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4-Amino-5-benzoyl-2-(4-methoxyphenylamino)thiazole (DAT1): a cytotoxic agent towards cancer cells and a probe for tubulin-microtubule system

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1 Microtubule binding drugs are of special interest as they have important roles in the modulation of cellular functions and many of them act as anticancer agents. 4-Amino-5-benzoyl-2-(4-methoxyphenylamino)thiazole (DAT1) was identified as one of the active compounds from a series of diaminoketothiazoles in a cell-based screening assay to discover cytotoxic compounds.

2 DAT1 shows cytotoxicity with GI_{50} values ranging from 0.05 to 1 μM in different malignant cell lines with an average value of 0.35 μM. It blocks mitosis in the prometaphase and metaphase stages. In HeLa cells, DAT1 blocks the spindle function by disturbing spindle microtubule and chromosome organization.

3 The drug also inhibits assembly of brain microtubules and binds tubulin specifically at a single site with induction of fluorescence. The dissociation constant of DAT1 binding to tubulin was determined as 2.9 ± 1 μM at 24°C. The binding site of DAT1 on tubulin overlaps with that of the conventional colchicine-binding site.

4 DAT1 can thus be considered as a lead compound of a new class of small molecules and this study can be used as a step to develop potent antimitotic agents for the control of cytoskeletal functions and cell proliferation. It would also be an interesting probe for the structure–function studies of tubulin–microtubule system.
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Abbreviations: DAT1, 4-amino-5-benzoyl-2-(4-methoxyphenylamino)thiazole; MTP, microtubular protein; s.d., standard deviation

Introduction

Dynamic microtubules play a crucial role by maintaining proper spindle function during mitotic cell division. Many of the anticancer agents act on microtubules and arrest mitosis. The target of majority of these compounds is tubulin, which is the major component of microtubules (Jordan *et al*., 1998; Jordan & Wilson, 2004). Microtubule effectors work in two ways: they interfere with microtubule dynamics (Jordan *et al*., 1998; Wilson *et al*., 1999) and at relatively higher concentrations, and they shift tubulin–microtubule equilibrium in the cell by either inducing or inhibiting microtubule assembly. There are three major classes of microtubule effectors (Li *et al*., 1999) that bind tubulin in different sites. Taxanes stabilize microtubules by blocking disassembly. The other two classes are vinca alkaloids and colchicine analogues. These compounds destabilize microtubules by the inhibition of assembly of tubulin molecules.

Apart from taxanes, vinca alkaloids and colchicine analogues, some other compounds with widely different structures have also been discovered to date that bind to the sites targeted by these three class of compounds (Beckers *et al*., 2002; Holwell *et al*., 2002; Jimenez-Barbero *et al*., 2002; Gupta & Bhattacharyya, 2003; Hadfield *et al*., 2003).

Paclitaxel, docetaxel and vinca alkaloids like vincristine and vinblastine are well characterized and widely used clinically in the treatment of different types of malignancies (Rowinsky, 1997; Duflos *et al*., 2002; Beer *et al*., 2003; Gautam & Koshkina, 2003). Drugs targeting to the colchicine-binding site, however, have not shown much promise due to toxicity. However, E7010 (*N*-[2-[(4-hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide) (Yoshimatsu *et al*., 1997), combretastatins and their analogues (Cirla & Mann, 2003; Cooney *et al*., 2004), which bind tubulin at the colchicine site, have recently attracted a great deal of attention in the preclinical and clinical studies due to their promising antitumour effects and inhibition of angiogenesis. An interesting point is that the structure of E7010 is unrelated to colchicine. Therefore, it appears to be important to continue drug discovery efforts for colchicine derivatives or compounds binding to the colchicine site of tubulin with increased antitumour activity and decreased toxicity. As part of our search to find new antimitotic compounds that would have

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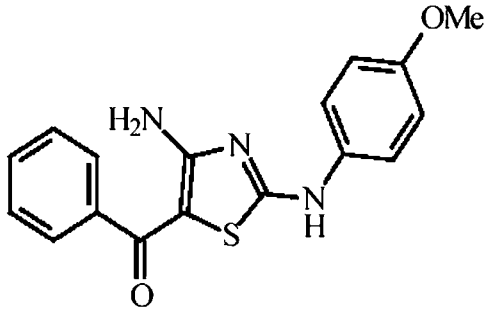

Figure 1 Structure of 4-amino-5-benzoyl-2-(4-methoxyphenylamino)thiazole (DAT1).

potential as anticancer agents, we became interested in the marine alkaloid dendrodoine. This compound was reported earlier to have cytotoxicity (Helbecque *et al*., 1987), but was not explored further. A series of synthetic diaminoaroylthiazole analogues of dendrodoine (Rajasekharan *et al*., 1986; Binu *et al*., 1998) were synthesized, and DAT1 (4-amino-5-benzoyl-2-(4-methoxyphenylamino)thiazole) (Figure 1) was selected in a preliminary screen based on its cytotoxic activities (Patent Application no. PCT Int. PCT/IN04/00108; Indian 789/DEL/2004). Recently, diamino thiazoles have been shown to act as inhibitors of certain protein kinases (Buolamwini, 2000; Bowler *et al*., 2001; Chu *et al*., 2003). In this report, we show that DAT1 exhibits cytotoxicity in different types of cancer cell lines, causes mitotic arrest and disrupts the spindle morphology of mitotic cells. It also inhibits the *in vitro* assembly of microtubular proteins (MTPs). It binds to tubulin at a site, which is either the same or overlapping to the colchicine-binding site. Moreover, it develops fluorescence upon binding to tubulin, making it a suitable probe for the structure–function studies of tubulin–microtubule system. Thus, DAT1 can be considered as a lead diaminoketothiazole to develop potent antimitotic agents of a new class.

Methods

The cell lines HeLa (human cervical epidermoid carcinoma), CaSki (human cervical adenocarcinoma), SW620 (human colon adenocarcinoma) and A549 (human lung carcinoma) were obtained from National Centre for Cell Sciences, Pune, India. HCT 116 (human colon carcinoma) was a kind gift from Dr Bert Vogelstein of The Johns Hopkins University School of Medicine, Baltimore, U.S.A. L929 (mouse connective tissue fibrocarcoma) was from ATCC, Manassas, U.S.A. Antibodies, paclitaxel, vinblastine, podophyllotoxin, colchicine and other fine chemicals were from Sigma, St Louis, U.S.A. [^{3}H] colchicine was from Amersham Biosciences Ltd, U.K. The other chemicals used were of reagent grade.

DAT1 was synthesized by adopting an improved solid phase approach (unpublished results) of the solution phase synthesis as reported earlier (Rajasekharan *et al*., 1986; Binu *et al*., 1998).

Cytotoxicity assay

MTT assay (Hansen & Brünner, 1994) was used to determine the number of proliferating cells upon drug addition. Cells were seeded in microtitre plates (generally 5×10^3 cells well^{-1}) and were incubated with different concentrations of the cytotoxic agents for 48 h. Subsequently, 100 μl of MTT solution (0.6 mg ml^{-1}) was added per well and incubated at 37°C for additional 2 h. The amount of formazan salt was quantified in quadruplicates by recording the absorbance at 570 nm using a Biorad Plate reader. The drug concentrations, which inhibited cell growth by 50% of the control (GI_{50}), were calculated from the semilogarithmic dose–response plots using the nonlinear regression program Origin. All the experiments were repeated at least three times.

Mitotic index analysis

Mitotic index was determined as described previously (Huang & Lee, 1998). Briefly, HeLa cells were plated at a density of 1×10^4 cm^{-2} in 35 mm dishes. Next day, they were treated with different concentrations of DAT1. After 24 h incubation, both attached and rounded-up cells were harvested by trypsinization and washed with PBS. Subsequently, they were treated with 0.5% KCl for 12 min at room temperature. After centrifugation, they were fixed with methanol/acetic acid solution (3 : 1, v v^{-1}). Two to three drops of the cell suspension were spread on clean slides, air-dried and stained with a 10% Giemsa solution in Sörensen buffer. At least 200 cells were counted for each concentration and mitotic index was calculated as described (Huang & Lee, 1998).

Immunofluorescence microscopy

HeLa cells were grown on coverslips in a 24-well plate and incubated with drugs for 24 h. Cells were then washed with PBS and fixed with 3.8% paraformaldehyde for 30 min at room temperature and treated for 10 min with methanol chilled at −20°C. Subsequently, the cells were washed with PBS and treated with PBS containing 2% BSA for 10 min. Microtubules were stained with a mouse monoclonal antibody against β-tubulin in 1 : 100 dilution in PBS containing 2% BSA for 2 h at 37°C followed by a Rhodamine-conjugated goat anti-mouse antibody in 1 : 100 dilution for 1 h at 37°C. Coverslips were then washed with PBS and incubated with DAPI (0.5 μg ml^{-1}) for 45 s at room temperature. Cells were viewed in a Nikon Eclipse TE300 microscope with a Plan Fluor $\times$40/0.75 objective and images were captured.

Protein preparation

MTP was prepared from goat brains by two cycles of temperature-dependent assembly–disassembly process in PEM buffer (100 mM PIPES, pH 6.9, 1 mM $MgSO_4$ and 1 mM EGTA) with 1 mM GTP at 37°C (Slobada & Rosenbaum, 1982), except in assembly experiments, where three cycles of polymerization were carried out. Tubulin was purified from 2 $\times$ MTP using glutamate buffer for assembly (Hamel *et al*., 1981).

Polymerization assay

MTP polymerization, in the presence or absence of different concentrations of DAT1, was followed in PEM buffer by two methods. In one, time course of the turbidity at 37°C was measured at 345 nm in a Shimadzu UV-1601 double beam spectrophotometer fitted with a temperature-controlled circulating water bath. Equal amounts of DAT1 were added to the

reference cuvette to nullify the effect of the absorbance of DAT1. In another method, different concentrations of DAT1 were incubated with 10 μM MTP and 1 mM GTP at 37°C for 20 min, followed by centrifugation for 10 min at 63,000 $\times$ g in a Sigma 3K30 centrifuge. Subsequently, the pellets were dissolved in 0.5 ml of 0.1 N NaOH and the protein concentrations were measured by Bradford's method (Bradford, 1976) to quantitate polymerized microtubules.

Electron microscopy

MTP was polymerized at 37°C in PEM buffer with 1 mM GTP in the presence or absence of DAT1. The polymerized microtubules were fixed with glutaraldehyde and negatively stained with uranyl acetate. Subsequently, they were viewed under a Hitachi H-600 electron microscope.

Fluorescence measurements

All fluorescence measurements were performed in a Perkin-Elmer LS50B Luminescence spectrometer. Excitation and emission band passes were 2.5 nm each and the fluorescence values recorded were uncorrected. Emission spectra of DAT1 in the presence of tubulin were recorded from 400 to 600 nm using an excitation wavelength of 374 nm.

The experiment to show the specificity of DAT1 binding to tubulin was performed with tubulin and certain other proteins by recording the fluorescence of DAT1 in the range of 400–600 nm. In all, 5 μM of different proteins were added to 5 μM of DAT1 and the spectrum of each one was recorded with an excitation wavelength of 374 nm.

The binding parameters of DAT1 binding to tubulin were measured from fluorescence data by the standard Scatchard analysis (Cantor & Schimmel, 1980): $r = (F_D\ F_0^{-1})\ (D_0\ P_0^{-1})$, where r = moles of DAT1 bound per mole of tubulin, F_D is the fluorescence of a given solution of DAT1–tubulin complex and F_0 is the fluorescence of an equal concentration of DAT1 in excess tubulin, such that all the DAT1 is bound. D_0 is the total DAT1 concentration, and P_0 is the total protein concentration. Free DAT1 concentration D_f was calculated from the relation $D_f = D_0 - rP_0$. The binding constants and stoichiometries were determined from Scatchard plot using 2 μM tubulin and varying DAT1 over 0.2–20 μM. Fluorescence values were recorded at 450 nm using an excitation wavelength of 350 nm to reduce the absorbance of DAT1. Inner filter effect corrections were performed using the following equation

$$F_{\text{corrected}} = F_{\text{observed}}\,[\text{antilog}\{(A_{\text{ex}} + A_{\text{em}})/2\}].$$

Effect of podophyllotoxin or vinblastine on DAT1 binding to tubulin

The effect of podophyllotoxin or vinblastine on DAT1 binding to tubulin was noted by the incubation of tubulin with excess of podophyllotoxin or vinblastine (at concentrations where near saturation was achieved as calculated from the dissociation constants of the tubulin–drug complexes) and the fluorescence spectra were recorded after the addition of DAT1 to these complexes. For the competition experiment, tubulin (5 μM) was added to a solution containing 5 μM DAT1 and varying concentrations of podophyllotoxin. Subsequently, fluorescence spectra were recorded.

Competition of [^{3}H] colchicine binding to tubulin by DAT1

The [^{3}H] colchicine-binding assay was performed by the filter disc method (Banerjee & Bhattacharyya, 1979).

Stability of DAT1 was checked from time to time by thin-layer chromatography or recording its absorption spectrum.

Results

Effect of DAT1 on different tumour cell lines

To investigate the effect of DAT1 on tumour cell growth, we treated human tumour cells from cervix, colon and lung and mouse fibrosarcoma cells with different concentrations of DAT1. The potent cytotoxic agents paclitaxel, vinblastine and colchicine were also used for comparison. Cytotoxicity was measured by the cellular metabolic activity using MTT assay. Growth of most of the cell lines was inhibited by DAT1 in a concentration-dependent manner. The growth inhibition constants (GI_{50}) of DAT1 varied considerably for different cell lines (Table 1). The GI_{50} values for the different cell lines ranged from 0.05 to 1 μM for DAT1, from 0.007 to 0.3 μM for paclitaxel, from 0.001 to 0.2 μM for vinblastine and 0.02 to 0.5 μM for colchicine. The results indicate that DAT1 is an effective cytotoxin against different cancer cell lines. In some cell lines, it is significantly less potent than the widely used antimitotic drugs, whereas in other cell lines it is only slightly less potent.

Mitotic indices

To check whether the antiproliferative activity of DAT1 is anyway related to its effect on mitotic phase of cell cycle

Table 1 Cytotoxic activity of DAT1 against different tumour cell lines

Cell lines (source)	*DAT1* (μM)	*Paclitaxel* (μM)	*Vinblastine* (μM)	*Colchicine* (μM)
HCT 116 (human colon)	0.3 ± 0.0947	0.012 ± 0.005	0.005 ± 0.003	0.03 ± 0.011
HeLa (human cervix)	0.054 ± 0.007	0.034 ± 0.008	0.001 ± 0.0001	0.024 ± 0.011
L929 (mouse connective tissue)	1 ± 0.256	0.3 ± 0.07	0.2 ± 0.078	0.55 ± 0.081
CaSki (human cervix)	0.2 ± 0.06	0.02 ± 0.007	Not done	0.25 ± 0.07
SW620 (human colon)	0.2 ± 0.06	0.007 ± 0.002	0.2 ± 0.05	Not done
A549 (human lung)	0.352 ± 0.131	0.007 ± 0.001	0.0014 ± 0.0005	0.056 ± 0.003

Different concentrations of DAT1, paclitaxel and vinblastine or colchicine were incubated at 37°C with the different cell lines. After 48 h, drug containing media were removed and MTT assay was carried out as described in the 'Methods' section. GI_{50} values were calculated using the nonlinear regression program Origin. The average of at least three experiments is shown.

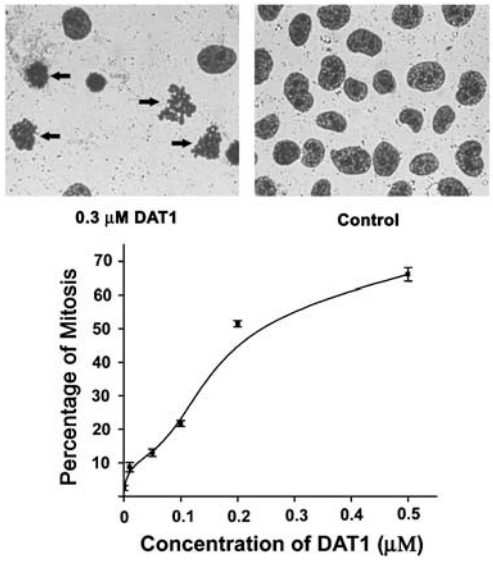

Figure 2 Mitotic arrest by DAT1 in HeLa cells. Microscopic view of cells treated with DAT1 and control cells (upper panel). Mitotic nuclei are shown by arrows. HeLa cells were treated with 0.3 μM DAT1 for 24 h and then prepared by chromosome spreading technique as described in the Methods section for mitotic index analysis. Lower panel: HeLa cells were treated with various concentrations of DAT1 (0–0.6 μM) for 24 h. Subsequently, their mitotic indices were analysed as described above. Bar represents the s.d. of three independent experiments.

progression, we checked the mitotic arrest of HeLa cells after 24 h of DAT1 treatment by Giemsa staining procedure. A typical field of 0.3 μM DAT1-treated cells showing the mitotic and interphase nuclei under a microscope is shown in Figure 2 (upper panel, left). The right picture of the upper panel of Figure 2 shows a typical field for control cells. The figures show clearly that a considerable number of cells are arrested in the mitotic phase following the treatment with 0.3 μM DAT1. A plot of the percentage of mitotic cells against different concentrations of DAT1 (Figure 2, lower panel) showed the IC_{50} of mitotic arrest as 0.24 μM. These data show the antimitotic property of DAT1.

Effect of DAT1 on the microtubule organization in cells

We also checked the effect of DAT1 on the organization of spindle microtubules and chromosomes as well as in the interphase cells. HeLa cells were treated with DAT1 for 24 h. After fixing and permeabilization, microtubules were visualized by indirect immunofluorescence microscopy using an antibody against β-tubulin. Figure 3a shows the control cells (DMSO treated) with the microtubule network constituting the cell shape at interphase and normal spindle morphology for cells at metaphase of mitosis. The corresponding mitotic chromosomes are well aligned at the metaphase plate (part b of Figure 3). In contrast, cells treated with 0.08 μM DAT1 showed clear abnormalities with broken bipolar spindle, star-like spindle, etc. with uncongressed chromosomes at the metaphase plate (parts c and d of Figure 3; mitotic spindles and chromosomes are shown by arrowheads). Cells treated with 7.5 times the GI_{50} concentration of DAT1 showed tripolar, scattered and other abnormal spindle and chromosome

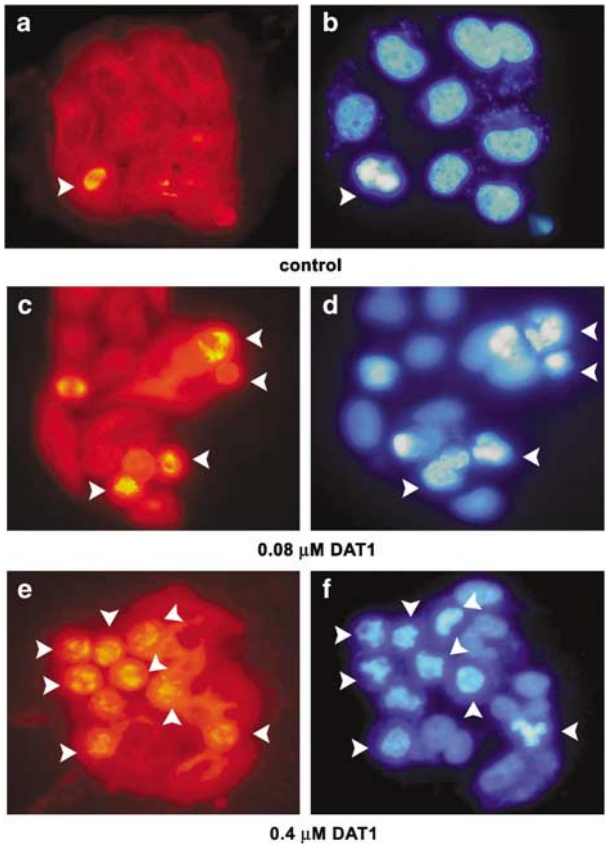

Figure 3 Effect of DAT1 on the microtubule and chromosome organization. HeLa cells were exposed to DMSO or 0.08 and 0.4 μM DAT1. After 24 h, cells were fixed, permeabilized and microtubules were visualized by indirect immunofluorescence microscopy using an antibody against β-tubulin. Microtubules are shown in (a) control, (c) 0.08 μM and (e) 0.4 μM DAT1 and corresponding chromosome organizations are shown in (b, d and f). Mitotic cells and their corresponding chromosomes are shown by arrowheads.

organization (parts e and f of Figure 3) similar to those observed with colchicine (data not shown). At these concentrations, DAT1 did not appear to disrupt the microtubule organization of interphase cells significantly, a property typical of more potent antimitotic drugs (Jordan *et al*., 1992).

Effect of DAT1 on microtubule assembly

From the above experiments, it is clear that DAT1 has a prominent effect on mitotic cells and spindle microtubules. Hence, we tested the effect of DAT1 on the *in vitro* microtubule assembly. A spectrophotometric assay was performed where the turbidity of the microtubules was measured at 345 nm. This wavelength setting was used instead of the usual 350 nm as DAT1 had high absorbance at 350 nm. Figure 4a shows that there is a concentration-dependent inhibition of microtubule assembly by DAT1 with 72% inhibition achieved by 40 μM DAT1. The half-maximal concentration (IC_{50}) of polymerization inhibition was determined from a typical plot of the percent inhibition of microtubule assembly against the concentration of DAT1 and the value was found as 23 μM (Figure 4b). A sedimentation assay was also performed where the amount of polymers formed in the presence of different

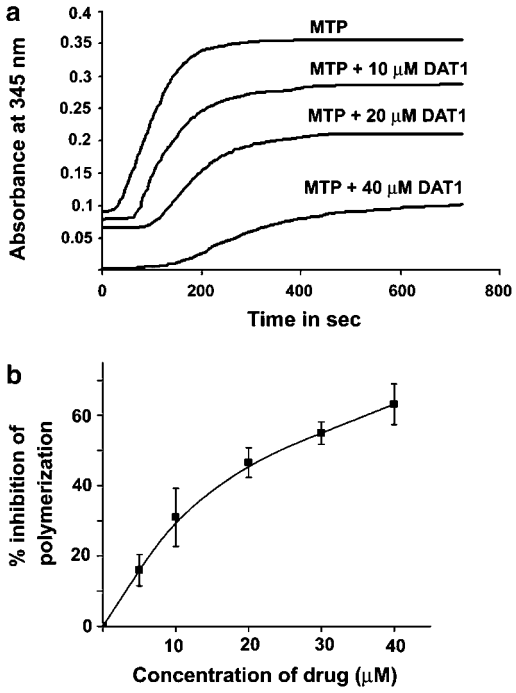

Figure 4 Inhibition of *in vitro* microtubule assembly by DAT1. (a) Time course of inhibition. In all, 1.2 mg ml^{-1} of 3 × MTP was incubated with different concentrations of DAT1 for 3 min at 24°C in PEM buffer (100 mM PIPES, pH 6.9, 1 mM $MgSO_4$ and 1 mM EGTA). Subsequently, 1 mM GTP was added and polymerization was followed by the turbidity at 345 nm for 15 min at 37°C. (b) Inhibition of microtubule assembly plotted as a function of DAT1 concentration.

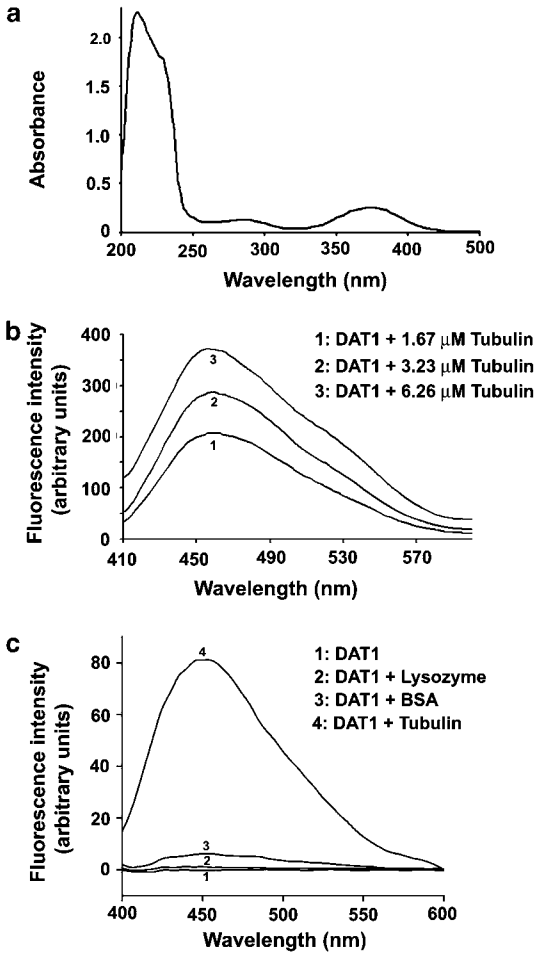

Figure 5 Spectra of DAT1. (a) Absorption spectra of DAT1 in methanol; (b) fluorescence emission spectra (uncorrected) of 1 μM DAT1 in the presence of different concentrations of tubulin in PEM buffer; and (c) fluorescence emission spectra of 5 μM DAT1 in the presence of different proteins. In both cases, excitation wavelength was 374 nm and band passes were 2.5 nm each.

concentrations of DAT1 were measured. Similar inhibition of microtubule assembly was found in this case also (data not shown). Compared to the control, where normal filamentous structures were observed, no significant change in the morphology was observed in the microtubules formed in the presence of 20 and 40 μM DAT1 by electron microscopy (data not shown).

Binding of DAT1 to tubulin

As tubulin is the major component of microtubules, we wanted to test whether the effects of DAT1 on microtubule network and microtubule assembly are due to its binding to tubulin. DAT1 absorbs light with absorption maxima at 212, 283 and 374 nm in methanol (Figure 5a). Although DAT1 does not exhibit any fluorescence by itself in aqueous solution, when incubated with purified tubulin, it showed fluorescence with an emission maximum of 457 nm upon excitation at 374 nm. The fluorescence intensity increased with the increase in concentration of tubulin (Figure 5b), showing that it bound to tubulin. The fluorescence of tubulin alone in that wavelength range was negligible even at the highest concentration tested. To check if this binding was nonspecific in nature, we compared the fluorescence increase of 5 μM DAT1 in the presence of tubulin, lysozyme or BSA, each at the concentration of 5 μM (part c of Figure 5). Considering the increase with tubulin at 457 nm as 100%, the increase with lysozyme and BSA were 1 and 7%, respectively. This rules out the possibility of nonspecific binding of DAT1 to tubulin.

Binding parameters of DAT1 binding to tubulin

The binding affinity and stoichiometry of DAT1 binding to tubulin were calculated by the titration of tubulin with DAT1 at 24°C. Data for the Scatchard plot for one such experiment are presented in Figure 6. A K_d value of 2.9 ± 1 μM was calculated (mean of three experiments). The *x*-axis intercept of 0.85 shows that DAT1 has only one binding site on tubulin.

Binding site of DAT1 on tubulin

The above results suggest that DAT1 binds to tubulin and inhibits microtubule polymerization. Vinblastine and colchicine are two classical microtubule-destabilizing agents, which are known to bind to tubulin at different sites. To get an idea of the binding site of DAT1 on tubulin, we checked if these drugs could affect the binding of DAT1 using the fluorescence of DAT1 bound to tubulin. However, colchicine also exhibits fluorescence upon binding to tubulin (Bhattacharyya & Wolff, 1974) with an emission maximum of 430 nm when excited at 350 nm. We found that the fluorescence emission spectrum of

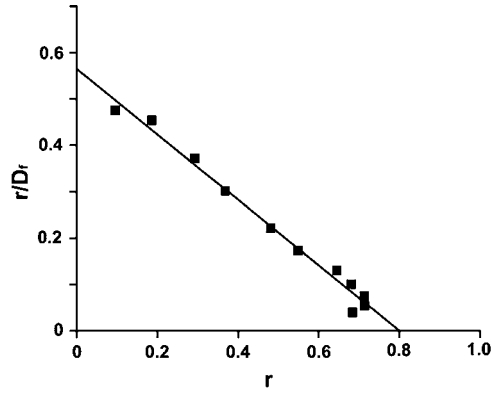

Figure 6 Scatchard plot of DAT1 binding to tubulin. Tubulin (2 μM) was titrated with 0.2–20 μM of DAT1 at 24°C in PEM buffer. Fluorescence values were recorded at 450 nm upon excitation at 350 nm. r/D_f and r values were calculated as given in the Methods section. Three experiments were performed. The plot for one typical experiment is shown.

DAT1 upon binding to tubulin (part b of Figure 5) partially overlaps with that of tubulin-bound colchicine. So, we selected podophyllotoxin, a drug known to bind reversibly to tubulin at the colchicine-binding site and inhibit tubulin polymerization but which does not induce fluorescence (Cortese *et al*., 1977). Part a of Figure 7 shows that a 20 μM podophyllotoxin can reduce the fluorescence of 2 μM DAT1 bound to 2 μM tubulin by 73%. Vinblastine (5 μM), a concentration that could almost saturate its binding to 2 μM tubulin, had no effect on DAT1 fluorescence, indicating that the binding sites of DAT1 and vinblastine are independent. A detailed competition study shown in the inset of part b of Figure 7, using 5 μM DAT1 and varying podophyllotoxin concentrations, demonstrate that podophyllotoxin effectively inhibits DAT1 binding, with 77% inhibition achieved at a concentration of 40 μM. This indicates that DAT1 binds to tubulin to a site either identical or overlapping to the podophyllotoxin-binding site and hence tentatively to the colchicine-binding site. To confirm the binding to the colchicine-binding site, a competition experiment with [^{3}H] colchicine was performed by varying concentrations of DAT1 (part b of Figure 7). Inhibition (78%) was obtained with 40 μM DAT1. For comparison, podophyllotoxin was also used under the same conditions and it gave similar type of competition.

All these observations place DAT1 in a suitable position for considering it as a good antimitotic agent, microtubule inhibitor, ideal probe for studying tubulin–microtubule system and a promising cytotoxic agent.

Discussion

In the last two decades, antimitotic agents took a major place as chemotherapeutic agents to control cell proliferation and induce cytotoxicity (Jordan *et al*., 1998; Jordan & Wilson, 2004). The mechanism of their action often involves an interaction with microtubules, and in particular, tubulin. Antimitotic agents include a surprisingly diverse group of compounds, most of which are natural products.

In our investigations, we have found that DAT1, which is a substituted diamino thiazole, exhibits cytotoxicity in cancer

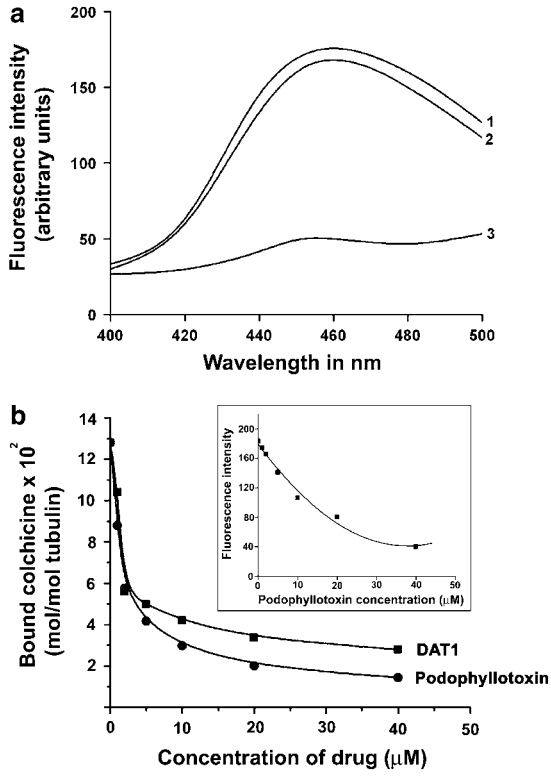

Figure 7 Competition experiments of DAT1 to determine the binding site. (a) DAT1 binding to tubulin in the presence of (1) none, (2) vinblastine and (3) podophyllotoxin. Tubulin (2 μM) was added to a mixture of 2 μM DAT1 and 5 μM vinblastine or 20 μM podophyllotoxin. (b) Competition of [^{3}H] colchicine binding by DAT1 or podophyllotoxin. Tubulin (2.5 μM) was added to a mixture of 5 μM [^{3}H] colchicine and DAT1 or podophyllotoxin in concentrations as shown in the figure. After 1 h of incubation, bound colchicine concentration was measured as described in the Methods section. The *inset* shows a competition of DAT1 binding by podophyllotoxin. Tubulin (5 μM) was added to a mixture of 5 μM DAT1 and podophyllotoxin in the concentrations as shown in the graph and fluorescence spectra were recorded at 450 nm upon excitation at 374 nm. Solution conditions were same as in Figure 4. The experiments for both inset and (b) were repeated three times from which one typical plot is shown.

cell lines from different malignant tissues with GI_{50} values, which are three- to 55-fold higher than the widely used anticancer agents paclitaxel and vinblastine. It is noteworthy that DAT1 is a synthetic compound and it was selected by screening compounds, which were obtained by modifications of some parts of the parent compound dendrodoine. More active compounds could be found by structure–activity studies of other modified compounds. Here, we found that DAT1 induces mitotic block at the prometaphase and metaphase stage. In case of HeLa cells, the IC_{50} of mitotic arrest is 0.24 μM, which is somewhat higher than its GI_{50} value (0.054 μM). A probable reason for that could be the existence of some other target for DAT1, which is important in the cell cycle progression and thus in cell survival. A support for this comes from the recent reports where diaminoketothiazoles have been shown to inhibit the kinases like CDKs (Buolamwini, 2000), GSK-3 (Bowler *et al*., 2001) and tyrosine kinases (Chu *et al*., 2003). It has been found that the success of the more potent antimitotic compounds like taxanes and vinca alkaloids towards clinical applications partially depends on

their ability to activate some signaling pathways, some of which ultimately increase the rate of apoptosis (Bacus *et al*., 2001; Ding *et al*., 2001; Hu *et al*., 2002; Shingu *et al*., 2003). However, the opposite effect is also seen, that they stimulate some compounds that promote cell survival. It is not understood yet whether these effects occur independent of the effects on microtubules. One popular hypothesis in the recent drug development strategy is that the compounds that would have more than one target in the cell are likely to be more successful and less susceptible to drug resistance. Diaminoketothiazoles thus have the potential to be interesting drugs in the future.

DAT1 did not depolymerize microtubules in the cells during interphase in its half-maximal inhibitory concentration of cell proliferation or mitosis. This is a common phenomenon exhibited by other potent antimitotic drugs (Jordan *et al*., 1992). However, in a concentration somewhat above the GI_{50} value, it could perturb the spindle microtubule organization (parts c and d of Figure 3). At a concentration higher than the IC_{50} of mitotic arrest, however, the effect was more pronounced and the organization of the chromosomes was also strongly disrupted (parts e and f of Figure 3). The effect of DAT1 on microtubules was confirmed by the fact that it inhibited the assembly of brain microtubules in a concentration-dependent manner.

DAT1 shows fluorescence upon binding to tubulin, although the drug itself does not have fluorescence in aqueous solution. This omits the cumbersome stage of separation of free drug from the complex and makes it an interesting agent for the structure–function studies of tubulin–microtubule system. The binding is quite specific as evidenced by the negligible fluorescence of DAT1 in the presence of BSA and lysozyme. It binds to tubulin at a single site with fairly high affinity. A point to be noted here is that the DAT1 concentration required for the 50% inhibition of polymerization, 23 μM, is quite high compared to K_D value of DAT1 binding to tubulin (2.9 ± 1 μM). This is quite different than that observed in the cases of colchicine or vinblastine whose affinities towards tubulin and polymerization inhibition activity are almost parallel. So, it can be anticipated that DAT1 inhibits assembly by a different mechanism than these more potent drugs. Considering similar antimitotic effect of DAT1 and colchicine, it appears to be interesting to study the effect of DAT1 on microtubule dynamics and other effects of tubulin binding.

An initial competition experiment with podophyllotoxin and then with [^{3}H] colchicine confirmed that it bound to the same or overlapping site to that of the colchicine-binding site. This strengthens the fact that the colchicine-binding domain of tubulin is a prominent domain where many antimitotic compounds bind. DAT1 exhibits fluorescence upon binding to tubulin. An interesting fact can be noted in this connection. Colchicine also does not exhibit fluorescence in aqueous solution, but upon binding to tubulin, it generates fluorescence (Bhattacharyya & Wolff, 1974). This is attributed to the immobilization of the chromophoric group (Bhattacharyya & Wolff, 1984) and induction of conformational change in both the drug and the protein (Garland, 1978; Detrich *et al*., 1981; Andreu & Timasheff, 1982). This analogy between the tubulin binding properties of colchicine and DAT1 poses a contrast to the possible difference in their mechanism of inhibition of microtubule assembly. Further study on the binding of DAT1 to tubulin is needed to throw light on this aspect.

Considering all these facts, DAT1, with its small size and simple method of synthesis, can be a pilot compound for developing highly potent diaminoketothiazoles in diseases where cell proliferation is a problem. It can also be a promising agent for structural studies of tubulin and microtubules.

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