Abstract

Purpose: The design and development of synthetic small molecules to disrupt microtubule dynamics is an attractive therapeutic strategy for anticancer drug discovery research. Loss of clinical efficacy of many useful drugs due to drug resistance in tumor cells seems to be a major hurdle in this endeavor. Thus, a search for new chemical entities that bind tubulin, but neither are a substrate of efflux pump, P-glycoprotein 170/MDR1, nor cause undesired side effects, would potentially increase the therapeutic index in certain cancer treatments.

Experimental Design: A high-content cell-based screen of a compound library led to the identification of a new class of compounds belonging to a thiencyanpyrimidine series, which exhibited significant antitumor activities. On structure-activity relationship analysis, R-253 [[N-cyclopropyl-2- (6- (3,5-dimethylphenyl)thieno [3,2- d]pyrimidin-4-yl)]hydrazine carbothioamide] emerged as a potent antiproliferative agent (average EC$_{50}$ 20 nmol/L) when examined in a spectrum of tumor cell lines.

Results: R-253 is structurally unique and destabilizes microtubules both in vivo and in vitro. Standard fluorescence-activated cell sorting and Western analyses revealed that the effect of R-253 on cell growth was associated with cell cycle arrest in mitosis, increased select G$_2$-M checkpoint proteins, and apoptosis. On-target activity of R-253 on microtubules was further substantiated by immunofluorescence studies and selected counter assays. R-253 competed with fluorescent-labeled colchicine for binding to tubulin, indicating that its binding site on tubulin could be similar to that of colchicine. R-253 neither is a substrate of P-glycoprotein 170/MDR1 nor is cytotoxic to nondividing human hepatocytes.

Conclusion: Both biochemical and cellular mechanistic studies indicate that R-253 could become a promising new tubulin-binding drug candidate for treating various malignancies.

Microtubules are the principal components of the cytoskeleton network of eukaryotic cells and have been shown to be involved in various cellular functions, including cell division, organelle transport, cytokinesis, maintenance of cell morphology, and signal transduction (1, 2). Microtubules are intracellular, filamentous, polymeric structures composed of two structurally similar protein subunits, namely, α- and β-tubulin (molecular weight, 50 kDa each), arranged head-to-tail at 80-Å intervals to form a linear protofilament (3). During mitosis, microtubules undergo rapid polymerization and depolymerization to enable movement of the chromosomes. As cell division approaches metaphase, microtubules are disrupted and form a spindle surrounding the centrosome, thereby facilitating chromosomal alignment on the metaphase plate.

In this process, tubulin subunits freely exchange on the microtubules. If such free exchange of tubulin subunits is disrupted, the mitotic spindle is compromised and the cell cannot divide. Certain drugs have already been discovered which bind tubulin (e.g., Vinca alkaloids and the colchicine-site binders), thereby preventing them from being incorporated into growing microtubules, whereas others bind the microtubule itself, preventing additional tubulin subunits from binding (e.g., taxanes). As a consequence of either activity, cells undergoing division, and particularly those cells showing rapid division (i.e., cancer cells), are killed.

Among various anticancer drug targets known to date, microtubules are one of the most successful targets for anticancer therapies, and have become the target of choice, due to their multifunctional role in cell genesis (4). Most of the known drugs that target tubulin fall essentially into three major classes, such as taxanes, the Vinca alkaloids, and the colchicine-site binders. Drugs belonging to first two classes (e.g., paclitaxel and vincristine) are derived from natural products, whereas the drugs from the third category are composed of a collection of small molecules that are related by the fact that they all bind to a common site on tubulin, known as colchicine site, and prevent the normal polymerization of microtubules (5). Despite their structural diversity and mode of action, the consequence of disrupting tubulin and microtubule dynamics with these three classes of drugs leads to the same end result.
(i.e., metaphase arrest in dividing cells and induction of apoptosis; ref. 6). Although the commonly used anticancer drugs, such as taxanes and the Vinca alkaloids, are effective in the treatment of different malignancies, their potential is limited by the development of drug resistance (7) mediated by overexpression of transmembrane efflux pumps [i.e., P-glycoprotein 170, MDR1 (8), and MRP (9)]. Resistance is also mediated by the expression of tubulin isotypes and mutants that show impaired taxane-driven tubulin polymerization (10, 11). Another major problem associated with direct inhibitors of tubulin is their lack of specificity for dividing cells. Microtubules provide important structural and transport functions in neurons and other nonmitotic cells. Drugs that target the microtubule cytoskeleton without discriminating between dividing and nondividing cells may thus lead to undesired toxicities. Other significant drawbacks of tubulin inhibitors used for human cancer therapy include marginal oral bioavailability and poor solubility. To circumvent these problems, efforts are under way to identify new drug candidates with superior characteristics, minimal side effects, and improved pharmacologic profiles (4). It is noteworthy that there are many recent reports that highlight the discovery of various classes of synthetic antimitotic small molecules that in fact show potent antitumor activity with minimal side effects (12–18). These molecules are still either in preclinical evaluations or undergoing further chemical modifications to improve their potency, oral bioavailability, and efficacy.

We report here on thienopyrimidines as a new class of antitumor agents that exhibit the property of destabilizing microtubules in multiple tumor cell lines. Among various thienopyrimidine analogues synthesized and tested, R-253 seems to potently destabilize microtubules in both biochemical and cellular assays. As a consequence of this activity, we have shown that R-253 triggers mitotic cell cycle arrest leading to apoptosis in a wide array of tumor cell lines. Its ability to compete with colchicine in a competitive binding assay implies that R-253 has tubulin-binding site similar to that of colchicine. R-253 does not exhibit nonspecific mitotic-related kinase and/or microtubule-activated kinesin motor protein inhibitor activity, thus showing its on-target activity. Further characterization also revealed that R-253 is not a substrate of the efflux pump, MDR1, and is not cytotoxic to nondividing human hepatocytes as well. Based on various biochemical and cell biology data presented here, R-253 has the potential to become a novel anticancer drug.

### Materials and Methods

**Materials.** The following materials were obtained from the indicated sources: paclitaxel, vincristine, nocodazole, colchicines, camptothecin, and doxorubicin as well as ultra-grade biological buffer preparation reagents, such as Tris, NaCl, nonionic detergent NP40, EDTA, β-mercaptoethanol, glycerol, and DMSO (Sigma, St. Louis, MO); nuclear staining reagent 4,6-diamidino-2-phenylindole (DAPI); Molecular Probes, Eugene, OR); Complete EDTA-free protease inhibitor cocktail tablets and ATP (Roche Molecular Biochemicals, Indianapolis, IN). All cell culture media, including sterile PBS, are from Cellgro-Mediatech, Inc. (Herndon, VA). Antibodies used in this study were obtained from the following sources: –anti-cyclin B, anti-p53, and horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); FITC-labeled mouse anti-tubulin monoclonal antibody, anti-actin (Sigma), anti-cyclin E and anti-p21 (Upstate Biotechnology, Lake Placid, NY); anti-p27 (BD Pharmingen, San Diego, CA); anti-ubiquitin (Zymed Laboratories, San Carlos, CA); anti-nSECURIN (MBL International Corp., Woburn, MA); and phospho-p53Ser6 antibody (Anaspec, Inc., San Jose, CA).

**Chemical synthesis.** The most potent thienopyrimidine derivative, R-253, and its analogues with moderate biological activity (Table 1) used in this study were chemically synthesized and purified to homogeneity by conventional silica gel column chromatography using appropriate organic solvent systems. Detailed synthetic strategy used in the preparation of R-253 and its analogues will be published elsewhere (19). Purity and integrity of the final products as well as their synthetic intermediates were ascertained by liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopic methods. For the evaluation of biological activities, test compounds were dissolved in DMSO at final stock concentrations of 10 mmol/L and stored at –80°C.

**Cell culture.** The human tumor cell lines used in this study, such as HeLa (cervical carcinoma), A549 (lung carcinoma), U2OS (bone osteosarcoma), HCT116 (colon colorectal carcinoma), DLD-1 (colon colorectal adenocarcinoma), H1299 (lung carcinoma, non–small cell lung carcinoma), and SAOS2 (bone osteosarcoma), were from American Type Culture Collection (Manassas, VA). All cell lines were cultivated at 37°C in an incubator supplemented with 5% CO2 in respective culture medium as stated on the American Type Culture Collection 35-mm dishes.
Collection product specification sheet or as published. Tetracycline-regulatable paclitaxel-resistant HeLa cells overexpressing MDR1 used in this study were generated as described previously (20). Primary human umbilical vein endothelial cells and nondividing human hepatocyte cells were purchased from Cambrex Bioscience (Walkersville, MD) and cultured according to the vendor’s recommendations.

Image-based high-content proliferation assay. The proliferation-apoptosis-DNA content (PAD) assay is an image-based compound-screening assay developed at Riget that obtains three important variables of proliferating cells. The basic principle of the assay involves treatment of tumor or normal cells with small-molecule inhibitors for a long period of time and then fixing, staining, and imaging with an automated system and analyzing the result for the number of cells remaining, nuclear morphology, and cell cycle status. Typically, 2,000 cells per well were plated in 100 μL culture medium into sterile 96-well microtiter plates (Perkin-Elmer, Norwalk, CT) and incubated at 37°C and 5% CO2. After 24 hours, test compounds were serially diluted and added to each plate in 100 μL fresh medium and the plates were then incubated for an additional 48 hours. Normally, serial dilutions were done at six concentrations in duplicate. Cells were fixed using 2% paraformaldehyde in PBS for 1 hour and then stained with DAPI for 1 hour. Digital images were captured using a ×10 objective on a Zeiss Axiosvert S100 microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) equipped with a UV filter set and a Photometrics (Photometrics Inc., Tucson, AZ) camera. This system was controlled using the ImagePro ScopePro software package (Mediacy, Media Cybernetics Inc., Silver Spring, MD). The digital images were analyzed and results were summarized using a combination of ImagePro, Matlab (Mathworks, Mathworks Inc., Natick, MA), and MySQL (MySQL AB, Cupertino, CA) software packages. Number of positively stained cell nuclei remaining provided an EC50 after curve fitting and nuclear intensity indicated DNA content or cell cycle status. In addition, manual scoring of the nuclear morphology provided a percentage of nuclei exhibiting significant fragmentation, which is a characteristic indicator of late apoptosis.

Cytotoxicity assays. Unless otherwise stated, effects of test compounds on proliferation of various tumor cells were also determined using a standard bromodeoxyuridine incorporation cell proliferation assay. This assay was basically done on PAD-positive hits to confirm PAD antiproliferation EC50 values. Various tumor cell lines were cultivated in tissue culture-treated 96-well microtiter plates in 100 μL growth medium (∼10,000 cells per well) and incubated with different concentrations of thienopyrimidine test compounds along with 5% CO2. Alternatively, for measuring cytotoxicity of drugs in nondividing cells, a lactate dehydrogenase (LDH) assay was used. The LDH assay provides a fast and simple method for quantitating cytotoxicity based on the measurement of activity of LDH released from damaged cells in the presence of an inert dye. LDH oxidizes lactate to pyruvate, which then reacts with tetrazolium salt INT (2-[4-iiodophenyl]-3-[4-nitrophenoxy]-5-phenyltetrazolium chloride) to form formazan, which can be detected spectrophotometrically at 500 nm. For LDH cytotoxicity assay, nondividing human hepatocyte cells were cultured using hepatocyte basal medium in a collagen-coated 96-well microtiter plates according to the protocol provided by Cambrex-Clonetics. For calculating percent cytotoxicity in the LDH assay, DMSO and 1% Triton X-100 were used as low and high background controls, respectively. Both proliferation assay kit, bromodeoxyuridine and LDH were purchased from Roche Molecular Biochemicals. Assay measurements were carried out following the protocol provided in the kits. EC50 and inhibition curves connecting the data points were obtained using the nonlinear regression program GraphPad Prism (GraphPad, San Diego, CA).

Immunofluorescence microscopy studies. For immunostaining experiments, A549 cells were seeded in six-well plates containing sterile circular microscope coverslips (18 mm diameter; WVR Scientific, San Francisco, CA). Cells were treated with R-253 along with known microtubule-interacting agents, such as paclitaxel and nocodazole, at the indicated concentrations for 24 hours. Compound-treated cells were washed twice with PBS and fixed in 3.7% formaldehyde for 30 minutes. After fixing, cells were again PBS washed and blocked with “Super Block” solution (Pierce, Rockford, IL) containing 0.5% Triton X-100 for 2 hours at room temperature. Cells were stained for microtubules using FITC-conjugated mouse anti-tubulin antibody (Sigma) used at 1:300 dilution overnight in the same blocking solution at 4°C. Coverslips carrying microtubule-stained cells were carefully removed out of six-well plate and flipped onto glass slides preloaded with a drop of Vectashield mounting medium containing DAPI for nuclear staining (Vector Laboratories, Burlingame, CA). The slides were air-dried and examined under a fluorescent microscope, Axiosvert S100 (Carl Zeiss MicroImaging, Inc., Thornwood, NY), equipped with a 63 × 1.4 numerical aperture oil immersion objective lens, and the single fluorochrome filter sets for either DAPI or fluorescein were used for visualization and recording of the images. The images were captured with a CCD camera (model C4742-95-12ER, Hamamatsu Photonics KK, Hamamatsu, Japan).

Flow cytometric analysis. Flow cytometric analysis was carried out as described previously (21). Briefly, cells were treated with compounds at varied concentrations for 24 hours and trypsinized. After centrifugation at 1,000 × g for 5 minutes, the cells were washed in PBS and fixed in 70% ethanol overnight at −20°C. After carefully removing the fixing solution, cells were resuspended in 100 μL PBS and treated with 150 μL ice-cold RNase A (Sigma)/propidium iodide (Sigma) staining solution mix (25 μL propidium iodide at 100 μg/mL and 125 μL RNase A at 1 mg/mL in PBS). After incubating for 1 hour at room temperature in the dark, DNA content was analyzed using a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA). Cell cycle profiles were analyzed using the FlowJo program (Treestar Software, San Carlos, CA). The number of cells in G2-M phase was calculated using ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME). Similarly, induction of apoptosis by R-253 was determined by immunostaining for activation of apoptosis marker, caspase-3, using phycoerythrin-conjugated monoclonal active caspase-3 antibody following the protocol provided in the apoptosis detection kit purchased from BD Pharmingen.

Immunoblot analysis. For Western blot analysis of cell cycle–related proteins, cells treated with and without modulators were washed in PBS and lysed in radioimmunoprecipitation assay buffer composed of 10 mmol/L Tris (pH 7.2), 150 mmol/L NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mmol/L EDTA, and complete protease inhibitor cocktail as well as 2 mmol/L sodium fluoride and 1 mmol/L sodium orthovanadate (phosphatase inhibitors). Cell lysates were briefly sonicated in a Branson cell disruptor (Danbury, CT) thrice in a pulse mode for 20 seconds on ice to obtain both cytoplasmic and nuclear proteins. After centrifugation (14,000 rpm, 20 minutes, 4°C), the protein content of the supernatants was determined using a Micro-BCA protein estimation kit (Pierce) and samples were normalized for total protein. Cell lysates were then boiled in 4 × NuPAGE sample buffer containing lithium dodecyl sulfate/DTT and subjected to SDS-PAGE separation on premade 4% to 12% bis-Tris gradient gels using MOPS/SDS as running buffer system as per the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). Proteins electrophoretically onto polyvinylidene difluoride membranes were probed with appropriate antibodies and the blots were developed using an Enhanced Chemiluminescence Plus reagent kit obtained from Amersham Pharmacia Biotech (Piscataway, NJ) followed by detection on Kodak Bio-MAX MR film (VWR Scientific).

Microtubule polymerization assay. In vitro tubulin polymerization assays were done using the fluorescence-based tubulin polymerization kit purchased from Cytoskeleton, Inc. (Denver, CO). The polymerization
assay is a one-step assay, which uses a low-volume 96-well plate (Corning Costar, Corning, NY) for measuring tubulin polymerization. A 5 μl aliquot of a 10× concentration of the test compound in 80 mmol/L PIPES (pH 6.9) was pipetted into each well of the pre-warmed 96-well plates at 37°C. Following the addition of compound, 45 μl of a solution of tubulin (100 μg) in GPEM [80 mmol/L PIPES, 1 mmol/L EGTA, 1 mmol/L MgCl₂, 1 mmol/L GTP, 10% glycerol (pH 6.8)] containing the fluorophore (DAPI) was added. Then, the plate was immediately subjected to kinetic measurements on a Bio-Tek FL600 fluorescence microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The kinetics of tubulin polymerization reaction was monitored for every 5 minutes over a period of 60 minutes at 37°C with medium shaking of the plate for 5 seconds before taking reading at each time point of the kinetics. The change in the intensity of fluorescence due to polymerization or depolymerization of tubulin was determined by setting the excitation wavelength at 340 nm and emission wavelength at 460 nm having a gain value of 80. Appropriate controls, such as paclitaxel (2 μmol/L) for polymerization event and nocodazole (3 μmol/L) or vinblastine (3 μmol/L) for depolymerization event, were included in each set of the experiment. Reaction without test compound (DMSO only) and tubulin protein served as negative controls.

**Competitive binding assays.** Competitive tubulin ligand-binding assays were done on the colchicine- and vinblastine-binding sites as described by Jiang et al. (23). For monitoring the R-253 competition to colchicine-binding site, fluorescein-colchicine served as a reporter probe. The BODIPY-vinblastine was used as reporter probe for vinblastine-binding site. The GTP/GDP site was probed using the endogenous bound nucleotide. Both fluorescent conjugates were purchased from Molecular Probes, whereas highly purified bovine brain tubulin (99%) was procured from Cytoskeleton. Binding reactions were composed of 100 μg tubulin, 2 to 20 μmol/L fluorescent conjugate, 50 to 100 μmol/L R-253, and 0.1 mmol/L GTP plus 1% DMSO in 100 μL PEM (20 mmol/L PIPES, 0.25 mmol/L EGTA, 0.25 mmol/L MgCl₂). This competitive binding assay was based on the gel filtration method (24), which has been shown to provide greater sensitivity because greater amounts of tubulin could be used in each condition. Fluorochrome molarity was determined by absorbance at 455 and 504 nm for fluorescein-colchicine (molecular weight, 747) and BODIPY-vinblastine (molecular weight, 1,043), respectively. The stoichiometry of fluorochrome binding was determined by dividing the fluorochrome molarity by the tubulin protein molarity based on a molecular weight of 110,000. The fluorescent probes were determined to bind to the correct site by competition with the appropriate nonconjugated version of parent ligand.

**Results**

**Compound screening and hit identification.** Our medium-throughput image-based high-content antiproliferation assay served as a primary compound-screening tool because it is a one-step assay that gives cell cycle profile as well as nuclear morphology for predicting the apoptotic status of the cells in addition to the measurement of antiproliferation EC_{50}. Figure 1 shows a typical PAD assay data output obtained for some of the standard control compounds, such as paclitaxel and nocodazole, as well as example compound hits. The EC_{50} values purchased from Molecular Probes, whereas highly purified bovine brain tubulin (99%) was procured from Cytoskeleton. Binding reactions were composed of 100 μg tubulin, 2 to 20 μmol/L fluorescent conjugate, 50 to 100 μmol/L R-253, and 0.1 mmol/L GTP plus 1% DMSO in 100 μL PEM (20 mmol/L PIPES, 0.25 mmol/L EGTA, 0.25 mmol/L MgCl₂). This competitive binding assay was based on the gel filtration method (24), which has been shown to provide greater sensitivity because greater amounts of tubulin could be used in each condition. Fluorochrome molarity was determined by absorbance at 455 and 504 nm for fluorescein-colchicine (molecular weight, 747) and BODIPY-vinblastine (molecular weight, 1,043), respectively. The stoichiometry of fluorochrome binding was determined by dividing the fluorochrome molarity by the tubulin protein molarity based on a molecular weight of 110,000. The fluorescent probes were determined to bind to the correct site by competition with the appropriate nonconjugated version of parent ligand.

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obtained from this method were found to be in excellent agreement with reported values as well as correlating well with the standard bromodeoxyuridine assay (data not shown). Based on the reliability and consistency of this assay, we screened our compound library using A549 (p53-positive) and H1299 (p53-negative) tumor cell lines. Thienopyrimidines were initially identified as potent, antiproliferative agents from this screening. Figure 2 shows the structures and average antitumor potency of representative thienopyrimidines. Among them, R-719 with an average EC\textsubscript{50} of 600 nmol/L was found to be the primary hit. When >100 thienopyrimidine analogues were synthesized and screened based on structure-activity relationship criteria, R-253 emerged as a lead structure. R-253 was found to be a potent molecule with an average antiproliferation EC\textsubscript{50} of 20 nmol/L against several tumor cell lines tested. R-253 is a thienopyrimidine with important substituents on either side of the core heterocyclic ring structure; the left-hand side bears a 3,5-dimethylphenyl ring, whereas the right-hand side has a cyclopropyl substituted hydrazine carbothioamide side chain. Structural variations of both left-hand and right-hand substituents were made. The optimal substituents on the left-hand side of the thienopyrimidine were found to be a 3-methylphenyl or a 3,5-dimethylphenyl ring, whereas small lipophilic groups, such as cyclopropyl or isopropyl, were favored for the right-hand hydrazine carbothioamide. Substitution of the hydrazine carbothioamide group with a phenyl (R-719) or tertiary amino group (morpholinoethyl, R-187; see Table 1) led to much less antiproliferative activity (12–18).

**R-253 inhibits microtubule polymerization in vitro.** An in vitro biochemical microtubule polymerization assay was carried out to investigate the activity of R-253 on microtubule function. Purified tubulin was allowed to undergo polymerization in the presence and absence of R-253 and its analogues in a cell-free environment. For measuring tubulin polymerization, a fluorescence-based assay system developed originally by Bonne et al. was used (22). When paclitaxel (2 μmol/L), a well-known microtubule stabilizer, was added to the assay, an increase in fluorescence intensity at 460 nm against solvent alone (DMSO) control was observed (Fig. 4A), indicating that paclitaxel is stabilizing the tubulin polymerization reaction. In contrast, when nocodazole (3 μmol/L), a well-known microtubule destabilizer, and R-253 (10 μmol/L) were added to the assay, a decrease in the fluorescence intensity against the solvent control (DMSO) was observed, which indicates that both nocodazole and R-253 are inhibiting the tubulin polymerization reaction in a similar mechanistic way. On the other hand, R-187, which is an inactive thienopyrimidine, showed no effect on fluorescence intensity similar to that of solvent alone control (DMSO). These data suggest that R-253 binds directly to tubulin and thereby prevents polymerization of tubulin. To obtain the IC\textsubscript{50} of inhibition of tubulin polymerization, R-253 was titrated in this assay (Fig. 4B) and R-253 blocks tubulin polymerization in a concentration-dependent manner with an IC\textsubscript{50} of ~1 to 2 μmol/L.

**R-253 competes with the colchicine-binding site.** To delineate the binding site of R-253 on tubulin, two well-known fluorescent-conjugated microtubule destabilizers, such as fluorescein-colchicine and BODIPY-vinblastine, were used to bind to their respective sites on tubulin. There are number of methods available to determine tubulin-binding sites, which low as 100 nmol/L, and in contrast, the cell cycle status of control cells (DMSO) and the inactive compound (R-187) remained unchanged. Increase in 4N DNA content (G\textsubscript{2}-M) followed by decrease in 2N DNA content and a slight increase in sub-G\textsubscript{0}-G\textsubscript{1} cells mediated by R-253 is an indicative of occurrence of programmed cell death or apoptosis. Consistent with an induction of apoptosis, we also observed activation of caspase-3, which is an apoptotic marker, in R-253-treated A549 cells by flow cytometry using caspase-3 immunostaining kit (data not shown). Such activation of caspase-3 was not observed in untreated (DMSO) and R-187-treated (inactive) A549 cells. It has been very well documented that microtubule inhibitors, including colchicine-like antimitotic compounds, are known to arrest cells in G\textsubscript{2}-M phase and induce apoptosis (1, 2). Based on this observation, we speculated that microtubules and their function could be a potential target of R-253.

**Effect on cell cycle and induction of apoptosis.** To determine the effect of thienopyrimidines on the cell cycle, A549 cells were treated with R-253 (potent) and R-187 (inactive) compounds for 24 hours and their cell cycle progression was followed by fluorescence-activated cell sorting analysis. Cell cycle profiles obtained for selected test compounds, including standard anticancer agents (nocodazole, paclitaxel, and vinblastine) and no compound control (DMSO), are shown in Fig. 3. DNA content was indicated as 2N for G\textsubscript{0}-G\textsubscript{1} cells and 4N for G\textsubscript{2}-M cells. Cells with DNA content between 2N and 4N are in the S phase. The sub-G\textsubscript{0}-G\textsubscript{1} population represents apoptotic cells with <2N DNA content. The results show that R-253 completely blocked the cells in G\textsubscript{2}-M phase at concentration as low as 100 nmol/L, and in contrast, the cell cycle status of control cells (DMSO) and the inactive compound (R-187) remained unchanged. Increase in 4N DNA content (G\textsubscript{2}-M) followed by decrease in 2N DNA content and a slight increase in sub-G\textsubscript{0}-G\textsubscript{1} cells mediated by R-253 is an indicative of occurrence of programmed cell death or apoptosis. Consistent with an induction of apoptosis, we also observed activation of caspase-3, which is an apoptotic marker, in R-253-treated A549 cells by flow cytometry using caspase-3 immunostaining kit (data not shown). Such activation of caspase-3 was not observed in untreated (DMSO) and R-187-treated (inactive) A549 cells. It has been very well documented that microtubule inhibitors, including colchicine-like antimitotic compounds, are known to arrest cells in G\textsubscript{2}-M phase and induce apoptosis (1, 2). Based on this observation, we speculated that microtubules and their function could be a potential target of R-253.

**In the light of the observation that microtubule plays crucial role in maintaining cell morphology and shape, disrupting such function as a result of R-253 intervention was undertaken.** Various reports have shown that cells undergo dramatic changes in their morphology when their microtubule dynamics are compromised (25). As a consequence of this activity, cells will start rounding up and eventually undergo apoptosis. Similar morphologic changes were observed when A549 cells were treated with R-253 for 24 hours. Such changes in morphology were also noticed in paclitaxel and nocodazole. These results clearly suggest that R-253 exhibits activities similar to most of the known microtubule inhibitors reported (12–18).
includes both radioactive and nonradioactive methods. In this study, we used a nonradioactive format due to the convenience and simplicity of the method. Among the two commonly used nonradioactive assay formats, such as DEAE filter binding method (26) and the size exclusion chromatography method (24), the later method provided a greater degree of sensitivity. Binding of Fluorescein-colchicine at 5 μmol/L was competed out close to 72% by 100 μmol/L colchicine and ~45% and 50% when R-253 was used at 50 and 100 μmol/L, respectively (Fig. 5A). On the other hand, binding of BODIPY-vinblastine at 5 μmol/L was inhibited 73% by 100 μmol/L vinblastine (Fig. 5B). BODIPY-vinblastine binding to tubulin was not affected by R-253 when used at 50 and 100 μmol/L (Fig. 5B). No significant difference in the amount of GTP/GDP was determined with and without the presence of 50 μmol/L R-253 on size exclusion chromatography (data not shown), implying that R-253 does not interfere with GTP/GDP binding. Similar observations have been made with other tubulin-interacting agents, such as 3-(iodoacetamido)-benzoylurea, a novel cancericidal tubulin ligand that inhibits tubulin polymerization (23). Results from this competition binding assay provided evidence that R-253 seems to bind at the colchicine-binding site on tubulin.

**Cellular mechanism(s) and on-target activity confirmation.** It has been well documented that DNA damage activates cell cycle checkpoints that prevent the progression to M phase while DNA repair is under way (27). Although there are several other mechanisms known to arrest cells at various phases of the cycle depending on the nature of the modulator involvement, DNA-damaging activity is frequently found in variety of synthetic small molecules. Thus, to explore the mechanism by which R-253 induces G2-M cell cycle arrest as opposed to potential DNA-damaging activity, we treated A549 cells with R-253 and a well-known DNA-damaging agent, doxorubicin. DNA damage induced by doxorubicin was documented by probing cell lysates using phospho-p53Ser6 (28). In contrast, phosphorylation of p53 was not observed in cells treated with R-253 as well as R-187 (data not shown). This observation provided evidence for two important activities of R-253, one is that it is not a DNA-damaging agent and the other is that it induces cell cycle arrest at G2-M phase by acting on targets that are involved in mitosis. To explore this hypothesis, R-253-treated A549 cells were probed for accumulation of G2-M- and G1-S-phase proteins (29). Figure 6A shows a R-253 concentration-dependent accumulation of cyclin B and securin, which are thought to play key regulatory roles during mitosis (30), thus supporting the possibility that R-253 is indeed blocking the cell cycle at G2-M phase. Furthermore, R-253 failed to alter expression of G1-S proteins, such as cyclin E and p27, as a consequence of cell cycle arrest at G2-M phase (Fig. 6B). It is noteworthy that proteasome inhibitors have been shown to block the degradation of most of the cell cycle-related proteins, including cyclin B, securin, and p53 (31). Based on this observation, one would argue that accumulation of cyclin B

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**Fig. 3.** Cell cycle progression of A549 cells after treatment with known tubulin inhibitors, such as nocodazole, paclitaxel, and vinblastine, as well as test thienopyrimidine compounds, such as R-253 (potent) and R-187 (inactive), for 24 hours at their indicated concentration. After treatment, cells were trypsinized, fixed, and stained with propidium iodide. The cell cycle distribution was measured by flow cytometry. R-253 clearly blocks cell cycle at G2-M phase similar to that of known microtubule inhibitors.
and securin could also occur as a consequence of proteasome inhibition rather than mitotic block. However, the inability of R-253 to block globally ubiquitinated proteins rules out the possibility of an nonspecific antiproteasomal activity as observed in a well-known proteasome inhibitor, MG132 (data not shown). These results strongly suggested that R-253 is neither a nonspecific DNA-damaging agent nor a proteasome inhibitor but seems to target mitosis. A close examination of cellular mechanism for mitotic arrest based on several reports in the literature as well as classic functional phenotype induced by R-253 begin to point to tubulin as its putative molecular target among other targets within the cell division cycle. Further studies subsequently showed that polymerization of bovine brain tubulin both in vivo and in vitro was greatly inhibited by R-253, which once again strongly supported that tubulin is in fact the most likely target of R-253.

R-253 disrupts microtubule structures in vivo. The ability of R-253 to bind to tubulin and disrupt microtubule polymerization under physiologic conditions was examined by immuno-fluorescence studies using HeLa cells treated with 1 and 0.1 μmol/L R-253 for 24 hours and stained for microtubules. Figure 7 shows immunostained images for HeLa cells treated with various compounds. Figure 7 (top) presents the view for HeLa cells treated with DMSO vehicle control followed by two control drugs, such as paclitaxel (30 nmol/L) and nocodazole (333 nmol/L), whereas Fig. 7 (bottom) depicts the view for HeLa cells treated with the inactive thienopyrimidine R-187 (1 μmol/L) and the active thienopyrimidine R-253 at two different concentrations (1 and 0.1 μmol/L). It is quite clear from the images that cells treated with vehicle control (DMSO) and the inactive compound (R-187) did not affect microtubule formation. B, dose-dependent effect of R-253 on in vitro microtubule formation. The level of the polymerization was measured by an increase in fluorescence emission intensity at λem = 460 nm and the SDs for each time point were within the 0.001 to 0.004 range. IC50 of nocodazole were found to be in 2 to 5 μmol/L range in this assay.

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Thienopyrimidines: New Potent Antimitotic Agents

R-253 kills drug-resistant tumor cells but not normal cells. To test the global antitumor activity of thienopyrimidines, we treated various human cancer cell lines, including HeLa cells, expressing high levels of drug-resistant protein MDR1 (20) and measured cell survival in the PAD assay (Table 2). It has been well documented that one major mechanism of multidrug resistance is mediated by the overexpression of the efflux pumps, P-glycoprotein170 and MDR1 (7–9). In this study, the panel of tumor cell lines was selected in such a way that the cell specificity covered both p53-positive and p53-negative or mutated protein. It is noteworthy that in most tumor cells p53 status is not uniform, thus justifying our rationale for panel selection. Tetracycline-regulatable HeLa cells overexpressing MDR1 were generated by selection in the presence of paclitaxel as described previously (20). Among various thienopyrimidines, R-253 showed greater potency (average EC50, 20 nmol/L) in all the cell lines tested (Table 2). Interestingly, paclitaxel was found to be more potent than R-253 against a spectrum of cell lines tested but exhibited significant loss in its potency when tested against the drug-resistant cell line, HeLa-MDR1 (Table 2). In contrast, R-253 was much more potent against HeLa-MDR
cell that expresses high levels of efflux pump, MDR1 (Table 2). These data suggest that R-253 is efficacious against a variety of human cancer cell lines, including cancer cells expressing high levels of drug resistance protein MDR1. To rule out the possibility of undesired side effects stemming from the lack of specificity against dividing and nondividing cells, which has been a bottleneck in anticancer drug development, R-253 was subjected to cytotoxic evaluation using nondividing human hepatocyte cells. Dose-dependent cytotoxic effect of R-253 (up to 10 μmol/L concentration) was measured using a LDH cytotoxicity assay kit. On treating nondividing human hepatocyte cells with R-253 and its inactive counterpart, R-187, no adverse cytotoxic effects were seen (<20%) in contrast to known cytotoxic drug, camptothecin, indicating that R-253 may not be lethal to normal cells (data not shown). Notably, camptothecin showed significant cytotoxicity (40%) at higher dose (10 μmol/L), but it was less pronounced when used at the pharmacologic doses (<1 μmol/L). Our preliminary studies also revealed a 10- to 20-fold differential antiproliferation effect mediated by R-253 when tested in normal primary human umbilical vein endothelial cells compared with that of tumor cells (data not shown).

**Discussion**

The property of uncontrolled cell growth and division makes cancer cells extremely dependent on microtubule dynamics and vulnerable to antimitotic drugs that target structural and functional integrity of microtubules. The different susceptibilities of tumor and nontumor cells to microtubule-active agents could be possibly due to (a) inactivation (phosphorylation) of Raf-1/Bcl-2 after disruption of microtubules (6), (b) abrogated function of G1 microtubule-dependent checkpoint in transformed cells (32), (c) deficiency of p53 and pRb tumor suppressor genes (33), and (d) structural differences of microtubule or microtubule-associated protein in normal and tumor cells (34). These differential activities underscore the fact that microtubules are indeed functionally important targets for the development of anticancer drugs (1–4). The mechanism of action of many antimitotic drugs is to interfere with the normal formation of the mitotic spindle by either increasing microtubule stability or depolymerization leading to cell cycle arrest at the mitotic checkpoint (35). As a result of this catastrophic event occurring at mitosis, cells undergo apoptosis (36). The major challenge in the design and development of drugs that target microtubules is to find compounds having superior characteristics, such as (a) lack of neurotoxicity at curative doses, which is a major drawback of taxanes and Vinca alkaloids in clinical use, (b) efficacy toward a broad range of tumor cells, including drug-resistant strains, and (c) should be orally bioavailable and possess good pharmacologic profiles. Research in this direction has successfully identified several lead drug candidates, which are currently at various stages of preclinical evaluations (12–18).

The efforts pertaining to antimitotic drug discovery to combat variety of cancers led to the identification of R-253, a thienopyrimidine derivative as a potent antiproliferative agent (Table 1). This lead compound was discovered via screening a compound library subset using our proprietary high-content cell-based PAD imaging assay (Fig. 1). Compounds belonging to this scaffold were found to be antiproliferative against a spectrum of human tumor cells (Table 2). Based on this finding, when >100 structure-activity relationship–based structural analogues belonging to this class were synthesized and tested, R-253 turned out to be the lead candidate. For further biological and pharmacologic evaluations, R-253 and its least active counterpart R-187 were chosen. Preliminary cellular studies revealed that the inhibitory effect of R-253 on tumor growth was associated with cell cycle arrest in mitosis (G2-M). Depending on the cell type, the mitotic block induced by many antimitotic compounds may persist for varying lengths of time;
however, most cells will exit the cell cycle and undergo apoptosis (35). Consistent with other antimitotic compounds, we observed a concomitant decrease in G1-S cells and an increase in G2-M cells with a few cells in the sub-G0-G1 phase, suggesting that cells arrested in mitosis with R-253 eventually became apoptotic. The process by which microtubule inhibitors induce apoptosis is poorly understood. It has been shown previously that apoptosis in cancer cells after paclitaxel treatment could occur directly from amitotic block (37). Other reports show that almost all microtubule-active drugs induce apoptosis may involve Bcl-2 and Raf-1 kinase phosphorylation, whereas DNA-damaging agents as well as alkylating agents do not (6). Chadbech et al. have shown that phosphorylation and degradation of Bcl-2 occurred in paclitaxel-treated cells arrested in mitosis (38). Basu and Haldar showed that the microtubule-interacting agents, such as colchicine, colcemid, and podophyllotoxin, caused Bcl-2 phosphorylation and apoptosis in cancer cells (39). Another mechanism one might think of involves both c-Jun NH2-terminal kinase-dependent pathways, because Wang et al. have shown that paclitaxel activated the c-Jun NH2-terminal kinase/stress-activated protein kinase signal transduction pathway in a variety of human cell lines (40).

Efforts to decipher the most likely target of R-253 to explain its mechanism of action led us to choose microtubules as tentative candidates. The rationale for this selection comes from the fact that most of these compounds exhibited a clear change in cell morphology that is similar to known antimitotic reference compounds, nocodazole and paclitaxel (25), and induced cell cycle at G2-M phase as well. This observation supported our hypothesis and prompted further evaluation of these compound activities in mitotic-related assays, including microtubule polymerization in vivo and in vitro.

It has been well documented that DNA damage activates a cell cycle checkpoint that prevents the progression to M phase while DNA repair is under way (27). To rule out the possibility of cell cycle arrest as a result of DNA-damaging activity, we compared such activity of R-253 against that of a well-known DNA-damaging agent, doxorubicin. The extent of DNA damage mediated by the R-253 was followed by a p53 phosphoblot. p53 is phosphorylated on its Ser6, Ser33, Ser46, and Ser392 when DNA damage occurs by cytotoxic agents or other external mediators (28). On exposing cells to doxorubicin, a well-known DNA-damaging agent, and R-253, p53 is phosphorylated only in doxorubicin exposure but not by R-253. These results suggest that R-253 does not induce apoptosis via DNA damage and indirectly provides evidence that it in fact causes cells to arrest at G2-M phase by selectively acting on a mitosis-related pathway. Slight induction of p53 phosphorylation noticed in paclitaxel and nocodazole could be explained based on the toxicity associated with usage of high doses particularly for this experiment, which is consistent with previous reports (28). R-253-mediated mitotic arrest was further supported by monitoring the stabilization of G2-M and G1-S phase protein markers. The rationale for conducting

Fig. 6. Western blot analysis of cell cycle-dependent protein expression levels following compound treatment in A549 cells. A, effect of known microtubule inhibitors and thienopyrimidines on expression of G2-M checkpoint proteins. R-253 showed dose-dependent accumulation of mitotic markers, cyclin B and securin, in contrast to its inactive counterpart, R-187, indicating the triggering of G2-M cell cycle arrest in A549 cells. B, effect of known microtubule inhibitors and thienopyrimidines on expression of G1-S checkpoint proteins. R-253, including known microtubule inhibitors, failed to alter the expression of both cyclin E and p27, further supporting that R-253 specifically mediates mitotic arrest. β-Actin served as loading control in all the blots.
this experiment is that during the cell cycle, which is an operating biochemical mechanism constructed from a set of regulatory enzymes (cyclins and cyclin-dependent kinases), activities of the enzymes alternate cyclically, concordant with the cell cycle downstream processes (42). Cell cycle arrest caused by drugs should therefore be reflected in the stabilization of their corresponding kinase substrates whose degradation is a prerequisite to complete the cycle during cell division (29). Accumulation of G2-M regulatory proteins, such as cyclin B and securin, further substantiated our observation that R-253 is indeed causing cell cycle arrest at mitosis. In contrast, no accumulation of G1-S proteins, such as cyclin E and p27, was found which once again supports our hypothesis that R-253 causes cell cycle arrest at the G2-M phase. To rule out the possibility of having a proteasome inhibitor, such as activity for R-253, which could also potentially up-regulate cell cycle-related proteins, Western analysis of R-253-treated cell lysates for accumulation of globally ubiquitinated proteins revealed that R-253 is completely devoid of such activity.

The mechanism of action of R-253 as a tubulin inhibitor was proven by immunofluorescence microscopic studies using an antibody against β-tubulin and in a cell-free tubulin polymerization assay. R-253 induced fragmentation of mitotic spindles and degradation of microtubule network at concentrations as low as 100 nmol/L (Fig. 7). Exposure of cells to nocodazole, a well-known microtubule-destabilizing agent, also revealed fragmented mitotic spindles similar to those shown for R-253. In contrast, paclitaxel, known as a microtubule-stabilizing agent, did not induce fragmentation of the spindle apparatus. This strongly suggests that R-253 arrests cells at metaphase because of modulating microtubule stability. The destabilizing effect of R-253 on microtubules was also seen in a cell-free environment using purified tubulin. Polymerization of tubulin was blocked by R-253 in a concentration-dependent manner with an IC50 of ~1 to 2 µmol/L, which may indicate a direct interaction of R-253 with tubulin. Such activity has been recently reported in several small-molecule drug candidates (12–18). The substoichiometric concentrations of the compound in relation to tubulin concentration (10 µmol/L) are sufficient to block tubulin polymerization, similar to vinblastine or other Vinca alkaloids. Exploration of tubulin-binding site of R-253 strongly favored a colchicine site versus the vinblastine and/or the GTP site. In this context, it is noteworthy that nocodazole, another known microtubule destabilizer, also binds at this site, but unlike colchicine it binds at two different

Fig. 7. Immunostaining of HeLa cells exposed to various compounds at the indicated dose for 24 hours using FITC-labeled mouse anti-tubulin monoclonal antibody. Cells exposed to either no compound or an inactive compound (R-187) retained their fibrous structures. However, R-253 (1 µmol/L/100 nmol/L) and nocodazole (333 nmol/L) completely disrupted microtubule structures, whereas paclitaxel (30 nmol/L) completely stabilized microtubules around the nucleus.
sites on a tubulin subunit (43). One site is thought to be a higher affinity site on β-tubulin and the other is a lower affinity site on α-tubulin (44). It is not clear whether R-253 also has similar affinity for two binding sites on tubulin subunits. Presumably because of the concentration of fluorescein-colchicine used in the experiment is less than the K_m for the lower affinity site, R-253 might interact at the high-affinity site. Reduction in the ability of R-253 to compete out fluorescein-colchicine at 50 μmol/L compared with unconjugated colchicine indicates the possibility of its lower affinity than the parent ligand.

Because R-253 causes mitotic arrest via microtubule dynamics blockade, the target specific activity of R-253 compounds was further examined and supported by several counter assays on targets, which are also primarily involved during mitosis. Such key targets include kinases (e.g., Aurora kinase; ref. 45) and mitotic kinesins (e.g., Eg5; ref. 46). Mitotic kinesins are a group of motor kinesins that play essential roles in assembly and function of the mitotic spindle and are required for cell division. During cell division, motor kinesins move cellular cargos on the microtubule cytoskeleton used as tracks. When R-253 was tested in microtubule-activated kinesin, ATPase end point assay using a panel of known motor proteins, such as Eg5, CENP-E, MKLP1, and KHC, did not show any marked inhibitory effect. In contrast, monastrol, a known Eg5 inhibitor, which served as a positive control in this assay, inhibited Eg5 activity by 83% at 100 μmol/L (data not shown). Among various kinases, the family of Aurora kinases has been implicated in mitotic spindle formation and centrosome maturation, ensuring faithful segregation of chromosomes into daughter cells (45). Inhibition of Aurora kinase activity leads to the generation of polyploid cells as a result of repeated rounds of DNA synthesis in the absence of cytokinesis as illustrated in known Aurora kinase inhibitor, VX-680 (47). In this context, failure to induce accumulation of cells with >4N DNA content (Fig. 3), which is a characteristic phenotype of Aurora kinase inhibition, implies that R-253 is not exhibiting a nonspecific Aurora kinase inhibition activity. In addition, R-253 was also found to be inactive in a panel of kinase biochemical assays developed in-house (data not shown). These observations clearly highlight the on-target activity of R-253 to tubulins. It is noteworthy that a recent report identified thienopyrimidines with varied substitutions on the core heterocyclic ring as potent inhibitors of vascular endothelial growth factor receptor-2 kinase (48). In addition, recent work from the same group of investigators suggested that thienopyrimidines could be used as potential chemotherapeutic agents to treat hyperproliferative disorders (49). This report once again describes the versatility as well as broad applicability of thienopyrimidines as druggable scaffold and opens up new avenues to drive the biological activity by altering specific substituents toward desired sites of action.

The use of cytotoxic agents is often accompanied by the development of MDR tumor phenotype. A major determinant of MDR is the overexpression of drug efflux pumps [i.e., P-glycoprotein 170 and MRP (MDR1)]. Our results show that sensitivity of R-253 to MDR1-expressing HeLa cells is >20-fold against paclitaxel (Table 2). These results indicate that R-253 is not a substrate of such drug efflux pump MDR1, thereby suggesting that it is superior to other antimitotic agents in this regard. In addition, the cytotoxic effects mediated by R-253 on nondividing human hepatocytes were found to be negligible as determined by a LDH assay, suggesting that R-253 may not pose any undesired toxicity to normal cells. In addition, we noticed a 10- to 20-fold differential antiproliferation effect mediated by R-253 in primary human umbilical vein endothelial cells versus tumor cells. However, further in vivo studies are required to confirm similar activity against other normal cells, including neuronal axons to obtain conclusive evidence to prove that R-253 is devoid of neurotoxicity and other undesired side effects. Although key experiments, such as pharmacokinetic profiling and animal studies, are pending, it seems that R-253 offers some unique properties and selectivity profiles over existing antimitotic agents.

In conclusion, we present a new class of antimitotic compounds that selectively binds to tubulin. The mode of binding of R-253, a potent lead compound, seems to be similar to that of colchicine, as it showed greater competition toward colchicine-binding site on tubulin. As a consequence, it inhibited microtubule assembly leading to blockage of cell division in tumor cell lines, including HeLa expressing efflux pump, MDRI, as determined by PAD assay.

### Table 2. Antitumor activity of select thienopyrimidines against a panel of tumor cell lines, including HeLa cells expressing efflux pump, MDRI, as determined by PAD assay

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>A549 EC50 (μmol/L)</th>
<th>U2OS EC50 (μmol/L)</th>
<th>HCT116 EC50 (μmol/L)</th>
<th>HeLa EC50 (μmol/L)</th>
<th>H1299 EC50 (μmol/L)</th>
<th>DLD-1 EC50 (μmol/L)</th>
<th>HeLa-MDR EC50 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.459</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.459</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>0.040</td>
<td>0.038</td>
<td>0.031</td>
<td>0.028</td>
<td>0.030</td>
<td>0.026</td>
<td>ND</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.003</td>
<td>0.006</td>
<td>0.002</td>
<td>0.003</td>
<td>0.001</td>
<td>0.004</td>
<td>ND</td>
</tr>
<tr>
<td>R-253</td>
<td>0.018</td>
<td>0.034</td>
<td>0.024</td>
<td>0.025</td>
<td>0.017</td>
<td>0.041</td>
<td>0.022</td>
</tr>
<tr>
<td>R-216</td>
<td>0.069</td>
<td>0.094</td>
<td>0.039</td>
<td>0.060</td>
<td>0.047</td>
<td>0.071</td>
<td>ND</td>
</tr>
<tr>
<td>R-404</td>
<td>0.078</td>
<td>0.113</td>
<td>0.183</td>
<td>0.174</td>
<td>0.042</td>
<td>0.147</td>
<td>ND</td>
</tr>
</tbody>
</table>

*p53 positive*  
*p53 negative and/or mutated*  
*MDRI positive*  

*Antitumor activity of the compounds against various cell lines are given in EC50 (μmol/L).*
cycle at mitosis and triggering of apoptosis. Based on the novel structural features offered on the parent thienopyrimidine scaffold, R-253 was shown to exhibit biochemical, molecular, and cellular mechanisms that are consistent with other natural and synthetic microtubule inhibitors. Importantly, R-253 kills tumor cells that are drug resistant, but it is not lethal to normal nonproliferating cells. These intriguing biological properties together with its unique structural features make R-253 even more attractive candidate for further development toward potential clinical applications.

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References

R-253 Disrupts Microtubule Networks in Multiple Tumor Cell Lines

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