Design and Biological Evaluation of Novel Tubulin Inhibitors as Antimitotic Agents Using a Pharmacophore Binding Model with Tubulin

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Although the structure has been elucidated for the binding of colchicine and podophyllotoxin as potent destabilizer for microtubule formation, very little is known about MDL-27048, a competitive inhibitor for colchicine and podophyllotoxin. The structural basis for the interaction of antimitotic agents with tubulin was investigated by molecular modeling, and we propose binding models for MDL-27048 against tubulin. The proposed model was not only consistent with previous competition experiment data between colchicine and MDL-27048, but further suggested an additional binding cavity on tubulin. Based on this finding from the proposed MDL−tubulin complex, we performed molecular design studies to identify new antimitotic agents. These new chalcone derivatives exerted growth inhibitory effects on all four human hepatoma and one renal epithelial cell lines tested and induced strong cell cycle arrest at G2/M phase. Furthermore, these compounds exhibited a strong inhibitory effect on tubulin polymerization in vitro. Therefore, we suggest that the validated MDL-27048 model would serve as a potent platform for designing new molecular entities for anticancer agents targeted to microtubules.

Introduction

Microtubules are ubiquitous, essential cytoskeletal polymers in all eukaryotic cells controlling various cellular functions—transport of material within the cell, movement of cytoplasmic organelles or vesicles, and proper progression through cell division.[1] The function of microtubules is strongly associated with their stability. The proteins that stabilize microtubules include the classic microtubule-associated proteins (MAPs)[a] that are thought to bind along the length of the microtubule polymer and enhance its stability. In the case of proteins that destabilize microtubules, Op18 protein, also referred to as stathmin, and XKCM1 have been recently identified.[2] Furthermore, exogenous ligands can also have dramatic effects on microtubules, often interacting directly with tubulin to either stabilize (such as paclitaxel)[3] or destabilize microtubules (such as colchicines, MDL-27048).[4,5] These ligands disrupt the dynamics of polymerization and depolymerization of microtubules involved in cell division,[1] and the interference with the dynamics of tubulin and cell division has been proven clinically useful for designing anticancer agents.[6] These agents can be grouped into three classes, as distinguished by the site of binding on the tubulin molecule. Representative examples are taxol, vinblastine, and colchicines binding sites, respectively (Figure 1). Among these binding sites, the taxol binding site has been well-characterized with the crystal structure of tubulin[7] and partially overlaps with that of other compounds such as epothilones[8] or eleutherobin.[9] The vinblastine binding site is not precisely known, except for a single cross-linking experiment that identified the residues 175−213 of β-tubulin as the binding region. Because these residues are located in the plus end of the microtubule, this result suggests that the vinblastine binding site is exposed to the plus end of a microtubule surface.[10] Colchicine is the first drug that is known to bind tubulin, and the colchicine binding site has been characterized recently from a complex with colchicine and a stathmin-like domain.[20] The development of ketones as tubulin-binding agents such as chalcones has been reviewed recently.[22]

Although the structure has been elucidated for binding with colchicine and podophyllotoxin as a potent destabilizer for microtubule formation, very little is known about the structural basis for other destabilizing agents such as MDL-27048, a competitive inhibitor with colchicine and podophyllotoxin.[11] In this study, we propose the binding model of MDL-27048 against tubulin by molecular modeling and validate the model by designing and testing new chalcone derivatives. A pharmacophore model was built by analyzing colchicine and podophyllotoxin interaction with tubulin, projected into the MDL-binding model and combined with the receptor shape information of the X-ray tubulin structure. The model was then used to screen new tubulin binding agents, potentially as antimitotic agents. The biological activity of these new chalcone derivatives was confirmed by tubulin polymerization inhibition assay and the cytotoxic activity against human hepatoma and kidney cell lines. The present MDL-27048 binding model and pharmacophore are

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[a] Abbreviations: MAPs, microtubule-associated proteins; RMSD, root-mean-square deviation; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagles medium; SBDD, structure-based drug design.

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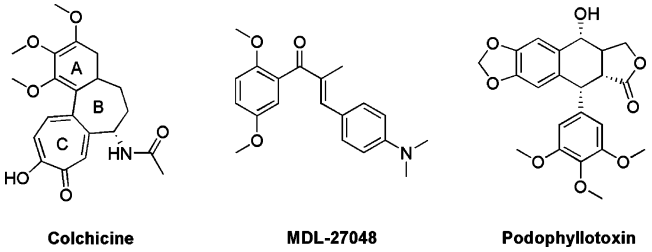


Figure 1. Chemical structures of tubulin inhibitors. MDL-27048 (*trans*-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one) and podophyllotoxin ([5R−(5α,5a*β*,8aα,9α)]-5,8,8a,9-tetrahydro[2,3-d]-1,3-dioxol-6(5aH)-one), competitive inhibitors of colchicine ((S)-*N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo-[*a*]-heptalen-7-yl)acetamide), interact with the colchicine binding site.

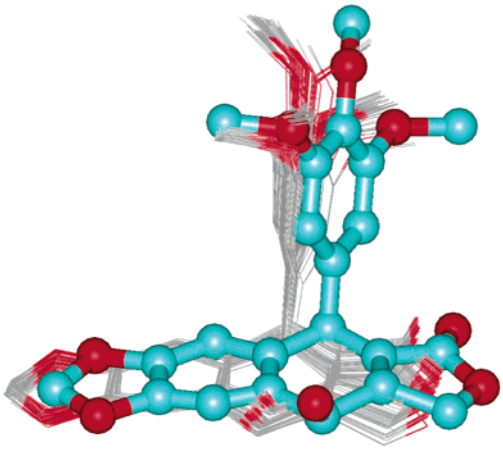


Figure 2. Comparison of podophyllotoxin in X-ray structure (PDB code 1SA1: cyan) and in AutoDock result (colored gray). X-ray structure of podophyllotoxin is represented by ball-and-stick. Only top cluster of AutoDock results (94 conformers) are shown with wire form.

expected to provide a useful basis for further designing of new derivatives intervening the dynamics of microtubule formation.

Results and Discussion

To test whether the AutoDock program is feasible for ligand binding to tubulin, the podophyllotoxin−colchicine complex structure (PDB code: 1SA1) was initially chosen and the docking structure of podophyllotoxin was compared with its crystallographic structure (Figure 2). The top configuration cluster among 100 independent podophyllotoxin conformers was composed of 94 conformers. This top cluster showed the highest frequency of occurrence, so we accepted top cluster configuration as podophyllotoxin binding model. The binding mode of the lowest energy structure in the top docking cluster and that of crystallographic structure to tubulin were very similar (RMSD: 1.07 Å). This result demonstrates that AutoDock analysis is suitable for the identification of the colchicine binding site in tubulin. Based on this result, the MDL-27048 binding model was generated using the same condition used for podophyllotoxin binding. The top configuration cluster among 100 independent MDL-27048 conformers was composed of 42 conformers, and the structure of lowest energy in the top docking cluster was shown in Figure 3A. As depicted in Figure 3B, MDL-27048 shared the binding pocket with colchicine, and the overlapping of the binding sites explained the competition among colchicine, podophyllotoxin, and MDL-27048 in tubulin binding.[11] Interaction of MDL-27048 involves amino acid residues Cys *β*241, Lys *β*352, Val *β*318, Leu *β*255, Leu *β*242, Leu *β*248, Ala *β*250, Ala *β*316, Ile *β*378, and Met *β*259, mostly through van der Waals interactions, similar to colchicine and podophyllotoxin interaction with tubulin.[20]

Except for colchicine, no information has been available on the interaction of the destabilizing agent, including MDL-27048, with tubulin. Therefore, we screened and tested new chalcone derivatives to validate our MDL-27048 binding model. First, we built a pharmacophore model by analyzing colchicine and podophyllotoxin interaction with tubulin. As depicted in Figure 4A, the derived pharmacophore consists of two hydrophobic centers (yellow sphere) and one polar interaction site (yellow cylinder). The hydrophobic center that is located in the middle of trimethoxyphenyl moiety of colchicine and podophyllotoxin is surrounded by Leu *β*242, Ala *β*250, Leu *β*255, Ala *β*316, Val *β*318, and Ile *β*378 residues. The other hydrophobic center makes contact with Met *β*259, Ala *β*316, and a carbon chain of Lys *β*352 residues. The yellow cylinder represents a possible polar interaction with the Cys *β*241 residue, and the distance between the cylinder and the sulfur atom in Cys *β*241 is 2.9 Å. These three characteristics represent the minimum interactions in the colchicine binding site. As shown in Figure 4B, the MDL-27048 binding model is matched properly with the proposed pharmacophore. The hydrophobic center located in the middle of trimethoxyphenyl moiety in podophyllotoxin corresponds to the 2,5-dimethoxyphenyl moiety in MDL-27048 and the other hydrophobic center matched with the 4-dimethylamino phenyl moiety in MDL-27048. The polar interaction between C2-oxygen in the A-ring of colchicine and Cys *β*241 residue also corresponds to the interaction between C5-oxygen in MDL-27048 and Cys *β*241.[20]

We observed an additional cavity within the colchicines binding site, as described with a red circle in Figure 4B, mainly composed of Val *β*238, Leu *β*242, and Leu *β*255 (Figure 3). After combining with receptor shape information of X-ray tubulin structure, the proposed pharmacophore was projected to the MDL-27048 binding structure and used to screen new tubulin binding agents.

To validate our proposed model, we virtually screened from our natural compound database of pharmacophores (total 9720 compounds) for the proposed colchicine binding site described in Figure 4B. Twenty-four compounds were selected. We then initially tested these compounds with cell proliferation assay on HL-60 cell line within the concentration range of 0.003 to 10 *μ*g/mL for each test compound (data not shown). We finally selected five compounds, **1** (CHA101), **2** (CHA102), **3** (CHA103), **4** (CHA105), and **5** (CHA106), which showed the highest activity against HL-60 growth inhibition. These new chalcone derivatives had different molecular sizes (Figure 5) and were found to be novel compounds hitherto without any previous reports as tubulin inhibitors.

For virtual screening of new derivatives, we considered primarily the two major interaction points (two hydrophobic interactions among three interaction points as discussed before) and an additional binding pocket (shown with red circle; Figure 4B). Therefore, all five compounds, **1**−**5**, fitted into the proposed MDL-27048 binding pocket comprising the two hydrophobic characteristics and an additional binding pocket. A representative binding model is shown for both **1** and **2**, respectively (Figure 6). It is interesting to note that **4** and **5** were similar in the sense that they carry almost the same size of moiety into the additional binding pocket, whereas **4** carried extra biphenyl moiety in the classic chalcone binding pocket (Figures 5 and 6).

Interestingly, the newly discovered compounds carry striking resemblance to the enone analogues of curcumin as a chalcone core that has been previously reported as an inhibitor of

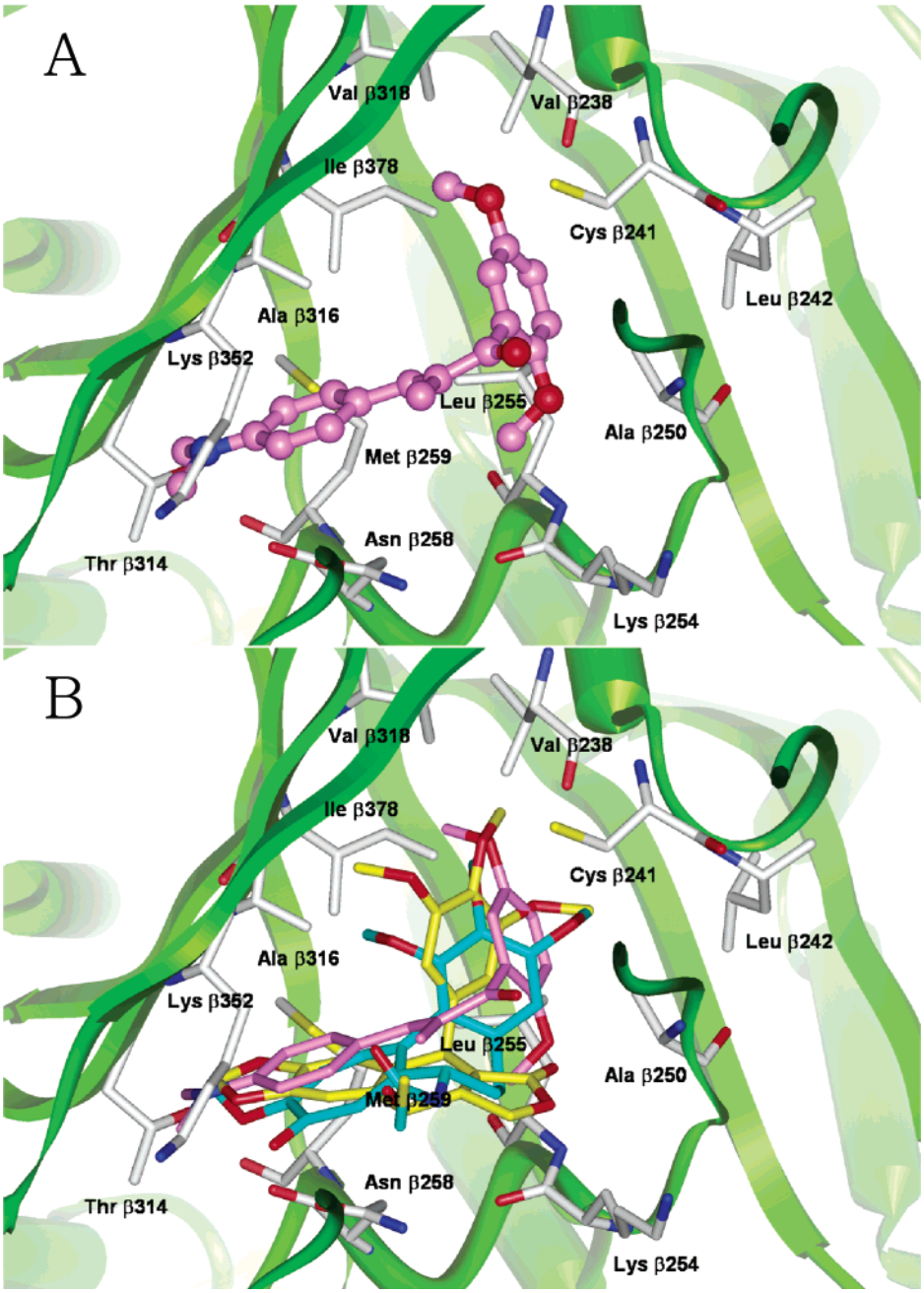


Figure 3. (A) Model of MDL-27048 and tubulin complex. (B) Comparison of colchicine, podophyllotoxin, and MDL-27048 binding model with tubulin. Cyan, yellow, and purple represent colchicine, podophyllotoxin, and MDL-27048, respectively.

endothelial cell proliferation.[23] This chalcone core, referred to as compound 6 in that paper, lacks the group that occupies the additional binding site (Figures 5 and 6). Therefore, our compound virtually screened in this study is expected to exhibit the improved activity over compound 6 lacking the group occupying the new binding site. For this reason, we used compound 6 as a control for the cell proliferation assay (Figure 7) and the in vitro tubulin polymerization assay (Figure 9).

A direct cell proliferation/cytotoxicity assay was performed using four independent human hepatoma and one renal epithelial cell lines within the range of 0.01 to 100 μM of test compounds, using compound 6 as positive control. As shown in Figure 7, strong inhibitory effects were observed with compounds **1**, **2**, and **5** for both Huh7 and 293T cell lines. Most importantly, these three compounds showed manifest improved activity over compound 6. We obtained similar results from the cell proliferation assay with other human hepatoma cell lines, HepG2, SNU-368, and SNU-398 (data not shown). The IC_{50} value of test compounds and control are summarized in Table 1. The antiproliferative activities of compounds **1**, **2**, and **5** were better than that of the control compound, compound 6, consistent with the interaction with the newly identified binding site in this study. However, the antiproliferative effects of **3** and **4** were much lower in all test cell lines. These results validate the newly discovered binding pocket in tubulin. Parallel analysis of MDL-27048, previously described as a potent antimitotic agent,[22] exhibited a relatively higher IC_{50} value on our cell proliferation assay (data not shown), consistent with previously observed resistance of human hepatoma cells to tubulin interfering agent.[24]

To determine whether the cytotoxic effects induced by the treatment of the three chalcone derivatives (**1**, **2**, and **5**) were caused by cell cycle arrest at a certain phase or not, we performed cell cycle analysis using flow cytometry. Indeed, the

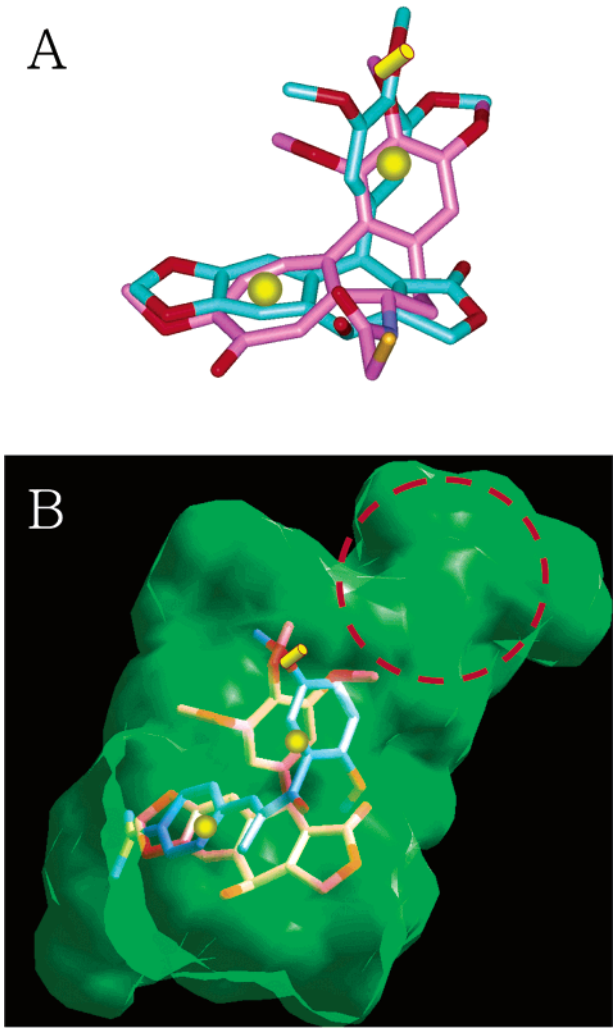


Figure 4. (A) Pharmacophore model of colchicine (pink) and podophyllotoxin (cyan). (B) Red circle proposes additional binding pocket. Pharmacophore binding model is shown under the backdrop of colchicine binding site shape information. MDL-27048 model and podophyllotoxin are represented with blue and pink, respectively.

flow cytometric analysis showed that all three compounds strongly induced G2/M arrest in SNU-398 cells, and the effect was observed in a dose-dependent manner in the test range of 50−5000 nM (Figure 8). These results confirmed and further extended the growth inhibitory effects of the three chalcone derivatives on hepatoma cells.

The results suggest that the additional binding pocket of tubulin identified in the present study can accommodate compounds **1**, **2**, and **5**. Compound **3** may not fit properly into this pocket because of a relatively long ethyl ether group extended from the phenyl moiety (Figure 5). We, therefore, suggest that, for further design of tubulin-targeted antimitotic agents, careful considerations should be given to the size of the moiety fitting the newly identified binding pocket.

Finally, we tested whether the compounds directly interact with tubulin and inhibit tubulin polymerization in vitro. As shown in Figure 9, strong inhibitory effect was observed with three derivatives. Compounds **1** and **2** and compound 6 showed 50% tubulin polymerization inhibition at 19.8, 15.7, and around 30 μM (IC_{50}), respectively. It was difficult to evaluate the IC_{50} value of compound **5** because of poor solubility in our experimental setting. It should be noted that the IC_{50} of compound 6 was higher than compounds **1** and **2**, further extending data in the cell proliferation assay (Figure 7) and Table 1. The results also confirm that the cytotoxic effect is mediated by direct interaction of the compounds with a new binding pocket in tubulin. The IC_{50} value for tubulin binding was higher than cell proliferation (Figure 9), reminiscent of previous studies.[22]

Conclusions

A molecular docking study suggests that MDL-27048 binding to tubulin involves Cys β241, Lys β352, Val β318, Leu β255, Leu β242, Leu β248, Ala β250, Ala β316, Ile β378, and Met β259, mostly through van der Waals interactions, in much the same way observed in colchicine and podophyllotoxin binding. This binding structure is in agreement with previous experimental data relevant to colchicine and MDL-27048 binding to tubulin.[11] Consistent with direct competition between the two compounds,[11] here we proposed a new binding model for MDL-27048 that overlaps with colchicines, and the binding involves three major interactions between MDL-27048 and tubulin (Figure 4B). The major interaction in this model is first of all in good agreement with the known interactions with classic ligands (colchicine and podophyllotoxin in this study). We further validated the binding model by designing new chalcone derivatives and testing the cytotoxic activities, cell cycle analysis, and the inhibition of tubulin polymerization in vitro. Among these, compounds **1**, **2**, and **5** consistently exhibited potent activities and merits for the further evaluation as novel antimitotic agents. Furthermore, the present MDL-27048 binding model and the proposed pharmacophore would provide a useful guideline for the future design of new chemical entities of microtubule-targeted anticancer agents.

Experimental Section

Materials. Compounds **1** and **2** were purchased from Sigma-Aldrich (www.sigmaaldrich.com), and compound **3** was purchased from Timtec (www.timtec.net). Compounds **4** and **5** were purchased from Asinex (www.asinex.com). All test compounds used in this study were dissolved in DMSO as 10 mM stock. The solubilities of **1**, **2**, and compound 6 were higher than 100 μM, whereas those of **3**, **4**, and **5** were near 20 μM in aqueous solution.

Synthesis of 1-Phenyl-3-pyridin-2-yl-propenone (Compound 6). Compound 6 was synthesized by the method of Robinson and co-workers.[23] An amount equal to 20 mL of 10% aqueous sodium hydroxide solution and 2 mL of methanol were added to a flask, and the mixture was cooled to 10 °C with stirring. An aliquot of 6.2 mL (0.065 mol) of pyridine-2-carboxaldehyde was added in one portion. Acetophenone in the amount of 3.8 mL (0.032 mol) was then added in small portions over a period of an hour, maintaining the temperature at around 10 °C. The contents of the flask were stirred for 5 h, and the reaction mixture was poured onto 30 mL of water. The resulting solid was filtrated and washed with 20 mL of water. The solid was then dried and recrystallized three times from hexane and diethyl ether to give 2.7 g (75%) of 1-phenyl-3-pyridin-2-yl-propenone. ^{1}H NMR ($CDCl_3$): 7.30 (t, 1H, CHPhH-*m*), 7.50 (m, 3H, COPhH-*m* and CHPhH-*m*), 7.60 (t, 1H, COPhH-*p*), 7.75 (m, 2H, CHPhH-*o*,*p*), 8.11 (m, 3H, COPhH-*o* and CH), 8.69 (dd, J = 4.2 Hz, 1H). MS(FAB) m/z 210 ($M + H^+$).

Molecular Modeling. Molecular modeling studies were conducted on several R12000 SGI Octane workstations with the software package InsightII (Accelrys, Inc.), and the structure-based virtual screenings were performed using Equispharm's in-house software packages on a Linux workstations (http://www.equispharm.com; the in-house programs, PharmoMap and PharmoScan, will be published elsewhere). The molecular visualization tool was performed by InsightII package.

Molecular Docking Study. To find out the docking structures of MDL-27048 into the previous defined colchicine binding region of tubulin,[20] automated docking simulation was implemented with

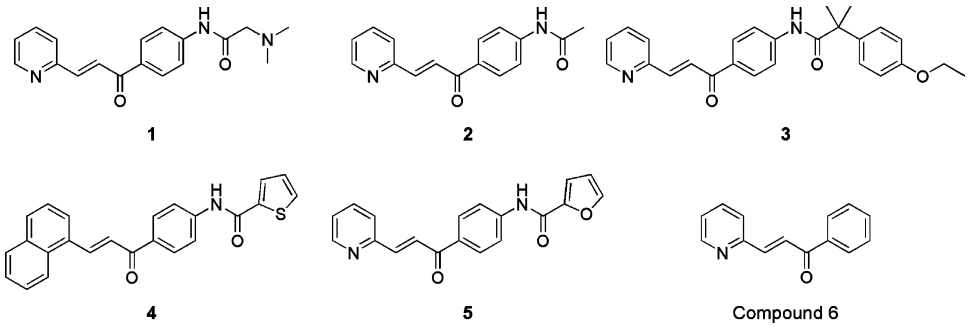


Figure 5. New chalcone derivatives as screened from chemical DB based on the MDL-27048 binding model. The enone analogue of curcumin as a chalcone core (compound 6 in the text) was presented. Compound 6 was reported as an inhibitor of endothelial cell proliferation.[23]

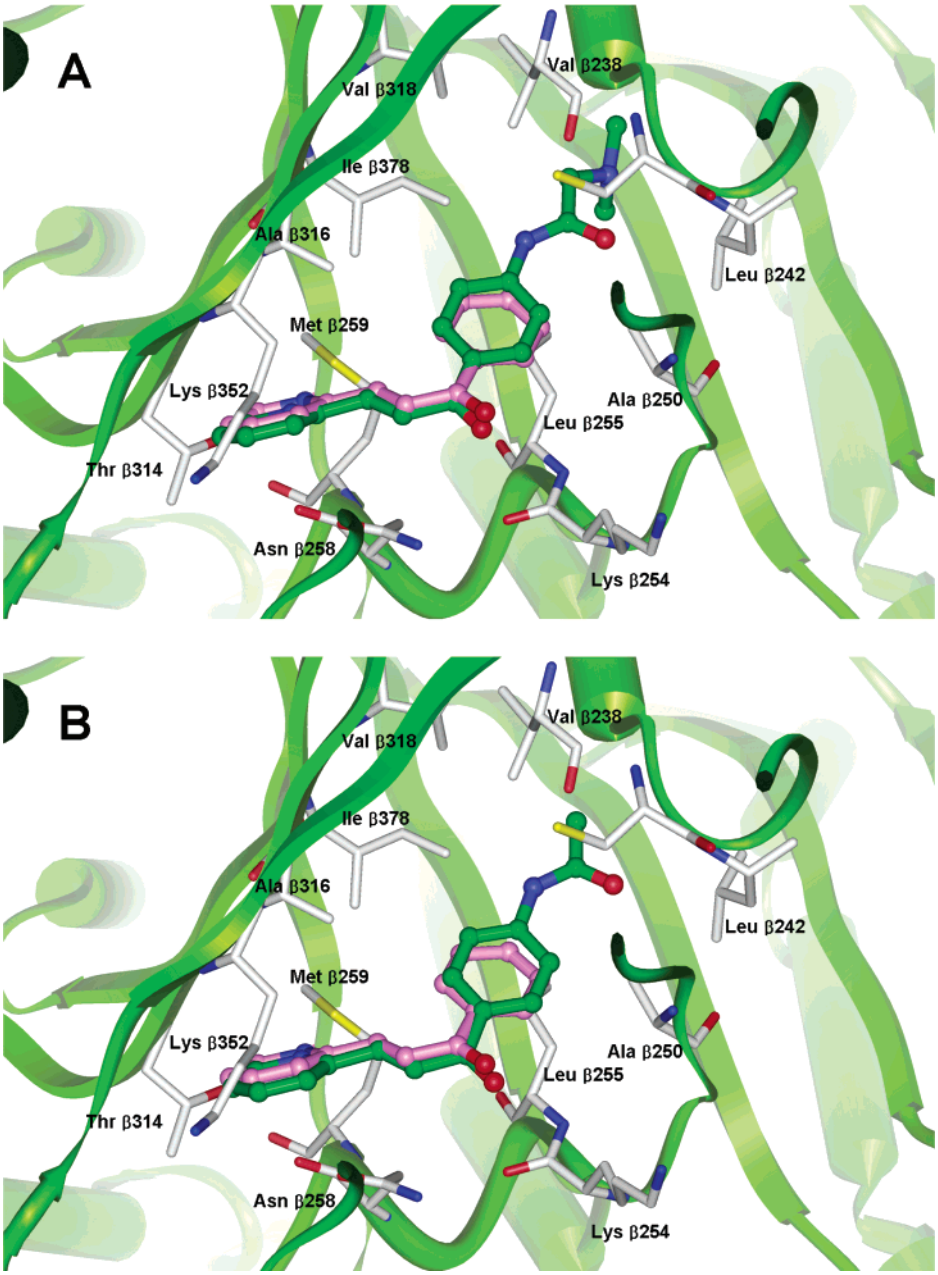


Figure 6. Model of new chalcone derivatives and tubulin complex. **1** (A) and **2** (B), shown in green, interact with both the classic colchicines binding site and the additional pocket newly identified in this work (see Figure 4). Binding model of compound 6 (purple) was presented for comparison.

the program AutoDock version 3.0.[12−14,19] The starting conformations of MDL-27048 were optimized with quantum mechanical calculations using Gaussian 98 (6-31g* base set).[21] All ligand atomic charges were assigned using the Gasteiger−Marsili formation, which is the type of atomic charges used in calibrating the AutoDock empirical free energy function.[15] Atomic solvation parameters and fragmental volumes were assigned to the protein atoms using the AutoDock utility, AddSol. All ligand atoms but

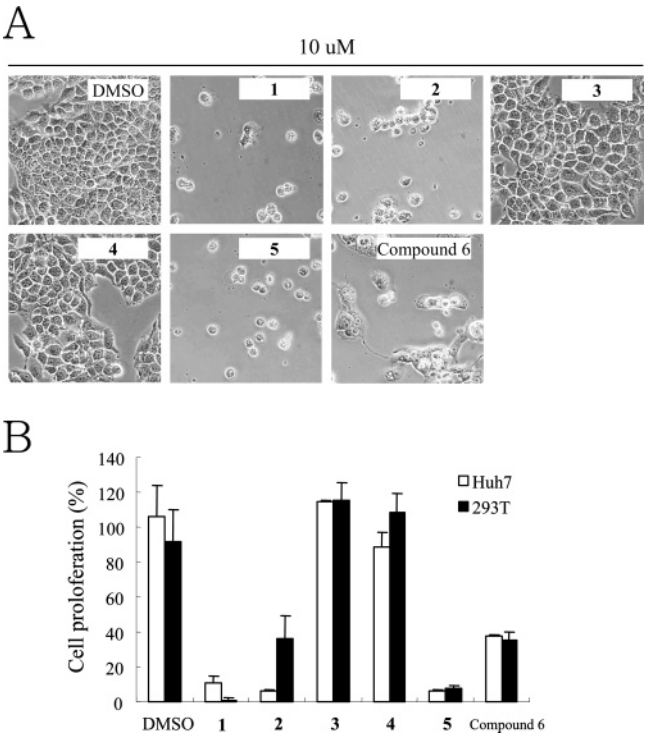


Figure 7. Cytotoxic effect on human hepatoma and renal epithelial cell lines by chalcone derivatives. (A) Huh7 cells grown in 24-well plates were treated with 10 μM of indicated compounds for 48 h. The phase-contrast microscopic examination of treated cells was shown (200× magnification). Results are representative of three independent experiments. (B) The effects of chalcone derivatives on cell growth were evaluated by cell proliferation assay, as described in Experimental Section. Huh7 and 293T cells were treated with 20 μM of indicated compounds for 48 h and a proliferation assay was performed. Results are an average of triplicate experiments, and the standard deviation is shown in a bar.

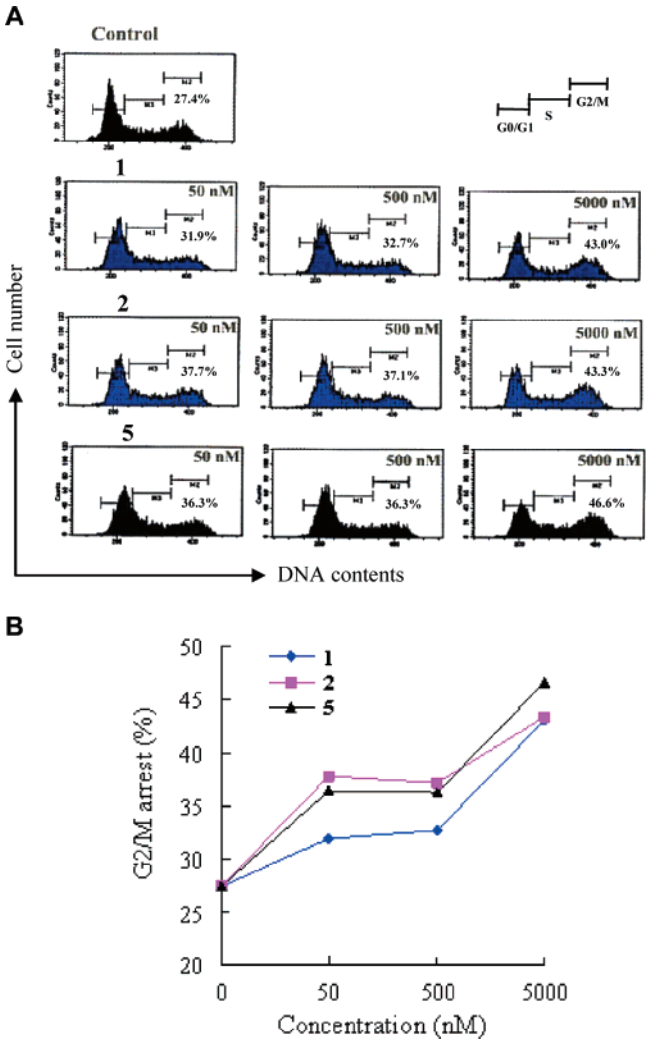


Figure 8. Inhibition of cell cycle progression by chalcone derivatives. (A) Cell cycle analysis after treatment of chalcone derivatives using flow cytometry. SNU-398 hepatoma cells were treated with chalcone derivatives for 48 h and subjected to cell cycle analysis. Each cell cycle phase and percentages of G2/M phase cells are indicated. (B) Cell cycle arrest at G2/M phase by chalcone derivatives treatment. Percentages of G2/M phase cells obtained from panel A were plotted.

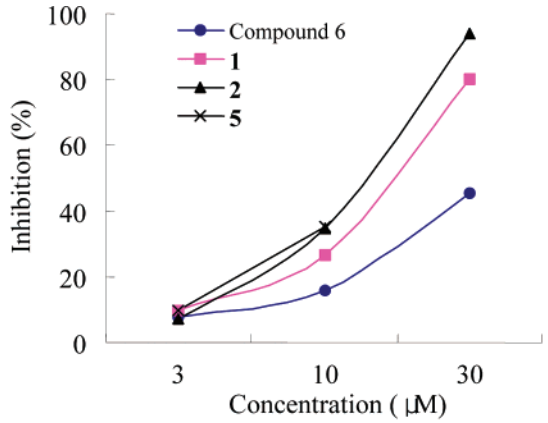
no protein atoms were allowed to move during the docking simulation, and the rotatable bonds in the ligand were defined using an AutoDock utility, AutoTors. The interaction energy between ligand and protein was evaluated using atom affinity potentials that are precalculated on a grid.[16] The grid maps were calculated using AutoGrid. We used this grid map with 60 points in each x, y, and z direction, equally spaced at 0.375 Å. Docking was performed using the Lamarckian genetic algorithm in AutoDock 3.0. Each docking experiment was performed 100 times, yielding 100 docked conformations. Parameters used for the docking were as follows: population size of 50; random starting position and conformation; maximal mutation of 2 Å in translation and 50 degrees in rotations; elitism of 1; mutation rate of 0.02 and crossover rate of 0.8; and local search rate of 0.06. Simulations were performed with a maximum of 1.5 million energy evaluations and a maximum of 27 000 generations. The pseudo-Solis and Wets local search method was included with the default parameters. Final docked conformations were clustered using a tolerance of 1 Å RMSD.

Virtual Screening. The MDL-27048 docking model and the podophyllotoxin−colchicine complex structure (PDB code: 1SA1) with tubulin were utilized for rapid virtual screening of the compound database. The pharmacophore was analyzed using PharmoMap, an in-house software package developed by Equispharm for structure-based virtual screening. The constructed PharmoMap is the feature-based pharmacophore in which the pharmacophoric points are represented by chemical features, such as hydrogen bond acceptors/donors or hydrophobic features. The pharmacophore models were employed as a search query to identify inhibitors targeted to a colchicine binding site from a 3D small molecule database, which comprises commercially available compounds of chalcone moieties (total 9720 compounds). The virtual screening for colchicines binding pocket of tubulin was carried out using the PharmoScan system, a structure-based virtual screening tool developed by Equispharm. Compounds of unfavorable interactions with the binding site or adopting unrealistic conformations were screened against the process of pharmacophore mapping into the colchicines binding site of tubulin. The twenty-four compounds were finally selected for cell proliferation assay in vitro.

Cell Lines. Human hepatoma cell lines (HepG2 and Huh7) and renal epithelial (293T) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). SNU-398 and SNU-368 hepatoma cell lines were obtained from the Korean Cell Line Bank, Seoul, Korea.[17,18] These cell lines were maintained in Dulbecco's modified Eagles medium (DMEM; Flow Laboratories), supplemented with 10% fetal bovine serum/glutamine/antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell Proliferation Assay. Cells (2×10^4 cells) were seeded into 96-well plates. After 24 h, the cells were grown in the presence or absence of drugs for 48 h. After removing 100 μL supernatant from each well, 20 μL of CellTiter 96Aqueous solution (Promega, USA) was added into each well of the 96 well plate containing 100 μL of culture medium, and the cells were further incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO_2. The reduction of absorbance at 490 nm was measured.

Cell Cycle Analysis. The effects of compounds on cell cycle were analyzed using flow cytometry. Briefly, hepatoma cells (5 ×



Compound	1	2	5	Compound 6
IC_{50} (uM)	19.8	15.7	NA	>30

Figure 9. Inhibition of tubulin polymerization by chalcone derivatives. The effects of chalcone derivatives on tubulin polymerization were evaluated as described in Experimental Section. Compounds **1**, **2**, and **5** and compound 6 (control)[23] were tested at different concentrations (0, 3, 10, and 30 μM). IC_{50} value was calculated based on the tubulin polymerization assay. The solubility of compound **5** was near 20 μM in aqueous solution, and the inhibition assay could not be performed at higher concentration. NA, not applicable. Compounds **1**, **2**, and **5** are newly tested in this report, whereas compound 6 has previously been reported.[23]

Table 1. Antiproliferative Activities of Newly Identified Chalcone Derivatives

	cell proliferation IC_{50} (μM)	
compounds	Huh7	293T
1	8.4	8.2
2	9.6	13.7
3	>100	>100
4	>100	>100
5	9.9	9.8
compound 6	14.3	13.8

10^5) were seeded into 12-well plates, grown for 24 h and further grown in the presence of drug or carrier alone for 48 h. Cells were harvested, washed twice with PBS, stained with the cell cycle analysis kit (Becton Dickinson, Mountain View, CA), and analyzed with flow cytometry (Becton Dickinson). The percentages of cells in different phases of the cell cycle were estimated using the Modfit LT software (Becton Dickinson).

Tubulin Polymerization Assays. HTS-tubulin polymerization assay kit (cat. No. BK004/ CDS01, Cytoskeleton, USA) was used. All components were added to microwells held at 0 °C, and baselines were established at 340 nm. Assembly was followed sequentially at 0 °C and 37 °C, and tubulin assembly was measured turbidometrically in Molecular Device Spectra Max Plus model (Molecular Devices, USA), equipped with electronic temperature controllers.

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