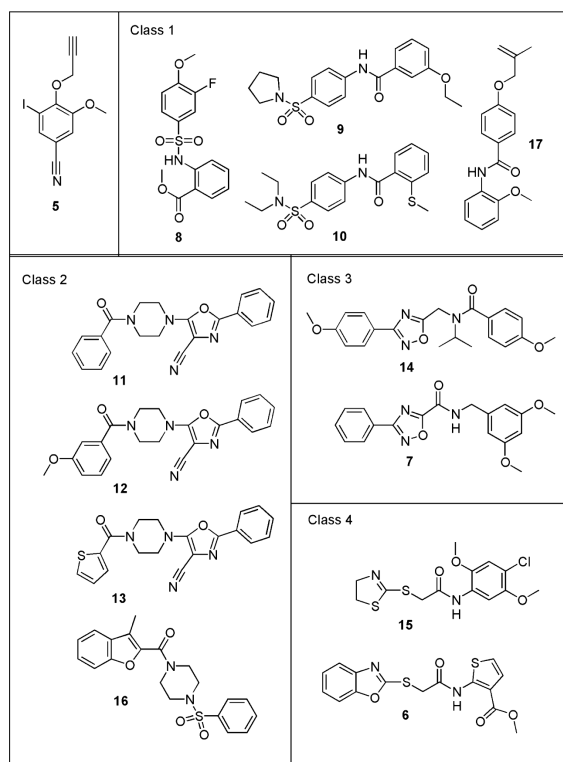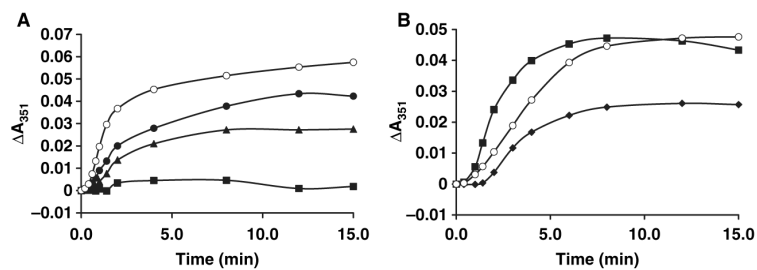
Figure 3.
Structures of the 13 hits.

Figure 4.
Inhibition of the assembly of *Leishmania* tubulin (A) and mammalian tubulin (B) by compound **5**. The assembly of 1.5 mg/mL purified *Leishmania* and mammalian tubulin was assessed as described previously (15,22,29) in the absence (open circles) or presence of compound **5** at 5 (closed circles), 10 (triangles), 20 (squares) or 40 μ_M (diamonds). Data shown are from a representative experiment performed on three separate occasions.
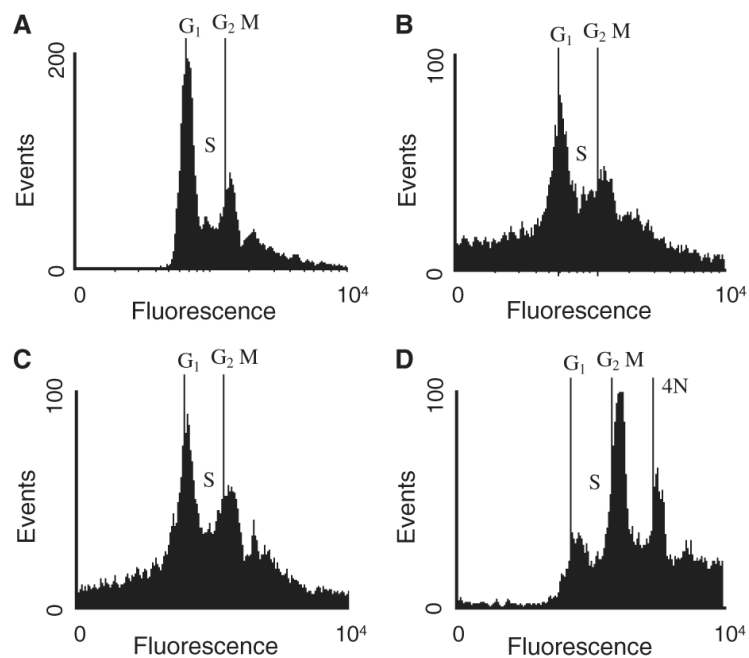
Figure 5.
Cell cycle analysis of *Leishmania donovani* promastigotes treated with compounds **3** and **5**. After 48 h incubation in the presence of 1% DMSO (A) **5** at 20 μ_M (B), 5 at 10 μ_M (C) or **3** at 2 μ_M (D), parasites were fixed, stained with propidium iodide, and analysed by flow cytometry as described in the Experimental section.
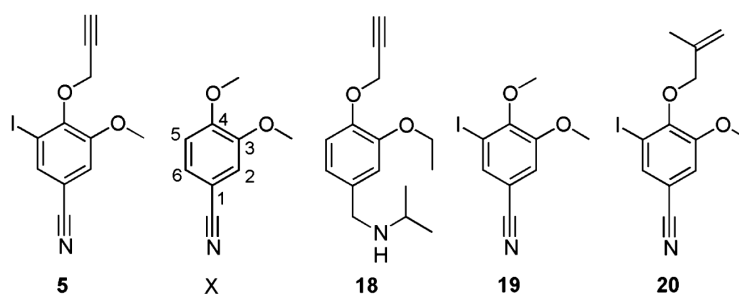
Figure 6.
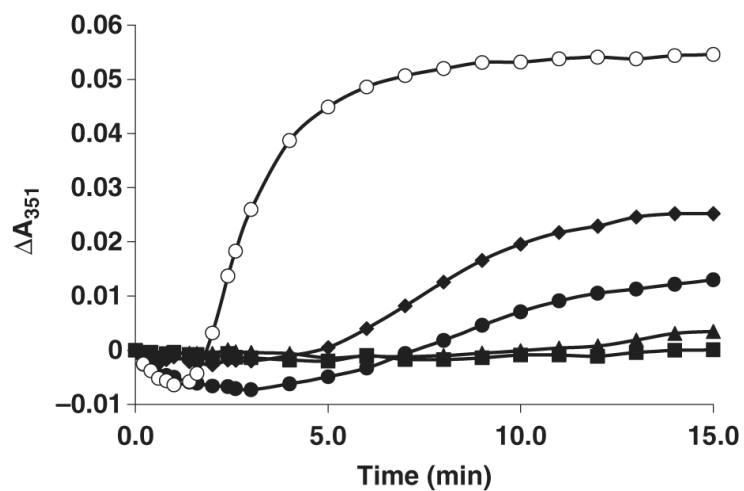Selected analogues of compound **5** present in the compound library.

Figure 7.
Inhibition of mammalian tubulin assembly by compound **8**. The assembly of 1.5 mg/mL purified mammalian tubulin was assessed as described previously (13) in the absence (open circles) or presence of compound **8** at 2.5 (diamonds), 5 (closed circle), 10 (triangles) or 20 μ_M (squares). Data shown is from a representative experiment performed on three separate occasions.
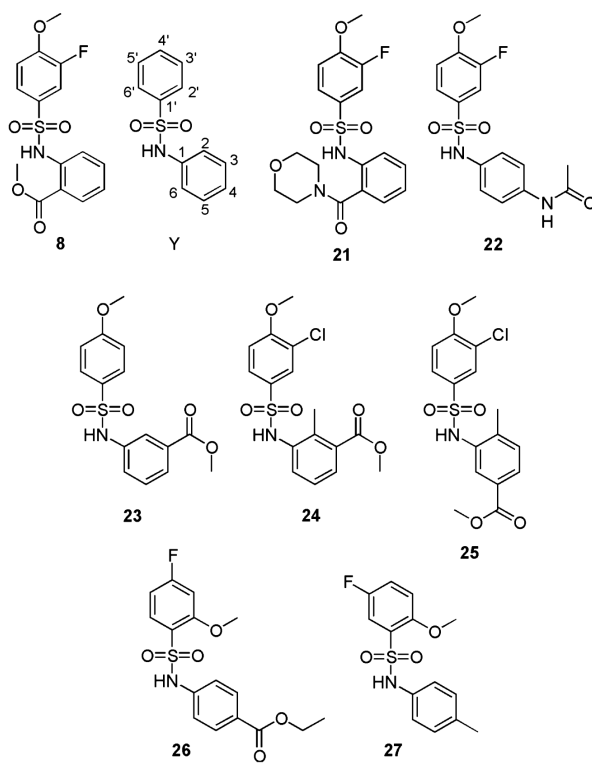
Figure 8.
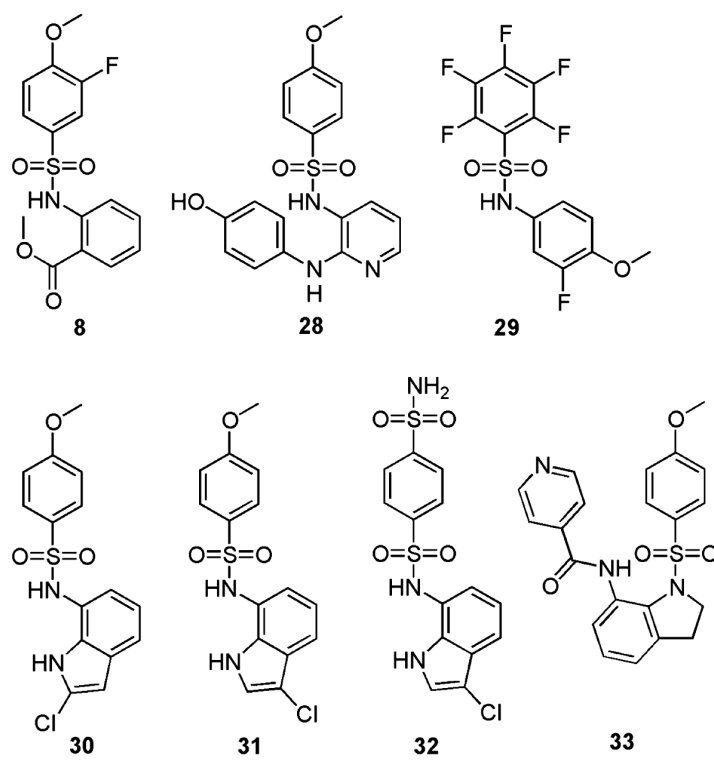Structures of analogues of compound **8** present in the library.

Figure 9.
Structures of sulphonamides with known antitubulin activity (33,42,43,45).

Table 1

In vitro activity of the 13 hits identified from the screen (μ_M)

Compound	IC_{50} versus *Leishmania tarentolae* tubulin assembly	IC_{50} versus porcine brain tubulin assembly	IC_{50} versus *L. donovani* axenic amastigotes	IC_{50} versus Vero Cells
1	>40 (14)	>40 (14)	72 ± 10 (14)	
3	6.4 ± 1.0	>40 (16)	2.3 ± 0.5 (15)	9.7 ± 1.1 (16)
4	6.6 ± 0.7 (17)	>50[a] (17)	4.4 ± 0.09 (17)	16 ± 1 (17)
5	13 ± 1	38 ± 4	11 ± 5	29 ± 15
6	81 ± 18	120 ± 40	>50	>100
7	78 ± 14	140 ± 30	>50	>100
8	55 ± 5	5.2 ± 0.7	>100	>50
9	58 ± 7	67 ± 11	>100	31 ± 5
10	64 ± 4	68 ± 10	>100	37 ± 17
11	68 ± 6	63 ± 13	>100	>100
12	66 ± 3	61 ± 50	>50	>100
13	60 ± 11	63 ± 30	>100	>100
14	89 ± 4	111 ± 33	>100	>50
15	107 ± 12	72 ± 13	>100	>100
16	95 ± 11	71 ± 13	>100	>100
17	70 ± 7	70 ± 6	>100	>100
Podophyllotoxin	NT	1.4 ± 0.6	NT	0.05 ± 0.03
Pentamidine	NT	NT	3.2 ± 1.5	NT

IC_{50} values represent the mean ± standard deviation of at least three independent experiments.

NT, not tested.

[a] Compound not soluble at higher concentrations.

Table 2

The activity of compounds **8, 23** and **25** against several mammalian cancer cell lines

Compound	Cancer cell line IC$_{50}$ (μM)					
	HT29	**LNCaP**	**PPC1**	**PC3**	**MCF7**	**TSU-Pr1**
8	1.9 ± 0.2	1.6 ± 0.4	1.2 ± 0.3	3.5 ± 1.1	1.3 ± 0.2	1.0 ± 0.6
23	0.71 ± 0.03	0.20 ± 0.02	0.37 ± 0.04	0.79 ± 0.07	0.18 ± 0.00	0.33 ± 0.02
25	5.0 ± 0.1	0.88 ± 0.10	1.5 ± 0.2	5.8 ± 0.5	0.74 ± 0.03	1.2 ± 0.2
Vinblastine (nM)	2.4 ± 0.2	3.4 ± 0.9	1.0 ± 0.4	1.4 ± 0.3	1.3 ± 0.4	1.6 ± 0.08

Table 3

Displacement of colchicine site ligand binding of mammalian tubulin by competitors

	Inhibition (%) of MTPT binding in presence of competitors		
	2 μM	**20 μM**	**50 μM**
Dihydrocombetastatin A4	30 ± 1	69 ± 2	74 ± 2
8	12 ± 1	47 ± 1	51 ± 1
5	0 ± 1	0 ± 2	0 ± 2