Reversible Action of Diaminothiazoles in Cancer Cells Is Implicated by the Induction of a Fast Conformational Change of Tubulin and Suppression of Microtubule Dynamics

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Abstract

Diaminothiazoles are novel cytotoxic compounds that have shown efficacy toward different cancer cell lines. They show potent antimitotic and antiangiogenic activity upon binding to the colchicine-binding site of tubulin. However, the mechanism of action of diaminothiazoles at the molecular level is not known. Here, we show a reversible binding to tubulin with a fast conformational change that allows the lead diaminothiazole DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino)thiazole] to cause a reversible mitotic block. DAT1 also suppresses microtubule dynamic instability at much lower concentration than its IC50 value in cancer cells. Both growth and shortening events were reduced by DAT1 in a concentration-dependent way. Colchicine, the long-studied tubulin-binding drug, has previously failed in the treatment of cancer due to its toxicity, even though it generates a strong apoptotic response. The toxicity is attributable to its slow removal from the cell due to irreversible tubulin binding caused by a slow conformational change. DAT1 binds to tubulin at an optimal pH lower than colchicine. Tubulin conformational studies showed that the binding environments of DAT1 and colchicine are different. Molecular dynamic simulations showed a difference in the number of H-bonding interactions that accounts for the different pH optima. This study gives an insight of the action of compounds targeting tubulin’s colchicine-binding site, as many such compounds have entered into clinical trials recently.

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Introduction

A major role of microtubules is to form the spindle leading to segregation of chromosomes during mitotic cell division. Because of this critical function, microtubules have become a popular target to stop cancer cell proliferation and indeed many microtubule-targeting compounds have shown high capability to kill and arrest the progression of tumors (1). Successful microtubule-binding agents shift tubulin–microtubule equilibrium and alter microtubule dynamics, which are extremely important in the formation of the mitotic spindle and the capture and segregation of chromosomes. The major constituent of microtubules, tubulin, has become one of the most validated targets for cancer chemotherapy. It harbors 3 important drug-binding sites. Compounds binding to the taxol- and vinblastine-binding sites are already being used in the clinic although many of them invariably suffer from drug resistance and neurotoxicity (2–4). The colchicine-binding site is another important drug-binding site in tubulin. The parent compound colchicine shows potent cytotoxic and apoptosis-inducing properties (5). Although studied extensively at the biochemical level, colchicine has failed in the clinic due to toxicity reasons (6). The toxicity can be attributed to its slow removal from the cell due to very slow dissociation from tubulin. This is indicated by the fact that when an antibody with higher affinity toward colchicine, compared with colchicine–tubulin affinity, was added to the cell after 6 hours of colchicine treatment, the colchicine-induced toxicity was reversed (7). A slow and poorly reversible binding to tubulin giving rise to a conformational change (8) can be implicated for this action of colchicine in the cell. However, in the last few years, many colchicine site–binding compounds have shown promise in preclinical studies. These compounds have also attracted attention as some of them showed potent antiangiogenic activity, which is an important aspect of cancer chemotherapy. Compounds

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like 2-methoxyestradiol, sulphonamide E7010, combrestatin A4 are now in clinical trials (9–12). These compounds show promise in resistant tumor lines, because their small size permits their escape from transmembrane pumps (13). It is thus important to understand the mechanism of action of colchicine site binders with respect to the colchicine–tubulin pharmacophore (the minimum structural requirement for a drug-binding site involving atoms or groups from both the protein and the ligand), so that new chemotherapeutic agents can be developed with improved efficacy and less toxicity.

Diaminothiazoles are novel cytotoxic compounds that have shown efficacy toward different cancer cell lines (14, 15). They block cells in the mitotic phase by destroying spindle structure and chromosome organization and inhibit microtubule assembly. They induce apoptosis by both the intrinsic and extrinsic pathways and are able to induce apoptosis in cells where many other compounds fail due to the blockage of the mitochondrial pathway (16). They also display potent antiangiogenic activity at very low concentrations (17). Diaminothiazoles have also shown an inhibitory effect on cyclin-dependent kinases (18) indicating multiple modes of action. The lead compound of this class, DAT1 (structure given in Fig. 1), binds to the colchicine-binding site of tubulin with exhibition of fluorescence (14). However, unlike colchicine, DAT1 binding is fast. The mechanism of action of diaminothiazoles at the molecular level is not known. Here, we report that DAT1 suppresses microtubule dynamic instability in live cells even at a concentration where there is little depolymerization of microtubules. We have also shown that DAT1 induces reversible mitotic block and the depolymerization of the microtubule network in the cell could also be reversed after removal of DAT1. Looking further, we have found that DAT1 binding to tubulin is reversible and gives rise to a fast conformational change in tubulin. The binding environment of DAT1 in tubulin has been discussed in comparison with colchicine.

Materials and Methods

DAT1 was synthesized as reported earlier (19). Taxol (paclitaxel), PIPES, GTP, EGTA, DTNB, and Sephadex G-50 were from Sigma. Dulbecco’s Modified Eagle’s Media (DMEM) with or without pH indicator and antibiotic/antimycotic solutions were from Sigma or Invitrogen. Antibody against α-tubulin (T6199) was from Sigma and secondary antibody conjugated with Alexa 488 was from Invitrogen. All other chemicals used were of reagent grade. HCT116 and HeLa cell lines were from American Type Culture Collection (ATCC), who authenticated the cell lines by short tandem repeat analysis (intraspecies). HCT116 cells stably expressing GFP tubulin (20) was a kind gift from Dr Gary K. Schwartz, Sloan-Kettering Cancer Centre, New York. GFP tubulin expressing LLCPK cell line (21) was a kind gift from Dr. P. Wadsworth, University of Massachusetts, Boston, MA. Frozen stocks of cells from the reference stock were made within passage 3 and stored in liquid nitrogen. For experiments, cells were never used beyond 3 months after revival. No further authentication of the cell lines was conducted. Transfected cell lines, while not directly verified, had maintained their morphologic characteristics and maintained stable expression of the GFP fusion protein.

Live cell imaging

The live cells were imaged in DMEM without phenol red indicator on a Zeiss LSM meta or Nikon Eclipse Ti (AIR) confocal microscope with a stage maintained at 37°C and 5% CO₂.

Microtubule dynamics

LLCPK cells stably expressing GFP tubulin were used for the dynamics studies (21) in the presence or absence of DAT1. The cells were grown at 37°C and 5% CO₂ in high-glucose DMEM supplemented with 10% FBS, antibiotic/antimycotic, 1 mmol/L sodium pyruvate, and 0.4 mg/mL G418 (to maintain the GFP-expressing cells).
Images were done for each clearly distinct and visible microtubule tip in a field for about 120 seconds. About 30 frames were acquired at an interval of 4 seconds. Meta morph imaging software (Molecular Devices) was used to track the length changes of individual microtubules as reported previously (22, 23). Dynamic Instability calculations were done for about 50 microtubules for the control and drug-treated sets as described previously (24). For each parameter of dynamic instability, statistical significance was analyzed by unpaired t test using GraphPad Software (www.graphpad.com/quickcalcs/ttest1.cfm).

Cell synchronization and cell-cycle analysis
Cells were seeded in 35-mm dishes in DMEM, and after attachment, they were synchronized with 2 μmol/L aphidicolin for 18 hours. After washing out the aphidicolin, the cells were released into 0.5 μmol/L DAT1-containing media and incubated for 12 hours. Subsequently, the drug-containing medium was removed and fresh medium was added. The cells were then trypsinized at 6, 12, and 24 hours following washing and then fixed in ice-cold 70% ethanol. After RNase treatment, fixed cells were stained with 40 μg/mL propidium iodide (PI) and analyzed in a BD FACSAria flow cytometer using the software FACSDiva.

Reversible depolymerization of microtubules in HCT116 cells
HCT116 cells stably expressing GFP tubulin were seeded in 35-mm glass-bottomed dishes. After imaging control cells, 5 μmol/L DAT1 was added to the cells and imaged at different time points. When the microtubule fibers appeared to be almost depolymerized, the drug-containing medium was replaced by fresh medium without drug and imaged again under the same conditions.

Tubulin purification
Microtubular protein (MTP) was prepared from goat brain homogenate by 3 cycles of temperature-dependent assembly and disassembly in PEM buffer (100 mmol/L PIPES, 1 mmol/L EGTA, 1 mmol/L MgSO4, pH 6.9) in the presence of 0.5 mmol/L GTP (25). Pure tubulin was purified from MTP by glutamate-induced polymerization in presence of 1 mmol/L GTP (26).

Binding reversibility
Tubulin was incubated with DAT1 and then passed through a sephadex G-50 column (1 × 20 cm) equilibrated with PEM buffer containing 0.1 mmol/L GTP at 4°C. The protein collected in the void volume was monitored by measuring the absorbance at 280 and 374 nm and the fluorescence at 455 nm. To measure the off-rate of DAT1 dissociation, 50 μmol/L podophyllotoxin was added to the DAT1–tubulin complex (1:1), and the emission spectrum was recorded immediately in the time scan mode at 435 nm using an excitation wavelength of 374 nm. The fluorescence values were plotted against time and fitted exponentially. The off-rate constant (koff) was calculated using the exponential equation $y = A_1 e^{-x/t_1} + y_0$ where $A_1$ is the pre-exponential factor and $1/t_1$ is the off-rate constant.

Measurement of GTPase activity
The standard method of malachite green ammonium molybdate assay was used to estimate the amount of inorganic phosphate released during the hydrolysis of GTP (27). Tubulin was incubated with drug or PEM buffer (control) for 30 minutes at room temperature and 0.1 mmol/L GTP was added and further incubated. The reaction was stopped at specific time intervals by adding 70% perchloric acid. The quenched samples were stored on ice until all time points were collected. The samples were then incubated with malachite green ammonium molybdate reagent at room temperature for 1 hour in the dark and then the absorbance at 650 nm was measured.

Measurement of DTNB kinetics
The formation of a thionitrobenzoate anion by DTNB reaction with free sulfhydryl groups of tubulin was monitored by measuring absorbance at 412 nm ($\epsilon_{412} = 13,600$ (mol/L cm)$^{-1}$) over time (28). Tubulin–colchicine and tubulin–DAT1 complexes were prepared separately (1:15) in PEM buffer and then incubating at 37°C for 60 minutes. For comparison, control tubulin was also incubated at 37°C for 60 minutes. Immediately after the incubation, each sample was mixed with DTNB at a final concentration of 400 μmol/L and the absorbance was measured at 412 nm continuously over time. The number of cysteine residues per molecule of tubulin reacting with DTNB was calculated using the equation $(A_{Tub-DTNB} - A_{DTNB})/13,600 \times$ tubulin concentration.

pH studies
Tubulin (22.2 mg/mL) in PEM buffer, pH 6.95, was added to respective pH buffers to a final concentration of 5 μmol/L. The stock protein concentration was maintained high so that the volume required to be added in the buffers of different pH was within 2.4%. In this condition, the pH change due to dilution was within 0.05 units. Ten μmol/L DAT1 or colchicine was then added and the fluorescence readings were recorded after 5 minutes for DAT1 and 50 minutes for colchicine. Emission values at 457 nm for DAT1 and 435 nm for colchicine were plotted. Excitation wavelengths used were 374 and 350 nm for DAT1 and colchicine, respectively.

Spectrophotometric and spectrofluorometric experiments were carried out in Perkin Elmer Lambda 25 UV/VIS spectrophotometer and LS50B luminescence spectrometer, respectively.

Molecular dynamic simulation
The ligands were docked in the colchicine-binding pocket of tubulin (29) using the crystal structure of dama colchicine-bound tubulin (PDB id 1SAO) using Glide program (Schrodinger). The final energy evaluation was done with Glide Score, and the best poses were generated.
as the output for the ligands. The simulation was conducted in GROMACS 4.5.4 software package (29) using GROMOS43a forcefield (30). Molecular dynamic simulation was done starting from the coordinates of tubulin crystal structure (PDB-1SAO) complexed with colchicine as well as with DAT1 obtained from docking. The PRODRG server (31) was used to generate the ligand topology. Upon analysis of the amino acid composition of tubulin, all the titrable amino acids and ionizable groups of ligands were identified and protonated appropriately at pH 7.0 and 6.0 after comparing their pKa values using PROPKA server (32). A total of 10 nanoseconds molecular dynamics simulation was conducted at 310 K in the NPT ensemble with temperature velocity rescaling coupling (33) and at 1 atm pressure with Berendsen isotropic coupling (34). For protein–ligand interaction studies and other analyses, representative structure was extracted using the closest frame from the native structure and visualized using PyMol (35). Hydrogen bonds have been considered within 2.5 Å donor–acceptor distance.

Results

Effect of DAT1 on microtubule dynamics in living cells

Dynamic turnover of microtubules is involved in many functions within cells, including a critical role during mitosis. Successful anticancer drugs, including taxol and vinblastine, and some other potential drugs affect the dynamic properties of microtubules and typically lead to mitotic arrest. We hypothesized that DAT1 would also disrupt normal microtubule turnover in cells. Because the density of microtubules is very high within the mitotic spindle, we examined the turnover of interphase microtubules and focused our analysis on microtubules extending to the cell periphery. Individual microtubule ends can be easily detected in this location in cells expressing GFP-α-tubulin. An LLCPK cell line stably expressing GFP-α-tubulin (21) was used for all experiments. DAT1 was added to the culture medium at 2 different concentrations: 80 nmol/L, which was much less than the average IC_{50} of DAT1 in cancer cells (0.3 μmol/L; ref. 14) and at 1.6 μmol/L, a concentration 5-fold higher than the IC_{50}. Cells were then imaged beginning approximately 5 minutes after drug addition and images were collected up to an hour after drug addition. At 80 nmol/L DAT1, the microtubule cytoskeleton remained intact and little, if any, microtubule depolymerization was apparent (Fig. 2). Microtubules remained dynamic but showed slightly slower depolymerization rate (Table 1). The microtubules also spent more time in a pause state, where they showed no detectable growth or shortening. These microtubules also showed less frequent catastrophes (the switch from growth to shortening states) and rescues (the switch from shortening to growth states; Table 1). At the higher DAT1 concentration (1.6 μmol/L), microtubule depolymerization was apparent from the increased fluorescence of soluble tubulin (visible in Fig. 2, outlining the cell margin). Microtubules also did not polymerize all the way to the plasma membrane. For those microtubules that remained in 1.6 μmol/L DAT1, the dynamics were altered more significantly than in 80 nmol/L DAT1. Both growth and shortening rates were reduced and the time spent in pause was increased. Catastrophe and rescue frequencies were also reduced. Net assembly, as measured by dynamicity (the total micrometers of polymer gained and lost per unit time) and drift velocity (net gain or loss of micrometers of polymer over time), was reduced at both DAT1 concentrations tested (Table 1). Taken together, the microtubules were generally less dynamic in DAT1 compared with dimethyl sulfoxide (DMSO)-treated cells, consistent with previous reports examining other tubulin-targeted drugs, vinblastine (36) and nocodazole (37). In each case, observation of cells shortly after adding the drug revealed a general dampening of microtubule turnover, at least for the microtubules that remain, whereas at longer incubation times and/or higher concentrations, these drugs depolymerize microtubules. Therefore, we next examined whether DAT1 could reversibly arrest mitosis and depolymerize microtubules following longer incubations in the drug compared with the 1-hour window explored above.

Reversible G2–M arrest by DAT1

Irreversible effects and slow removal of drugs in cells may lead to toxicity. Colchicine, whose therapeutic use has been discarded in cancer treatment due to toxicity, was earlier shown to arrest mitosis efficiently. But its effect is not reversed resulting in polyplody and chromosomal abnormalities (7). As DAT1 was earlier found to bind to the colchicine-binding site of tubulin and arrest mitosis efficiently, we analyzed the DNA content of cells by flow cytometry for populations of cells fixed before or at time points after drug washout. Synchronized HCT116 cells were treated with 0.5 μmol/L DAT1 (2 times the IC_{50} of mitotic arrest; ref. 14) for a period of 12 hours and then DAT1 was washed out and cells maintained in drug-free media. After 12 hours of drug treatment, 55% of cells were arrested in G2–M phase (Fig. 3). Cells recycled back from the mitotic arrest within 6 hours of drug removal and maintained normal profile of cell-cycle phases, as measured by their DNA content, till 24 hours of follow-up. The reversible action of DAT1 on the induction of the mitotic block was also confirmed by immunocytochemistry. Multipolar spindles and depolymerization of tubulin were seen following 12 hours of DAT1 treatment (Supplementary Fig. S1). Following drug removal, the cells regained their normal morphology, including normal spindle morphology, within 6 hours. The reversible mitotic block by DAT1 was also observed in HeLa cells (Supplementary Fig. S2) proving that the reversible action of DAT1 is not cell line specific.

Reversible depolymerization of microtubules in cells by DAT1

DAT1 was earlier shown to inhibit microtubule polymerization in vitro with an IC_{50} of 23 μmol/L (14). We
thus tested whether the effect of DAT1 on cellular microtubules was reversible in colon cancer cell line HCT116 expressing GFP tubulin. The cells were plated onto glass bottom dishes and were allowed to attach and spread on the surface. Microtubules were clearly visible in these cells. Five μmol/L DAT1 was added into the dish and the cells were then imaged at 10, 20, and 30 minutes, respectively. The microtubules showed a gradual depolymerization, and at about 30 minutes after drug addition, the microtubules almost fully depolymerized and the cells became rounded (Fig. 4A, top). The repolymerization was then tracked after removing the drug-containing medium and adding fresh medium. The depolymerized microtubules slowly began to polymerize, and within 2 hours, the cells started regaining their normal shape and size (Fig. 4A, bottom). However, a full microtubule array was not present until 6 to 12 hours after drug washout as was seen by immunocytochemistry (Supplementary Fig. SIC and S1D).

Reversible binding of DAT1 to tubulin

To check for causes of the reversible effect of DAT1 in the cell, we further studied the reversibility of DAT1 binding to tubulin. DAT1 binding to tubulin was complete within the time of mixing manually. To detect the nature of binding, the DAT1–tubulin complex showing fluorescence emission maximum at 457 nm (14) was passed through a sephadex G-50 column. Protein collected in the void volume showed absorbance at 280 nm only and much reduced absorbance at 374 nm. It showed only 5% to 10% fluorescence compared with the initial complex of same concentration, consistent with release of DAT1. Under similar conditions, colchicine cannot be removed from the colchicine–tubulin complex, which is poorly reversible (38). These data indicate that DAT1 binding to tubulin is noncovalent and DAT1 can be removed easily from tubulin.

Reversibility of binding of DAT1 to tubulin was checked using excess podophyllotoxin as it was found to be a competitive inhibitor of DAT1 (14). The addition of...
molar podophyllotoxin reduced the fluorescence of the DAT1–tubulin complex at 455 nm in a time-dependent manner. Within 3 minutes, 90% fluorescence was reduced (Fig. 4B). The off-rate constant of the dissociation reaction was found to be $2.9 \times 10^{-2}$ s$^{-1}$ showing that DAT1 binding to tubulin was reversible.

This is in contrast to the action of colchicine, which has an off-rate constant of $1.8 \times 10^{-5}$ s$^{-1}$ (38), even though DAT1 shares the same binding site with colchicine on tubulin (14). To find an explanation of this difference in behavior, we compared the binding of DAT1 and colchicine with tubulin.

### Table 1. Effects of DAT1 on the MT dynamic instability in LLCPK cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DMSO control cells</th>
<th>80 nmol/L DAT1</th>
<th>1.6 (\mu\text{mol/L}) DAT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of microtubules</td>
<td>37</td>
<td>51</td>
<td>28</td>
</tr>
<tr>
<td>Growth rate, (\mu\text{m/min})</td>
<td>8.4 ± 5.0</td>
<td>7.2 ± 3.8</td>
<td>5.7 ± 2.5$^a$</td>
</tr>
<tr>
<td>Shortening rate, (\mu\text{m/min})</td>
<td>9.9 ± 5.9</td>
<td>8.1 ± 5.3$^b$</td>
<td>5.7 ± 1.7$^c$</td>
</tr>
<tr>
<td>Percent time spent in growth</td>
<td>24.9</td>
<td>13.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Percent time spent in shortening</td>
<td>16.7</td>
<td>10.7</td>
<td>11.9</td>
</tr>
<tr>
<td>Percent time spent in pause</td>
<td>58.5</td>
<td>76.2</td>
<td>79.4</td>
</tr>
<tr>
<td>Dynamicity, (\mu\text{m/min})</td>
<td>3.8 ± 2.0</td>
<td>2.0 ± 2.4$^a$</td>
<td>1.5 ± 1.3$^c$</td>
</tr>
<tr>
<td>Drift velocity, (\mu\text{m/min})</td>
<td>0.8 ± 1.7</td>
<td>0.2 ± 0.8$^c$</td>
<td>-0.2 ± 0.3$^c$</td>
</tr>
<tr>
<td>Catastrophe frequency, s$^{-1}$</td>
<td>0.054 ± 0.005</td>
<td>0.024 ± 0.003$^c$</td>
<td>0.032 ± 0.004$^c$</td>
</tr>
<tr>
<td>Rescue frequency, s$^{-1}$</td>
<td>0.058 ± 0.006</td>
<td>0.029 ± 0.003$^c$</td>
<td>0.024 ± 0.004$^c$</td>
</tr>
</tbody>
</table>

**NOTE:** Microtubule dynamics were measured in LLCPK cells expressing GFP-\(\alpha\)-tubulin as described in Materials and Methods. For the control cell population, DMSO was added at 1:1,000 dilution. Data are given as mean ± SD. Data between groups were compared by unpaired t tests as described in Materials and Methods. Growth and shortening velocities are averages of all events (\(n\)) for all microtubules analyzed. For growth rates, \(n = 126, 94, 47\) (DMSO, 80 nmol/L and 1.6 \(\mu\text{mol/L}\) DAT1, respectively) and for shortening rates, \(n = 100, 80, 63\) (DMSO, 80 nmol/L and 1.6 \(\mu\text{mol/L}\) DAT1, respectively).

$^a$\(P < 0.001\).

$^b$\(P < 0.05\).

$^c$\(P < 0.0001\).

50 \(\mu\text{mol/L}\) podophyllotoxin reduced the fluorescence of the DAT1–tubulin complex at 455 nm in a time-dependent manner. Within 3 minutes, 90% fluorescence was reduced (Fig. 4B). The off-rate constant of the dissociation reaction was found to be $2.9 \times 10^{-2}$ ± 0.5 s$^{-1}$ showing that DAT1 binding to tubulin was reversible.

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Characterization of DAT1 binding to tubulin

Colchicine is known to give rise to a slow conformational change in tubulin. Earlier report showed that DAT1, which did not have fluorescence of its own, induced fluorescence upon binding to tubulin. This indicated a conformational change upon binding. To test whether tubulin experienced any conformational change upon binding of DAT1, we examined tubulin’s GTP hydrolysis and sulfhydryl exposure as measures of conformational change. The tubulin that we used for these experiments was in GTP–tubulin conformation as was confirmed by its assembly competence.

Tubulin is known to have a polymerization-independent GTPase activity that depends on tubulin conformation and certain changes in the conformation give rise to a change in its intrinsic GTPase activity. Colchicine induces GTPase activity and large conformational changes in tubulin (39). DAT1 also gave rise to a modest increase in the GTPase activity of tubulin indicating a conformational change (Fig. 5A).

Change in protein conformation might result in changes in sulfhydryl group exposure (40, 41). To test whether DAT1 changes the exposure of cysteine residues in tubulin, the formation of a thionitrobenzoate anion was monitored by measuring absorbance at 412 nm over time by DTNB reaction with free sulfhydryl groups of tubulin (Fig. 5B). As a positive control, the DTNB reaction was also measured for the tubulin–colchicine complex.
found that 18.6 cysteines reacted with DTNB in colchicine-bound tubulin and 18.4 reacted with DTNB in the DAT1–tubulin complex as compared with the 20.8 in free tubulin. This shows that DAT1, similar to colchicine, protects 2 sulfhydryl groups due to a conformational change in tubulin induced by drug binding. In addition, the reactivity of the sulfhydryl groups in DAT1 bound tubulin was faster than that of the tubulin–colchicine complex in the initial stages, for example, 15 cysteine residues of the DAT1–tubulin complex reacted within 5 minutes. For native tubulin, 18 cysteines reacted in the same time, whereas for the colchicine–tubulin complex, only 10 cysteine residues reacted in 5 minutes. These data indicate that DAT1-induced conformational change of tubulin makes the protein less compact than colchicine.

**pH dependence of DAT1 binding**

The pH dependence of protein–ligand binding arises from changes in the pKa values of ionizable groups upon complex formation that reflects a change in affinity (42) and thus can give important information about the binding site and the mechanism of binding. When tested in a range of pH 6 to 7.5, DAT1 exhibited maximum fluorescence intensity and consequently maximum binding with tubulin at pH 6 (Fig. 5C), whereas the colchicine-binding activity of tubulin showed a maximum at pH 7.0 (Supplementary Fig. S3). At these different pH buffers, DAT1 or colchicine alone did not have any fluorescence. Earlier reports also support that colchicine exhibits maximum binding to tubulin at pH 6.8 (43). This shows that even though DAT1 and colchicine bind to the same or overlapping binding sites, the pharmacophore for these 2 compounds might be exposed to somewhat different environments. The slow conformational change for colchicine and fast change for DAT1 also supports this conclusion. We thus did a molecular modeling study to get an overview of the DAT1–tubulin interaction and compared it with the colchicine-binding pharmacophore.
Molecular modeling
We conducted a systematic docking study of both DAT1 and colchicine into the colchicine-binding pocket of tubulin using GLIDE. As the conformational studies and the pH study indicated a difference in the binding environment of DAT1 and colchicine, we conducted molecular dynamic simulations of both the docked complexes in different pH conditions for 10 nanoseconds using GROMACS force field. The simulations were analyzed by plotting the number of hydrogen bonds as a function of simulation time (Supplementary Fig. S4).

We have observed a remarkable difference between the DAT1–tubulin and colchicine–tubulin complexes in the formation of intermolecular hydrogen bonds during the simulation length at different pH. During the analysis time, DAT1 showed maximum frequency of one H-bond at pH 7.0, whereas at pH 6.0, it could form two H-bonds with highest frequency (Supplementary Fig. S4A). The frequency of three H-bonds also increased. However, a reverse scenario was predicted for colchicine that showed maximum frequency of two H-bonds at pH 7.0, and at pH 6.0, the number of H-bonds showing highest frequency was one (Supplementary Fig. S4B). The protonation of the amino group of Lys 352 played an important role, which was found to be involved in the H-bond formation almost throughout the 10-nanosecond time of simulation in case of the DAT1–tubulin interaction at pH 6.0. Asn258 and Ala371 also showed high frequency of H-bond formation (Supplementary Movies SM1, SM2 for DAT1 and SM3, SM4 for colchicine). The models for DAT1 and colchicine bound to tubulin are shown in Fig. 6. The H-bonds that were found to be formed in the highest frequencies during simulation are shown in each case (Fig. 6, yellow dotted lines).

Discussion
Diaminothiazoles are an emerging class of antimitotic agents that are of interest due to their efficacy against different types of cancer cells and their antiangiogenic properties. They target the colchicine-binding site of tubulin. Colchicine binds to tubulin with high affinity but has slow binding kinetics and the interaction is poorly reversible. It is an efficient cytotoxic agent and apoptosis inducer (44), but its therapeutic use has been hampered because of high toxicity (6). The toxicity can be attributed to its slow removal from the cell due to very slow dissociation. Compounds that bind to the colchicine-binding site reversibly are likely to have a therapeutic advantage over colchicine. In an effort to study the mechanism of action of diaminothiazoles, we found that the lead diaminothiazole DAT1 induces a reversible mitotic block in cancer cells. Cells re-entered the cell cycle after DAT1 washout within 24 hours, indicating that the removal rate for DAT1 is high. The depolymerizing effect of DAT1 on cellular microtubules was also reversible showing that DAT1 treatment did not cause any long-term effect on cell morphology. Compared with colchicine, DAT1 binding to tubulin was fast and reversible with a much faster

Figure 6. Models of the DAT1–tubulin and colchicine–tubulin interaction. Molecular simulations were carried out with the docked structures using GROMACS. The α-tubulin subunit is shown in blue and the β-tubulin is shown in green. In the DAT1 structure, the atoms are indicated as carbon-cyan, oxygen-red, hydrogen-white, nitrogen-blue, and sulfur-yellow. In the colchicine structure, atoms are carbon-yellow, oxygen-pink, hydrogen-white, and nitrogen-blue. The H-bonds are shown as yellow dotted lines and marked with red arrows. A, DAT1–tubulin; B, DAT1–protonated tubulin; C, colchicine–tubulin; and D, colchicine–protonated tubulin. The nonexchangeable site GTP bound to the α-subunit of tubulin is shown in orange.
dissociation with an off-rate constant 1,600 fold higher than colchicine. Furthermore, DAT1 induced a fast conformational change in tubulin as opposed to colchicine, which was earlier reported to induce a slow conformational change (8). It should be noted that colchicine is a rigid molecule whereas DAT1 is a flexible molecule, which may partially explain the difference in kinetics. The colchicine–tubulin interaction is strongly influenced by pH. The pH profile of colchicine-binding activity to tubulin shows the optimum pH as 6.8 to 7.0. Over the pH range tested (6.0–7.5), DAT1 displayed maximum binding at pH 6.0, significantly different from colchicine binding. Modeling of DAT1 and colchicine to tubulin’s colchicine-binding pocket showed that Lys352 was in close proximity of the binding site and an extra H-bond with the protonated amino group of this lysine at pH 6.0 formed the basis of a stronger interaction of DAT1 at pH 6.0. Colchicine, however, underwent destabilization by the loss of one H-bond at pH 6.0 (Fig. 6).

Cells treated with DAT1 showed a significant dampening of microtubule dynamic instability at the periphery of interphase cells (Fig. 2, Table 1). DAT1 (1.6 µmol/L) also caused depolymerization of some microtubules within cells. While we did not measure mitotic microtubule dynamics directly, it is likely that DAT1 has similar effects on microtubules during mitosis. For several well-characterized microtubule-targeting drugs, it is thought that the drugs work through modulation of microtubule dynamics rather than through changes to the polymer mass of the microtubules (45). Consistent with this idea, DAT1 changed the dynamic properties of microtubules, the morphology of the spindles, and induced a mitotic block at concentrations lower than its IC50 for inhibition of polymerization (this report and ref. 14).

Thus, in short, DAT1 can be considered as an efficient drug modulating microtubule dynamics and with a highly reversible nature, thus lowering its toxicity in cells. This study places diaminothiazoles in a favorable position to be developed further for cancer treatment. A large number of antimitotic compounds showing efficacy against cancer cells bind to the colchicine-binding site of tubulin. The fact that the structure of these compounds may vary widely has raised interest about the pharmacophores involved in the colchicine–tubulin binding site. Our study brings an important insight into the environment and properties of the colchicine–tubulin interaction site, which would help in future drug development targeting this site.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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