D-24851, a Novel Synthetic Microtubule Inhibitor, Exerts Curative Antitumoral Activity in Vivo, Shows Efficacy toward Multidrug-resistant Tumor Cells, and Lacks Neurotoxicity

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ABSTRACT

N-(pyridin-4-yl)-[1-(4-chlorobenzyl)-indol-3-yl]-glyoxyl-amid (D-24851) is a novel synthetic compound that was identified in a cell-based screening assay to discover cytotoxic drugs. D-24851 destabilizes microtubules and blocks cell cycle transition specifically at G2-M phase. The binding site of D-24851 does not overlap with the tubulin binding sites of known microtubule destabilizing agents like vincristine or colchicine. In vitro, D-24851 has potent cytotoxic activity toward a panel of established human tumor cell lines including SKOV3 ovarian cancer, U87 glioblastoma, and ASPC-1 pancreatic cancer cells. In vivo, oral D-24851 treatment induced complete tumor regressions (cures) in rats bearing Yoshida AH13 sarcomas. Of importance is that the administration of curative doses of D-24851 to the animals revealed no systemic toxicity in terms of body weight loss and neurotoxicity in contrast to the administration of paclitaxel or vincristine. Interestingly, multidrug-resistant cell lines generated by vincristine-driven selection or transfection with the Mtr, 170,000 P-glycoprotein encoding cDNA were rendered resistant toward paclitaxel, vincristine, or doxorubicin but not towards D-24851 when compared with the parental cells. Because of its synthetic nature, its oral applicability, its potent in vitro and in vivo antitumoral activity, its efficacy against multidrug-resistant tumors, and the lack of neurotoxicity, D-24851 may have significant potential for the treatment of various malignancies.

INTRODUCTION

Compounds that interfere with the cell cycle have become a major interest in cancer research because they inhibit the proliferation of tumor cell lines derived from various organs (1). The well-characterized and clinically used antimitotic drugs, namely the taxanes (paclitaxel, docetaxel; Refs. 2–5) and the Vinca alkaloids (vincristine, vinblastine, vinorelbine; Refs. 6), bind to tubulin, one of the essential proteins for chromosomal segregation. Alternating α- and β-tubulins polymerize to microtubules, long dynamic tubular fibers, which constitute the mitotic spindles. Microtubule inhibitors interfere with the microtubule dynamics of tubulin polymerization and depolymerization, which results in the inhibition of chromosome segregation in mitosis and consequently the inhibition of cell division (7, 8). The three major classes of tubulin-binding agents are the taxanes, which stabilize microtubules by blocking disassembly, the Vinca alkaloids, and the colchicine-site binders (9). The latter two are microtubule destabilizing agents that act by blocking assembly of tubulin heterodimers. A major point is that nondividing cells are extremely resistant toward these drugs (10), whereas uncontrolled dividing tumor cells run into cell cycle arrest by cell cycle checkpoint pathways. Subsequently apoptosis of the cells may be initiated (11).

Although the taxanes and the Vinca alkaloids are effective in the treatment of different malignancies, their potential is limited by the development of drug resistance (8). One pathway leading to resistance is mediated by overexpression of transmembrane efflux pumps, namely the p-gp170 (12) and the MRPs (13). These efflux pumps are able to reduce the intracellular concentrations of taxanes and Vinca alkaloids to a nontoxic level. Resistance is also mediated by the expression of tubulin isotypes and mutants that showed impaired taxane-driven tubulin polymerization (14). Another major drawback of taxanes and Vinca alkaloids in clinical application is the development of neurotoxicity (15–19). The drugs interfere with the function of microtubules in axons, which mediate the neuronal vesicle transport (8). New chemical entities that bind to tubulin but neither are a substrate of transmembrane pumps nor interfere with the function of axonal microtubules would strongly increase the therapeutic index in the treatment of malignancies. In screening for a compound with these superior characteristics, a molecule named D-24851 was identified that destabilizes microtubules in tumor cells and cell-free systems. D-24851 does not interact with the tubulin-binding sites of vincristine and colchicine. Further characterization revealed that this microtubule inhibitor is not a substrate of p-gp170 nor of MRPs and, consequently, retains its antitumoral efficacy in cell lines with MDR or MRP resistance phenotypes. In addition, the administration of D-24851 to rats revealed no deficit in motor function and no change in NCV, which suggested a lack of neurotoxicity of D-24851.

MATERIALS AND METHODS

Materials and Cell lines. D-24851[N-(pyridin-4-yl)-[1-(4-chlorobenzyl)-indol-3-yl]-glyoxylic acid amide; ASTA Medica AG]3 was synthesized as follows. Reaction of indole with 4-chlorobenzylchloride in dimethylformamide yielded N-(4-chlorobenzyl)-indole in 97% yield. Treatment of N-(4-chlorobenzyl)-indole with oxalychlordie in tert-butylmethylether or diethyl ether as solvent gave, in 90% yield, 1-(4-chlorobenzyl)-indol-3-yl-glyoxylic acid chloride. Aminolysis reaction of the latter compound with excess of 4-aminoypyridine in dimethylformamide under cooling afforded the desired N-(pyridin-4-yl)-[1-(4-chlorobenzyl)-indol-3-yl]-glyoxylic acid amide in 64% yield.

General chemicals (paclitaxel, vincristine, vinblastine, and podophyllotoxin) were purchased from Sigma (Munich, Germany). Radiochemicals were obtained from Amersham Corp. P-glycoprotein (C219), and α-tubulin (B-5–1-2) antibodies, were purchased from Alexis Biochemicals Corp. (Grüningen, Germany) and Sigma (Munich, Germany), respectively. Cy3-conjugated and peroxidase-conjugated goat antinouse antibodies were obtained from Dianova (Hamburg, Germany). Tumor cell lines SKOV3 (ovary/human/HTB-77, KB/184).

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3 The abbreviations used are: p-gp170, M1, 170,000 P-glycoprotein; MDR, multidrug resistant; MRP, multidrug resistance protein; NCV, nerve conductance velocity; 5-FU, 5-fluorouracil; XTT, sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)-benzene sulfonic acid hydrate; FACS, fluorescence-activated cell sorting; SRB, sulforhodamine B, ILS, increased life span; GTP, guanosine triphosphate.

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HeLa (cervix/human/CCL-17), HT 29 (colon/human-HTB-38), A549 (lung/human/CCl-185), PC-3 (prostate/human/CRL-1435), DU145 (prostate/human-HTB-81), AsPC-1 (pancreas/human/CRL-1682), C6 (brain/rat/CCL-107), U 87 (brain/human/HTB-14), MDA-MB 231 (breast/human-HTB-26), and L1210 (leukemia/mouse) were obtained from ATCC. LT12 and LT12/mdr1 were a gift from Dr. K. Nooter (University Hospital Rotterdam, Rotterdam, the Netherlands). The detailed characteristics of human parental A2780/wt (ovarian), MCF-7/wt (breast), HT1080 (fibrosarcoma), HCT-8 (colon), HT-29 (colon) cell lines as well as the MDR p-gp170 overexpressing A2780/Dx5 and MCF-7/adr, the MRPs expressing MDR HT1080/Dx4 (20), the cisplatin-resistant A2780/CIP2, the 5-FU-resistant HT29-R1 (bolus) and HT29-R24 (continuous exposure), the raltitrexed-resistant HT29/ICD, cell lines have been published previously (21–25).

**Flow Cytometry.** KB/HeLa cells (1 × 10^6 cells) were incubated for 24 h with cytotoxic agents and then extracted for 3 min with ice-cold 0.5% Triton X-100 in PHEM containing 10 μM paclitaxel. This treatment removes unassembled tubulin while preserving microtubules (26). The cultures were then fixed by the addition of PHEM containing 8% paraformaldehyde and 0.5% glutaraldehyde at 4°C. Microtubules were visualized using a mouse monoclonal antibody against α-tubulin (Sigma) used at 1:1000 dilution and a Cy3-conjugated goat antimouse antibody used at 1:1000 dilution and a cooled AT200 CCD (charge coupled device) camera system (Photometrics Ltd., Munich, Germany). Fluorescent images were further processed using FluoroPro module for Image-ProPlus.

**Tubulin Polymerization Assay.** The assay was basically performed according to Bollag et al. (27). Microtubules from calf brain (Sigma) were depolymerized according to the manufacturer’s protocol. Tubulin heterodimers (10 μM) were incubated with different compounds (1 μM or as indicated) in PEMT buffer (100 mM PIPES, 1 mM EGTA, 1 mM MgCl2, and 0.05% Triton-X-100) containing 1 mM GTP in a total volume of 100 μl at 37°C for 1 h. Triton-X-100 strongly increased the solubility of D-24851 (data not shown). Samples (75 μl) were then transferred to a 96-Well Millipore Multi- screen Durapore hydrophilic 0.22-μm pore size filtration plate, which had been previously washed with 200 μl PIPES buffer (100 mM PIPES, 1 mM EGTA, and 1 mM MgCl2) under vacuum. Recovered microtubules on the filters were stained with 50 μl of amido black solution (0.1% naphthol blue black (Sigma), 45% methanol, and 10% acetic acid) for 2 min. Vacuum was applied, and unbound dye was removed by two additions of 200 μl of destaining solution (90% methanol and 2% acetic acid). The microtubule bound dye was then eluted by incubation with elution solution (25 mM NaOH, 0.05 mM EGTA, and 50% ethanol) for 10 min. The elution solution was then transferred to a 96-well plate and the absorbance measured at 600 nm.

**Spin Column Assay.** Tubulin heterodimers (3 μM) were obtained by depolymerization of microtubules purchased from Sigma as described above and were incubated either with 3 μM colchicine containing [3H]colchicine (4 × 10^5 dpm/nmol) in the presence of D-24851 or podophyllotoxin (A) or with 3 μM vincristine containing [3H]vincristine (4 × 10^5 dpm/nmol) in the presence of D-24851 or vinblastine (B) in PEMT buffer at 37°C for 1 h. Two aliquots (90 μl) of the incubation mixture (200 μl) were each loaded onto a 0.8-m1 Sephadex G25 column previously equilibrated in PEMT. The columns were then placed into 1.5-ml tubes and spun at 200 × g for 1 min, and radioactivity in the flow-through was analyzed by scintillation counting. Radioactivity that was found in the flow-through after centrifugation of the incubation mixture in the absence of tubulin was taken as background. Data points were connected and IC50 calculated using a nonlinear regression program (GraphPad Prism).

**XTT Assay.** The XTT assay (28) was used to determine proliferation by quantification of cellular metabolic activity. Tumor cell lines were cultivated in microtiter plates (1 × 10^3 cells per well in 100 μl) and were incubated with different concentrations of cytotoxic agents for 48 h. Subsequently, 50 μl of XTT solution (1 mg/ml XTT, 25 μM N-methylphenazonylazine methyl sulfate) was added per well, and mixtures were incubated for an additional 4 h. The amount of formazan salt was quantified in at least four replicates by absorbance at 490 nm using a Biomek Plate Reader (Beckman). IC50 and inhibition curves connecting the data points were obtained using the nonlinear regression program GraphPad Prism.

**SRB Assay.** Drug sensitivity was also assayed with the SRB-assy (29). Exponentially growing cells were seeded at a density of 600–1000 cells/well in 96-well microtiter plates (Falcon, Becton Dickinson Labware, Plymouth, United Kingdom) and allowed to attach overnight. After 24 h, cells were exposed for 24 h to either paclitaxel, vincristine, or D-24851, washed twice with PBS, and incubated in drug-free medium. At four cell-doubling times after the beginning of drug treatment, cells were fixed with trichloric acetic acid and were washed and stained with SRB as originally described. The absorbance was measured at 570 nm using a 96-well plate reader (340 EL, BIO Kinetics Reader, BIO-TEK Instruments Inc., Winooski, VT). The drug concentrations that inhibited cell growth by 50% (IC50) were determined from semilogarithmic dose-response plots.

**L1210 Mouse Leukemia Xenografts.** The MDR subline of mouse leukemia L1210 cells (L1210/VCR) was generated by long-term adaptation in a medium with stepwise increasing concentrations of vincristine. Expression of P-glycoprotein was analyzed by Western blotting using monoclonal antibody C219. L1210 and L1210/VCR cells were injected i.p. in male CD 2 F1 mice (20–25 g). The following day, mice were treated with maximally tolerated doses of D-24851 (150 mg/kg p.o.; d1–d4), vincristine (0.5 mg/kg i.p.; d1–d4), paclitaxel (15 mg/kg i.p.; d1–d4), or doxorubicin (1 mg/kg i.p.; d1–d4), and the ILS of the mice relative to the vehicle group was monitored. The survival time of the treated animals in percentage (ILS) of the vehicle-treated control group was calculated.

**Yoshida AH13 Rat Sarcoma Model.** Yoshida AH13 sarcoma cells were implanted i.p. in rats. After two passages, obtained ascites were grafted s.c. into female Sprague Dawley rats (250–290 g). Administration of the compounds or a vehicle control was started when mean tumor weights were approximately 0.5–1 g. D-24851 was given on days 1–5 and 8–12 (10 mg/kg p.o.; d1–5 × 2), paclitaxel was given on days 1–4 and 8–11 (2 mg/kg i.p.; d1–4 × 2), and vincristine was given on days 1, 4, 8, and 12 (0.6 mg/kg i.p.; d1, d4 × 2). Body and tumor weights as determined by comparison to the size of standard weights were monitored at day 1, 3, 7, 10, 14, and so forth. All of the studies were conducted in accordance with the local animal ethics regulatory requirements. In accordance with the animal ethics regulations, euthanization of tumor-bearing rats was required when the calculated tumor weight reached ±10% of the body weight.

**Coordination Test (Rota-Rod Test).** Rats (Wistar, 250–290 g) were treated with D-24851 (10 mg/kg p.o.; d1–5 × 2), paclitaxel (2 mg/kg i.p.; d1–4 × 2), vincristine (0.4 mg/kg i.p.; d1, d4 × 2), or a vehicle control. Rota-rod testing was performed at day 0, 5, and 10. The rod was set in motion at constant speed (2 rpm), and the rats were placed into individual sections of the apparatus. The rod was then accelerated from the rate of 2 rpm to 6 rpm. The animal’s performance was recorded as the time that had elapsed when the rats fell off the rotating rod. The data were analyzed using 1-way ANOVA, followed by Newman-Keuls test as appropriate. All of the results are expressed as mean ± SE with n = 6 animals. All of the studies were conducted in accordance with the local animal ethics regulatory requirements.

**NCV.** Rats were treated with the compounds as described above in the section on “Coordination Test.” At days 0, 5, and 10, the NCV was recorded in accordance with the method as described previously (30). The equipment used were the electrical stimulator ESU-2, amplifier of biological potentials, oscillograph, and photorecorder. The temperature in the room during the experiments was kept at the level of 28–30°C. NCV was measured retrospectively using the photos. The data were analyzed using 1-way ANOVA, followed by Newman-Keuls test as appropriate. All of the results are expressed as mean ± SE with n = 6 animals. All of the studies were conducted in accordance with the local animal ethics regulatory requirements.

**RESULTS**

**Effect of D-24851 on Cell Cycle.** D-24851 (chemical structure see Fig. 1A) was identified in a cytotoxic assay and first evaluated by cell
cycle analysis using flow cytometry. KB/HeLa cells were exposed to different concentrations of D-24851 for 24 h. At low concentration of D-24851 (8.5 nM), the main part of the cells were found at G1 phase of the cell cycle as observed with the solvent control, which indicated that the compound causes no detectable cell cycle effect at this concentration (Fig. 1A, upper diagram, peak at 300). At higher concentrations of D-24851 (850 nM), a complete shift from G1 to G2-M phase was observed (Fig. 1A, lower diagram, peak at 600). Thus, D-24851 induces an accumulation of KB/HeLa cells specifically in G2-M phase of the cell cycle. To compare D-24851 with the known G2-M cell cycle inhibitors vincristine and paclitaxel, we treated KB/HeLa cells with different concentrations of the compounds (Fig. 1A). The cells also completely shifted from G1 to G2-M phase (Fig. 1A, upper and lower diagrams, respectively). When the percentage of cells in G2-M phase were plotted against different concentrations of the compounds, D-24851, vincristine, and paclitaxel arrested the cell cycle in a concentration-dependent manner with IC50 values of 190, 3, and 15 nM, respectively (Fig. 1B). When the percentage of cells accumulated in G2-M phase were plotted against the concentration divided by their IC50 values of cell growth inhibition (Table 1), the resulting curves were nearly identical (Fig. 1B, inset). This indicates that the cell cycle arrest of all of the compounds correlates with the cell growth inhibition and that D-24851 may have a mode of action similar to that of paclitaxel and vincristine.

**Effect of D-24851 on Microtubule Organization in Mitotic Spindles.** To test whether D-24851 affects the microtubule organization in mitotic spindles, we treated human KB/HeLa cells or human SKOV3 ovarian carcinoma cells with D-24851 for 24 h and subsequently extracted the cells with Triton X-100 and paclitaxel. This treatment removes unassembled tubulin while preserving microtubules (24). Microtubules were then visualized by indirect immunofluorescence using an antibody against α-tubulin. Fig. 2, A and B, show solvent-treated (control) cells with microtubules constituting the cell shape. In contrast, microtubules were mainly found organized in mitotic spindles with abnormal structures after D-24851 treatment (45 or 80 nM) of the cells (Fig. 2, C and D). The abnormal spindles had extremely long astral microtubules and were seen monopolar or bipolar. When cells were exposed to higher concentrations of D-24851 (400 or 450 nM), we observed fragmented mitotic spindles (Fig. 2, E and F). This indicates that D-24851 destabilizes the microtubules of mitotic spindles in a concentration-dependent manner. Similar effects on tumor cells were observed by exposure to vincristine (1–15 nM; Ref. 31), a known microtubule-destabilizing agent (Fig. 2, G–J). Microtubules were also found fragmented over the entire cell (Fig. 2, I and J).

**Table 1** Cytotoxic activity of D-24851 against different tumor cell lines

All of the experiments were performed in at least four replicates using the XTT assay as described in “Materials and Methods.” IC50 values were calculated using a nonlinear regression program.

<table>
<thead>
<tr>
<th>Tumor cell line (tissue/species)</th>
<th>Growth inhibition constant (IC50) [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3 (ovary/human)</td>
<td>0.036 0.007 0.002</td>
</tr>
<tr>
<td>KB/HeLa (cervix/human)</td>
<td>0.115 0.007 0.001</td>
</tr>
<tr>
<td>HT 29 (colon/human)</td>
<td>0.072 0.010 0.005</td>
</tr>
<tr>
<td>A549 (lung/human)</td>
<td>0.164 0.013 0.027</td>
</tr>
<tr>
<td>PC-3 (prostate/human)</td>
<td>0.064 0.012 0.004</td>
</tr>
<tr>
<td>DU145 (prostate/human)</td>
<td>0.148 0.012 0.010</td>
</tr>
<tr>
<td>AsPC-1 (pancreas/human)</td>
<td>0.285 0.012 0.017</td>
</tr>
<tr>
<td>C6 (brain/rat)</td>
<td>0.200 0.047 0.007</td>
</tr>
<tr>
<td>U 87 (brain/human)</td>
<td>0.077 0.013 0.003</td>
</tr>
<tr>
<td>MDA-MB 231 (breast/human)</td>
<td>0.074 0.011 0.008</td>
</tr>
<tr>
<td>L1210 (leukemia/mouse)</td>
<td>0.089 0.140 0.021</td>
</tr>
</tbody>
</table>

**Effect of D-24851 on Polymerization of Purified Tubulin.** To investigate whether D-24851 induces destabilization of microtubules in a cell-free system, purified tubulin was allowed to polymerize in the absence or presence of different compounds. Microtubule polymers were separated from tubulin heterodimers by filtration as described previously (26). When paclitaxel (1 μM) was added to the assay, the amount of microtubule protein recovered was strongly increased as compared with the solvent (DMSO) alone (Fig. 3A, Lane 2 versus Lane 1). In contrast, no microtubule polymers could be recovered after the addition of vinblastine (1 μM) to the assay (Fig. 3A, Lane 3). This is consistent with the known destabilizing effect of vinblastine on microtubules (7, 8). D-24851 also completely blocked tubulin polymerization at a concentration of 1 μM (Fig. 3A, Lane 4). D-25552, a derivative of D-24851 that was not found to be active in our initial screening assay had no effect on tubulin polymerization (Fig. 3A, Lane 5). These data suggest that D-24851 binds directly to tubulin and thereby inhibits polymerization of tubulin. To compare the activity of vincristine and D-24851 on the inhibition of tubulin polymerization, different concentrations of the compounds were used in the assay. As shown in Fig. 3B, D-24851 blocked tubulin polymerization in a...
concentration-dependent manner with an IC\textsubscript{50} of 0.3 \textmu M, whereas the IC\textsubscript{50} value of vincristine was 10-fold lower.

**Competition of Binding of Vincristine or Colchicine to Tubulin by D-24851.** The microtubule destabilizing agents vincristine and colchicine are known to bind to tubulin at different sites. To address whether D-24851 binds to tubulin at one of those sites, binding of radiolabeled colchicine or vincristine to tubulin was tested in the presence or absence of unlabeled D-24851 using a spin column assay (32). Free radiolabeled compounds were retained in the column, whereas tubulin-bound compounds were found in the flow-through. When tubulin was incubated with \(^{3}\text{H}\)colchicine in the presence of different concentrations of unlabeled podophyllotoxin, a known competitor of colchicine binding to tubulin, and subsequently spun through the column, the amount of \(^{3}\text{H}\)colchicine found in the flow-through was strongly reduced (Fig. 4A). Thus, podophyllotoxin competes for the binding of colchicine to tubulin. In contrast, when different concentrations of D-24851 instead of podophyllotoxin were used in the assay, no effect on binding of \(^{3}\text{H}\)colchicine to tubulin was observed (Fig. 4A). Thus, D-24851 does not bind to the colchicine-binding site of tubulin. To test whether D-24851 might overlap with the vincristine binding site of tubulin, \(^{3}\text{H}\)vincristine was incubated with tubulin in the presence of the unlabeled vincristine derivative vinblastine or unlabeled D-24851. As shown in Fig. 4B, increasing concentrations of vinblastine reduced the binding of \(^{3}\text{H}\)vincristine to tubulin. In contrast, D-24851 was not able to compete for \(^{3}\text{H}\)vincristine binding to tubulin up to a concentration of 100 \textmu M. This indicates that D-24851 does also not bind to the vincristine-binding site of tubulin to induce disassembly of microtubules.

**Effect of D-24851 on Growth of Different Tumor Cell Lines.** To explore the effect of D-24851 on tumor cell growth we treated human and rodent tumor cell lines from prostate, brain, breast, pancreas, colon, lung, ovary, and cervix with different concentrations of D-24851, paclitaxel, or vincristine. Cytotoxicity was measured by cellular metabolic activity using the XTT assay. Growth of all cell lines was inhibited by all three compounds in a concentration-dependent manner. The growth inhibition constants (IC\textsubscript{50}) of the different tumor cell lines ranged from 0.002 to 0.027 \textmu M for vincristine, from 0.007 to 0.047 \textmu M for paclitaxel, and from 0.036 to 0.285 \textmu M for D-24851 (Table 1). This indicates that D-24851 inhibits growth of various human tumor cell lines at 10- to 20-fold higher concentrations than those of paclitaxel and vincristine.

**Cytotoxic Activity of D-24851 on MDR Tumor Cell Lines.** One major mechanism of multidrug resistance is mediated by the overexpression of the p-gp170 (8). The antitumoral efficacy of D-24851 was compared with vincristine, paclitaxel, and doxorubicin in the vincristine-selected MDR mouse leukemia cell line L1210/VCR using the cytotoxicity assay. Overexpression of p-gp170 in L1210/VCR was confirmed by Western blot analysis using monoclonal antibody C219 (Ref. 33; Fig. 5A). The resistant factors (RFs) as determined by the ratio of the growth inhibition constants (IC\textsubscript{50}) of the resistant cell line relative to those of its parental cell line were \(~1\) for D-24851 and \(>56\) for the other three anticancer agents (Fig. 5B; Table 2). Thus, L1210/VCR cells were resistant to vincristine, paclitaxel, and doxorubicin, whereas no cross-resistance to D-24851 was observed. In addition, three other p-gp170-overexpressing cell lines, including the mdrl cDNA-transfected acute myeloid leukemic rat cell line LT12, were tested. In all cases, no
A NOVEL MICROTUBULE INHIBITOR WITH POTENT ANTITUMORAL EFFICACY

Fig. 3. Effect of D-24851 on polymerization of purified tubulin. Purified bovine brain tubulin was incubated with GTP in the presence of DMSO alone as a control or different compounds dissolved in DMSO as indicated (A) or with different concentrations of D-24851 or vincristine (B). Polymerized microtubules were separated from heterodimeric tubulin by filtration and recovered on 0.22-µm pore size filters in a 96-well plate. Subsequently, microtubules were stained with naphthol blue black, and the amount of the dye was quantified. Data points are the means of duplicates and were connected using a nonlinear regression program. A single experiment of three independent experiments with similar results is shown.

cross-resistance of D-24851 to vincristine or paclitaxel was observed (resistance factors <2), whereas up to 1000-fold resistance to vincristine or paclitaxel was found (Table 2). Thus, in contrast to paclitaxel and vincristine, the cytotoxic efficacy of D-24851 against tumor cells is not altered by the MDR1 phenotype. In addition, the antitumoral efficacy of D-24851 was evaluated in resistance mediated by the MRCP and in human tumor cells with resistance to cisplatin, the topoisomerase-I-inhibitor SN-38 (7-ethyl-10-hydroxycamptothecin), and thymidylate synthase inhibitors (e.g., 5-FU and raltitrexed). Taken together, D-24851 retained unaltered cytotoxic efficacy toward all of the resistant sublines tested (Table 2). To test the cytotoxic efficacy of D-24851 toward MDR tumor cells in vivo, we used the L1210 leukemic mouse model. L1210 or L1210/VCR cells were implanted i.p. into mice. The mice were then treated with maximal tolerated doses (daily-times-four schedule) of D-24851, vincristine, paclitaxel, or doxorubicin and ILS of the mice relative to the vehicle control group was monitored. As shown in Fig. 5C, all of the three compounds were able to increase life span of mice grafted with parental L1210 cells (open bars). In contrast, only D-24851 was able to increase life span of the mice grafted with resistant L1210/VCR cells (closed bars). Moreover, the antitumoral efficacy of D-24851 was equal in the L1210 and L1210/VCR mouse model. Thus, the overexpression of P-glycoprotein in tumor cells does not influence the antitumoral in vivo efficacy of D-24851.

In Vivo Efficacy of D-24851 in Rat Yoshida AH13 Sarcoma Model. D-24851 affects the in vitro growth of different rodent and human tumor cell lines. To test whether D-24851 also affects growth of solid tumors in vivo, we used the Yoshida AH13 rat sarcoma model. Exponentially growing AH13 sarcoma tumor cells were s.c. grafted into rats. When tumors reached an initial weight of ~0.5–1 g, animals were treated with D-24851 (10 mg/kg p.o.; day 1–5 for 2 weeks) and maximal tolerated doses of paclitaxel (2 mg/kg i.p.; d1–4 × 2; Fig. 6B) and vincristine (0.6 mg/kg i.p.; d1, d4 × 2; Fig. 6B). D-24851 doses of the selected schedule exerted the maximal antitumoral efficacy and were ~1% of the acute toxicity LD50. Single D-24851 doses showed no antitumoral efficacy. The acute toxicity LD50 of p.o.D-24851 (single doses) to rats were higher than 850 mg/kg. As shown in Fig. 6A, D-24851 induced complete tumor remissions (cures) of animals. Curative doses of D-24851 were well tolerated with low or no systemic toxicity as indicated by an increase in body weight (Fig. 6B). Preliminary data also indicate that D-24851 exerts no hematological toxicities (data not shown). In contrast to D-24851, vincristine or paclitaxel administration resulted only in a modest inhibition of tumor growth when compared with the vehicle control group (Fig. 6A). The inhibition of tumor growth by vincristine or paclitaxel could be observed only when maximal tolerated (toxic) doses were administered coinciding with a loss of body weight >10% of the initial body weight (Fig. 6B). Thus, only D-24851 exerts potent antitumoral efficacy toward this solid tumor with low systemic toxicity.

Preliminary data on nude mice show that D-24851 also inhibits tumor growth in human xenografts, e.g., s.c. transplanted PL-3 prostate carcinoma.

Neurotoxicity. Administration of paclitaxel and vincristine is associated with a number of toxic side effects (16). One of the dose-
This suggests that D-24851 administration to rats revealed no deficit in motor function in contrast to paclitaxel or vincristine at antitumoral efficacious doses.

The determination of peripheral NCV might also serve as a reliable index of neurotoxicity (35). To this end, the effect of D-24851 in comparison with paclitaxel and vincristine on the NCV in rat tail was investigated at days 5 and 10 after the onset of treatment. When rats were dosed with D-24851 (10 mg/kg p.o.; d1–5 × 2) no change in NCV was observed as shown for the control group receiving only the vehicle (Fig. 7B). Treatment of rats with vincristine (0.6 mg/kg i.p.; d1, d4 × 2) or paclitaxel (2 mg/kg i.p.; d1–4 × 2) resulted in a strong decrease of NCV, which has been also reported previously (Fig. 7B; Ref. 18, 34). NCV of rats that have been treated with anticancer compounds strongly correlated with the performance of the rats on the rotating rod as well as with the body weight of the animals (Fig. 7C). These data demonstrate that D-24851 shows no neurotoxicity at antitumoral efficacious doses in vivo.

**DISCUSSION**

D-24851 is a novel synthetic anticancer agent with significant antitumoral activity in vitro and in vivo. It destabilizes microtubules in tumor cells as well as in a cell-free system. The binding site of D-24851 does not overlap with the tubulin-binding sites of the well-characterized microtubule destabilizing agents vincristine or colchicine. Furthermore, the molecule selectively blocks cell cycle progression at metaphase. In vitro, D-24851 exerts significant antitumoral activity against a variety of malignancies (e.g., prostate, brain, breast, pancreas, and colon). When compared with other microtubule-inhibiting compounds, D-24851 has a number of superior properties in vivo: (a) curative treatment of Yoshida AH13 rat sarcomas at almost nontoxic doses; (b) oral applicability; (c) lack of neurotoxicity at curative doses, which is a major drawback of taxanes and Vinca alkaloids in the clinical use; and (d) efficacy toward MDR tumor cells. Therefore, D-24851 may have significant potential as a therapeutic agent in cancer therapy.

The mode of action of the molecule as a tubulin inhibitor was shown by indirect immunofluorescence microscopy using an antibody against α-tubulin and in a cell-free tubulin polymerization assay.
D-24851 induced accumulation of cells with condensed nuclei (data not shown) and abnormal mitotic spindles. At higher concentrations, fragmentation of the spindle apparatus and degradation of microtubules were observed. The well-characterized Vinca alkaloids and colchicine interact with different binding sites on tubulin and were known to destabilize microtubules (7). In fact, exposure of cells to vincristine also revealed fragmented mitotic spindles similar to those shown for D-24851. Paclitaxel, known as a microtubule stabilizing agent, did not induce fragmentation of the spindle apparatus. This strongly suggests that D-24851 arrests cells at metaphase because of modulating microtubule stability.

The destabilizing effect of D-24851 on microtubules was also seen in a cell-free assay using purified tubulin. Polymerization of tubulin was blocked by D-24851 in a concentration-dependent manner with an IC50 of approximately 0.3 \( \mu M \), which may indicate a direct interaction of D-24851 with tubulin. The substoichiometric concentrations of the compound in relation to the tubulin concentration (10 \( \mu M \)) are sufficient to block tubulin polymerization, similar to vincristine or other Vinca alkaloids (36). D-24851 is a low-molecular-weight compound that shows no structural similarities to Vinca alkaloids or colchicine and did not compete for the binding of radiolabeled vincristine or colchicine to tubulin. This suggests that D-24851 may bind to a novel binding site on tubulin that results in inhibition of tubulin polymerization.

The IC50 value of vincristine for tubulin polymerization was \(~10\) fold lower than that of D-24851. The difference between both compounds in the inhibition of tubulin polymerization was also observed in inducing cell cycle arrest and in the inhibition of cell growth in a variety of different tumor cell lines. This suggests that D-24851 interferes with the function of tubulin, thereby inducing cell cycle arrest and consequently cell growth inhibition.

In vivo, D-24851 showed a remarkable antitumoral efficacy in the Yoshida AH13 rat sarcoma model. Oral application of D-24851 induced complete tumor regressions and resulted in curative treatment of the animals. Of great importance is that, at curative doses of D-24851, no systemic toxicity in terms of body weight loss or hematological toxicities were observed in vivo. In contrast, vincristine or paclitaxel treatment at their maximal tolerated doses resulted only in a moderate inhibition of tumor growth but in significant toxicity in terms of body weight loss. These data demonstrate that D-24851 is more potent than vincristine or paclitaxel in the treatment of Yoshida AH13 tumors in vivo.

In clinical studies it has been demonstrated that cumulative doses of paclitaxel or vincristine doses are associated with development of...
neurotoxicity (15, 16). The effect of these drugs on the nervous system of rats has also been shown previously (19, 35), i.e. administration of Vinca alkaloids significantly impaired coordination and NCV in the nerve tail. We also observed similar effects on rats after i.p. application of paclitaxel or vincristine. On a molecular level, drug-impaired microtubule function in axons seems to be responsible for the neurotoxic effects (8). Microtubules were found to accumulate in axons after the administration of paclitaxel, whereas Vinca alkaloids interfere with axonal transport, which induces spiralization of axonal microtubules. Although D-24851 also alters microtubule function, no neurotoxic effects on rats in terms of deficit in motor function or reduced NCV was seen at curative doses. One possible explanation for the lack of neurotoxicity of D-24851 could be that concentrations of D-24851 that are sufficient to block the cell cycle do not inhibit axonal vesicle transport. Alternatively, D-24851 may only interact with nonaxonal microtubules.

The use of cytotoxic agents is often accompanied by development of MDR tumor phenotype. A major determinant of MDR is the overexpression of drug efflux pumps, namely the p-gp170 and the MRP. The results reported herein suggest that D-24851 is a substrate neither of P-glycoprotein nor for MRP. Thus, D-24851 retains its cytotoxic activity toward MDR cells in vitro and in vivo. In contrast, paclitaxel and vincristine were shown to be actively transported by p-gp170 and, in part, by MRP. Of clinical importance is that D-24851 retains its antitumoral activity against cancer cell lines with resistance to cisplatin, the topoisomerase-I-inhibitor SN-38, and the thymidylate synthase inhibitors 5-FU and raltitrexed.

In summary, D-24851 is a novel tubulin-binding agent with significant antitumoral efficacy in vitro and in vivo. The lack of neurotoxicity and the potential in an oral formulation may provide an antimicrotubule drug with a significant therapeutic index. Clinical Phase I trials with D-24851 will be initiated.

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D-24851, a Novel Synthetic Microtubule Inhibitor, Exerts Curative Antitumoral Activity \textit{in Vivo}, Shows Efficacy toward Multidrug-resistant Tumor Cells, and Lacks Neurotoxicity

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