P-Glycoprotein-Independent Apoptosis Induction by a Novel Synthetic Compound, MMPT [5-[(4-Methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone]

Fuminori Teraishi,1 Shuhong Wu,1 Jiichiro Sasaki, Lidong Zhang, Hong-Bo Zhu, John J. Davis, and Bingliang Fang

Department of Thoracic and Cardiovascular Surgery, University of Texas M.D. Anderson Cancer Center, Houston, Texas (F.T., S.W., J.S., L.Z., H.-B.Z., J.J.D., B.F.); and Program in Gene Therapy and Virology, University of Texas Graduate School of Biomedical Sciences, Houston, Texas (J.J.D., B.F.)

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ABSTRACT

To develop new anticancer agents that are effective for treatment of chemoresistant tumors, we screened a chemical library for compounds that can effectively kill both paclitaxel-sensitive lung cancer cell H460 and P-glycoprotein-overexpressing paclitaxel-resistant cell H460/TaxR. A synthetic compound, MMPT (5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone), was identified to induce cytotoxic effects in both H460 and H460/TaxR cells but not in normal fibroblasts. MMPT effectively inhibited the growth of several human lung cancer cell lines in a dose-dependent manner, with 50% inhibitory concentrations ranging from 4.9 to 8.0 μM. The inhibitory effect on cancer cells is independent of the status of p53 and P-glycoprotein. Moreover, MMPT had no obvious toxic effects on normal human fibroblasts and mesenchymal stem cells at the 50% inhibitory concentration for lung cancer cell lines. Treating lung cancer cells with MMPT-induced apoptosis with caspase-3, -8, -9, and poly(ADP-ribose) polymerase cleavage and cytochrome c release from mitochondria. MMPT-induced apoptosis was abrogated when c-Jun N-terminal kinase (JNK) activation was blocked with a specific JNK inhibitor, SP600125. Furthermore, in vivo administration of MMPT suppressed human H460 xenograft tumor growth in nude mice. Our results suggest that MMPT may induce tumor-selective cell killing in both P-glycoprotein-negative and -positive cancer cells and could be a new anticancer agent for treatment of refractory tumors.

The emergence of drug resistance is a major challenge to the success of cancer chemotherapy. Because chemotherapeutic agents are usually administered to patients at an amount close to the maximum tolerated dose, dose escalation would lead to intolerable toxicity and is not acceptable for the treatment of refractory tumors. Consequently, resistance to treatment and dose-limiting toxicity are the two major reasons for the failure of anticancer therapies. Although chemoresistance can be caused by various mechanisms, the ultimate failure of chemotherapy often results from multidrug resistance (MDR), a phenotype of cross-resistance to multiple drugs with diverse chemical structures. One of the well-documented mechanisms responsible for MDR is the overexpression of the MDR-1 gene that encodes a 170-kDa P-glycoprotein, a transmembrane ATP-dependent drug efflux pump that prevents intracellular accumulation of these drugs.
compounds by increasing their efflux out of cells, leading to multidrug resistance (Gottesman et al., 2002; Jordan and Wilson, 2004).

Efforts have been made to overcome MDR by inactivating P-glycoprotein activity through P-glycoprotein inhibitors (such as verapamil, cyclosporine A, tamoxifen, and LY-335979) (Ferry et al., 1996; Samuels et al., 1997; Advari et al., 2001), antisense oligos (Cucco and Calabretta, 1996), ribozyme (Bouffard et al., 1996; Huesker et al., 2002), or siRNA (Wu et al., 2003). Although a growing body of evidence shows that the MDR phenotype can be reversed in cultured cells by these approaches, clinical trials of P-glycoprotein inhibitors to sensitize MDR-positive tumors to agents such as vinblastine revealed either no appreciable activity (Samuels et al., 1997) or no definitive conclusion (Ferry et al., 1996; Gottesman et al., 2002). Therefore, the discovery of new compounds that are not substrates of P-glycoprotein and are effective against drug-resistant cells but spare normal human cells is an important step for cancer therapy.

To develop new anticancer agents that are effective for refractory tumors, we screened a chemical library from ChemBridge Research Laboratories (San Diego, CA) for small molecules that can effectively kill paclitaxel-resistant, P-glycoprotein-overexpressing lung cancer cells, but not normal human fibroblasts. We found a synthesized compound, MMPT, can induce cytotoxic effects in both paclitaxel-susceptible and -resistant cancer cells but not in fibroblasts. In addition, MMPT had minimal effect on normal human bone marrow mesenchymal stem cells. The molecular mechanism of MMPT’s cytotoxic effect was further characterized on non-small cell lung cancer (NSCLC) cells that are either P-glycoprotein-positive or -negative. We found that MMPT-induced apoptosis, including cytochrome c release and caspase activation, was abrogated when JNK activation was blocked with a specific JNK inhibitor. Thus, MMPT and its analogs could be useful anticancer agents for treatment of refractory tumors.

**Materials and Methods**

**Cells and Culture Conditions.** The human NSCLC H1299, H322, and H460 cell lines, which have an internal homozygous deletion of the p53 gene, a mutated p53 gene, or the wild-type p53 gene (Nishizaki et al., 2001), respectively, were routinely propagated in monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Normal human fibroblasts (NHFb) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with the same supplements. Normal human mesenchymal stem cells (MSCs) were grown in α-minimal essential medium with 20% heat-inactivated fetal calf serum and the same supplements. All cells were maintained in the presence of 5% CO2 at 37°C.

**Chemicals and Antibodies.** A chemical library with 10,000 compounds, including MMPT, was obtained from ChemBridge Research Laboratories. The chemicals in the library are provided as 5 mg/ml in dimethyl sulfoxide (DMSO). MMPT has a molecular weight of 295.42 g/mol and was dissolved in DMSO to a stock concentration of 10 mM and stored at 4°C. Paclitaxel was purchased from Bristol-Myers Squibb Co. (Stamford, CT). Vinorelbine was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in DMSO to a concentration of 10 mM, stored at 20°C, and protected from light. The JNK-specific inhibitor SP600125, the ERK inhibitor PD98059, and the p38 inhibitor SB202190 were purchased from Calbiochem (San Diego, CA), dissolved in DMSO, stored at 20°C, and protected from light. An equal volume of DMSO (<0.1%) had no effects on cell viability and was used as a control. Antibodies to the following proteins were used for Western blot analysis: caspase-3 and P-glycoprotein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); caspase-8 (MBL International, Woburn, MA); COX-4, cytochrome c, and poly(ADP-ribose) polymerase (PARP) (BD Biosciences Pharmingen, San Diego, CA); JNK, phosphorylated JNK (p-JNK), ERK, phosphorylated ERK (p-ERK), p38, phosphorylated p38 (p-p38), and caspase-9 (Cell Signaling, Beverly, MA); and β-actin (Sigma-Aldrich).

**Cytotoxicity Studies.** The inhibitory effects of MMPT and other agents on cell growth were determined by the XTT assay. Cells (2–8 × 103 cells in 100 μl of culture medium/well) were seeded in 96-well flat-bottomed plates and treated the next day with the drugs at the indicated concentrations. After 72 h, cells were washed once with PBS, and cell viability was determined by XTT assay using a Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s protocol. The experiments were performed at least three times for each cell line. Cell viability was calculated using the following formula: cell viability = 100 × Areatment/Acontrol (percentage). The IC50 value, a dose that causes 50% reduction of surviving cells when compared with control, was determined by the CurveExpert Version 1.3 program. The cytotoxicity profile of MMPT was also evaluated at the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD) against 42 cell lines in the NCI drugs screening panel. The 50% growth inhibition (GI50) value, a dose that inhibits 50% of cell growth, was determined by sulforhodamine B assay 48 h after treatment as described previously (Monks et al., 1991).

**Flow Cytometry Analysis.** To analyze the intracellular DNA content, cells treated with various concentrations of MMPT were harvested in 0.125% trypsin, washed twice in PBS, and fixed in 70% methanol at 20°C for several hours. The cells were then suspended in PBS containing 10 μg/ml propidium iodide (PI) (Roche Diagnostics) and 10 μg/ml RNase A (Sigma-Aldrich) at 37°C for 30 min. Cell-cycle analysis was performed using an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL) with the Multicycle Phoenix Flow Systems program (Phoenix Flow Systems, San Diego, CA). All experiments were repeated three times.

**Western Blot Analysis.** For preparation of whole-cell extracts, cells were washed twice in ice-cold PBS, collected, and then lysed in lysis buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) containing 1x proteinase inhibitor cocktail (Roche Diagnostics). The lysates were spun at 14,000g for several hours. The cells were then suspended in PBS containing 10 μg/ml propidium iodide (PI) (Roche Diagnostics) and 10 μg/ml RNase A (Sigma-Aldrich) at 37°C for 30 min. Cell-cycle analysis was performed using an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL) with the Multicycle Phoenix Flow Systems program (Phoenix Flow Systems, San Diego, CA). All experiments were repeated three times.

**Animal Experiments.** Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23) and the institutional guidelines of M.D. Anderson Cancer Center. Subcutaneous tumors were established in 4- to 6-week-old female nude mice (Charles River Laboratories, Inc., Wilmington, MA) by inoculation of 1.5 × 106 H460 cells into the dorsal flank of each mouse. After the tumors grew to 3 to 5 mm in diameter, the mice (five or six per group) were treated with various concentrations of MMPT.
were treated with i.p. administration of 40 mg/kg/injection MMPT (six mice) (MMPT dissolved in 0.5 ml of solvent containing 5.7% DMSO, 9.6% Cremophore EL, and 9.6% ethanol), solvent alone (five mice), or 4 mg/kg/injection paclitaxel (dissolved in 0.5 ml of PBS; five mice) as previously described (Lin et al., 2003). Tumor volumes were calculated by using the formula $a \times b^2 \times 0.5$, where $a$ and $b$ represented the larger and smaller diameters, respectively (Lin et al., 2002). Mice were killed when the tumors grew to 15 mm in diameter.

To evaluate the toxicity of treatment, blood samples were collected from the tail vein before MMPT treatment and on day 21, 4 days after the last treatment, and serum alanine transaminase, aspartate transaminase, alkaline phosphatase, blood urea nitrogen, and creatinine levels were determined as described elsewhere (Gu et al., 2000, 2002). Hematopoietic toxicity was monitored by counting red blood cells, white blood cells, and platelets.

**Statistical Analysis.** Differences among the treatment groups were assessed by analysis of variance using statistical software (StatSoft, Tulsa, OK). Differences among the results for the experiment of tumor growth in vivo were assessed by analysis of variance with a repeated measurement module. $p < 0.05$ was regarded as significant.

**Results**

**Library Screening for Cytotoxic Compounds Effective for P-Glycoprotein-Overexpressing Cells.** To identify agents that are effective for chemoresistant cancer cells, we established paclitaxel- and vinorelbine-resistant lung cancer cells H460/TaxR and H460/VinR by repeated treatments of H460 cells with paclitaxel or vinorelbine. The cells were initially treated with 5 nM paclitaxel or vinorelbine. Surviving cells were then treated with increased doses of paclitaxel or vinorelbine, up to 100 nM. H460/TaxR10 cells had tolerance to 10 nM paclitaxel. As shown in Fig. 1a, H460 parental cells had little expression of P-glycoprotein; however, H460/TaxR10, H460/TaxR, and H460/VinR cells expressed high levels of P-glycoprotein. The expression of P-glycoprotein in H460/TaxR or H460/VinR cells were at least 10-fold greater than H460 parental cells.

We then used parental H460, H460/TaxR cells, and normal human fibroblasts to screen a chemical library from Chembridge, Inc. for compounds that can kill both H460 and H460/TaxR cells but not fibroblasts. For this purpose, $5 \times 10^{-5}$ and $1 \times 10^{-4}$ cells were seeded into each well of a 96-well plate and then treated with each compound at a final concentration of about 5 μg/ml. Cells treated with solvent (DMSO) were used as controls. Changes in cell morphology were then observed under microscope, and cell viability was determined by XTT assay 2 to 4 days after the treatment. The compounds that were initially observed to kill both H460 and H460/TaxR cells but not fibroblasts underwent two additional screenings to confirm the observation. After three rounds of screening, MMPT (Fig. 1b) was found to kill both H460 and H460/TaxR cells but not fibroblasts at the concentration of about 5 μg/ml.

**MMPT Inhibits Cell Proliferation of Cancer Cell Lines Independent of Their MDR and p53 Status.** We then determined the dose effect of MMPT on cell proliferation in other NSCLC cell lines using an XTT assay. H1299, H460, H460/TaxR, H460/VinR, and H322 cell lines were treated...
TABLE 1
IC_{50} for MMPT and some existing chemotherapeutic agents in various cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>p53 Status</th>
<th>MMPT IC_{50} ( \mu M )</th>
<th>Paclitaxel IC_{50} nM</th>
<th>Vinorelbine IC_{50} nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1299</td>
<td>Null</td>
<td>6.7 ± 2.9</td>
<td>6.3 ± 0.5</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>H322</td>
<td>Mutant</td>
<td>4.9 ± 0.9</td>
<td>2.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>H460</td>
<td>Wild</td>
<td>5.6 ± 1.9</td>
<td>3.3 ± 0.2</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>H460/TaxR</td>
<td></td>
<td>5.7 ± 1.3 (1.0)( ^{a} )</td>
<td>260.6 ± 11.7 (80.0)( ^{a} )</td>
<td>ND</td>
</tr>
<tr>
<td>H460/VinR</td>
<td></td>
<td>8.0 ± 0.8 (1.4)( ^{a} )</td>
<td>ND</td>
<td>513.3 ± 39.0 (213.9)( ^{a} )</td>
</tr>
<tr>
<td>NHFB</td>
<td></td>
<td>&gt;50</td>
<td>10.6 ± 4.8</td>
<td>5.9 ± 2.2</td>
</tr>
<tr>
<td>MSC</td>
<td></td>
<td>39.2 ± 4.3</td>
<td>3.9 ± 0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

\( ^{a} \)The relative resistant factors were calculated as follows: IC_{50} in resistant cells/IC_{50} in parental cells.

with MMPT at various concentrations, and cell viability was determined 72 h after the treatment. MMPT effectively inhibited growth of H1299, H460, H460/TaxR, H460/VinR, and H322 cells in a dose-dependent manner (Fig. 2). The IC_{50} values for these cells ranged from 4.9 to 8.0 \( \mu M \) (Table 1). In addition, the IC_{50} values at 48 h for H1299, H460, and H460/TaxR were 7.7, 7.8, and 7.2, respectively. Because p53 is the wild type in H460 cells but mutant or homologously deleted in H322 and H1299 cells, this result suggested that the action of MMPT is p53 independent. Moreover, the IC_{50} values of MMPT for parental H460 and P-glycoprotein-overexpressing H460/TaxR and H460/VinR cells were comparable, suggesting that MMPT-mediated cytotoxic effect is also P-glycoprotein independent.

We next determined the dose effect of cell killing by MMPT in NHFB and human bone marrow MSCs. The IC_{50} values in these two cell types were approximately 6 times higher than those observed in cancer cells. For comparison, IC_{50} values for paclitaxel in NHFBs and MSCs were 10.6 and 3.9 nM, respectively, similar to those observed in H1299, H460, and H322 cells (ranged from 2.9–6.3 nM). This result suggests that MMPT may have a larger therapeutic window than that of paclitaxel.

To further evaluate antitumor activity of the MMPT, we sent MMPT to the Developmental Therapeutics Program of the National Cancer Institute for testing its effect on a panel of cancer cells. A test on 42 cancer cell lines derived from leukemia, NSCLC, colon cancer, central nervous system (CNS) cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer by NCI’s Drug Discovery and Development Program showed that average concentration required to suppress GI_{50} after 48 h was 2.51 \( \mu M \). Twelve of the 42 cell lines had GI_{50} values lower than the average (Table 2), suggesting that MMPT may be effective for various other cancer cells.

**Induction of Apoptosis in NSCLC Cells by MMPT.** Numerous antineoplastic agents function by inducing apoptosis in cancer cells (Fisher, 1994). To assess the ability of MMPT to induce apoptosis, we examined the caspase activation and PARP cleavage in H460 cells treated with MMPT. Cells were treated with various concentration of MMPT for 48 h, and then cell lysates were subjected to 8 to 12.5% SDS-PAGE, followed by Western blotting with caspase and PARP antibodies. Cleavages of caspase-3, -8, -9, and PARP were easily detectable in H460 cells treated with 15 \( \mu M \) MMPT, a concentration close to the IC_{80} value (Fig. 3a).

Next, we determined the apoptotic cells by using FACs analysis. H1299 and H460 cells were treated with 15 \( \mu M \) MMPT for 12, 24, and 48 h. Cells were then harvested for quantification of apoptotic subdiploid cells by flow cytometry (Fig. 3b). At 48 h, 38% of H460 cells were in sub-G_{1} phase. The portion of sub-G_{1} cells in H460 cells was gradually increased in time-dependent manner; however, it reached a peak at 24 h in H1299 cells. In contrast, only background levels (less than 5%) of sub-G_{1} cells were seen in H1299 and H460 cells treated with solvent (data not shown).

We also tested apoptosis induction by MMPT in P-glycoprotein-overexpressing H460/TaxR and H460/VinR cells. For this purpose, H460, H460/TaxR, and H460/VinR cells were treated with 15 \( \mu M \) MMPT for indicated time periods and then activation of caspase-3, -8, and -9 was analyzed by Western blotting. Treatment with MMPT caused apparent cleavages of caspase-3, -8, and -9 in all three cell lines (Fig. 4a). Moreover, Western blotting for cytochrome c showed that treatment with MMPT resulted in release of cytochrome c to the cytosol in H460 and H460/TaxR cells after 24 h (Fig. 4b). These results clearly showed that MMPT could induce apoptosis in chemosensitive and chemoresistant cancer cells via caspase activation.

**JNK Activation Is Required for MMPT-Induced Apoptosis.** To further elucidate the mechanisms involved in MMPT-induced apoptosis, we tested MMPT-mediated activation of mitogen-activated protein kinases (MAPKs), including JNK, ERK, or p38, because many cellular stresses and stimuli induce apoptosis and modulate MAPK signaling pathways (Shtil et al., 1999; Stone and Chambers, 2000). Lysates of MMPT-treated cells were analyzed by Western blotting for MAPK phosphorylation, a hallmark of MAPK activation. At the indicated times following MMPT treatment, the protein levels of JNK, ERK, and p38 were not changed. However, activation of MAP kinases as determined...
by phosphorylation of these proteins was dramatically increased 24 h after MMPT treatment (Fig. 5a). It has been reported that JNK activation plays a critical role in induction of apoptosis by several anticancer drugs (Derijard et al., 1994; Xia et al., 1995; Zanke et al., 1996; Verheij et al., 1996). To examine whether the activation of JNK is required for MMPT-mediated apoptosis, H460 and H460/TaxR cells were exposed to MMPT for 24 or 48 h, and then cytosolic fractions were prepared. Lysates were subjected to 15% SDS-PAGE, followed by Western blotting with anti-cytochrome c antibody. Mitochondria fraction (M) was used as a positive control. COX-4 was used as a marker of mitochondrial proteins.

**Fig. 3.** Induction of apoptosis in NSCLC cells. a, caspase cleavage by MMPT in H460 cells. Cells were treated with MMPT at indicated concentrations for 48 h. Cleavage of caspase-3, -8, -9, and PARP was detected after 15 μM MMPT treatment. Arrowheads, cleaved proteins. b, percentage of cells in sub-G1 phase after treatment with 15 μM MMPT for the indicated times in H1299 and H460 cells. Values are means ± S.D. of three separate experiments.

**Fig. 4.** Apoptotic effects of MMPT on P-glycoprotein-overexpressing cells. a, caspase cleavage by MMPT in H460, H460/TaxR, and H460/VinR cells. Cells were treated with 15 μM MMPT for the indicated time periods. Cleavage of caspase-3, -8, and -9 were detected 24 or 48 h after MMPT treatment. Arrowheads, cleaved proteins. b, cytochrome c release from mitochondria in H460 and H460/TaxR cells. Cells were treated with 0 or 15 μM MMPT for 24 h, and then cytosolic fractions were prepared. Lysates were subjected to 15% SDS-PAGE, followed by Western blotting with anti-cytochrome c antibody. Mitochondria fraction (M) was used as a positive control. COX-4 was used as a marker of mitochondrial proteins.

Specific inhibitors for activation of ERK and p38 had no detectable effect in MMPT-induced apoptosis (Fig. 5d). These results suggested that MMPT-mediated JNK activation, but not ERK or p38, was required for MMPT-induced apoptosis.

**Antitumor Effect in Vivo.** To test in vivo effect of MMPT, we established subcutaneous H460 tumors in nude mice. When tumors reached 3 to 5 mm in diameter, mice were treated with three sequential i.p. injections of MMPT (40 mg/kg/injection), solvent, or paclitaxel (4 mg/kg/injection). Tumor volume was then monitored over time. In comparison with animals treated with solvent, tumor growth was significantly inhibited in mice treated with MMPT (p < 0.05) (Fig. 6). One week after the last treatment, the tumor volume in animals treated with solvent was 1435 ± 406 mm³ (n = 5), whereas the mean tumor volumes in animals treated with MMPT or paclitaxel were 755 ± 229 (n = 6) and 913 ± 424 (n = 5) mm³, respectively. We also monitored the changes of mouse body weight, red blood cells, white blood cells, platelets, serum alanine transaminase, aspartate transaminase, alkaline phosphatase, blood urea nitrogen, and creatinine levels before and after the treatment. No obvious change in body weight was found in all the animals among the groups. Blood cell counts and all the serum tests showed that the values were in normal ranges in all the animals tested. Together, these results indicated that MMPT effectively inhibits the growth of H460 human lung carcinoma models in vivo without noticeable acute toxicity.

**Discussion**

We have used paclitaxel-resistant and P-glycoprotein-overexpressing H460 cells to screening cytotoxic compounds effective for chemoresistant cancer cells. For each compound, 5 μg/ml was used in a primary screening. It is possible that
different concentrations will be required for different chemicals to elicit biological functions. However, for the screening test, using a fixed concentration is more practical. Most therapeutic agents also induce biological functions at concentrations around 1 to 10 \(\mu\)g/ml. Therefore, chemicals that cause dramatic cytotoxic effect at 5 \(\mu\)g/ml were used for further evaluation of dose-response effect. We also found that two to three rounds of screening of positive compounds are necessary to verify the initial screening results.

Our results showed that MMPT inhibited cell growth in various human NSCLC cell lines independent of P-glycoprotein and p53 status. Moreover, we sent the compound to the Drug Discovery and Development Program at the National Cancer Institute for test on cancer cell panels. The result showed that MMPT effectively suppressed growth of cancer cells of different origins. The average GI\(_{50}\) value for MMPT was 2.5 \(\mu\)M. According to the Standard AntiCancer Agent Database of the National Cancer Institute, this concentration is comparable with the mean GI\(_{50}\) value of chemotherapeutic agents cisplatin (2.0 \(\mu\)M) or 5-fluorouracil (20.0 \(\mu\)M). Interestingly, MMPT had little effect on cell growth of both NHFB and mesenchymal stem cells at the concentrations that were effective in NSCLC cells. We also compared the efficacy of MMPT with that of paclitaxel in NSCLC and normal cells in vitro. The range of IC\(_{50}\) values between NSCLC cells and normal cells are broader for MMPT than for paclitaxel. Although the mechanism by which the selectivity of MMPT action between normal cells and cancer cells remains unknown, these results suggest that MMPT could be a safe anticancer agent.

P-glycoprotein is known to be a major cause of chemoresistance to various drugs, including microtubule-interacting agents (Bosch and Croop, 1996; Smyth et al., 1998; Krishna and Mayer, 2000; Goldman, 2003). Our results show that MMPT can markedly inhibit cell growth in H460/TaxR and H460/VinR cells that have high P-glycoprotein expression. In addition, the testing result from the Drug Discovery and Development Program of the National Cancer Institute has shown that NCI/ADR-RES breast cancer cells are sensitive to MMPT. NCI/ADR-RES cells expressed high levels of P-glycoprotein and were resistant to several anticancer agents, including doxorubicin, paclitaxel, and docetaxel (Ogretmen and Safa, 1997; Chou et al., 1998; Naito et al., 2002). These results indicate that MMPT may not be a substrate of P-
glycoprotein and, thus, may be useful for treatment of P-glycoprotein-overexpressing multidrug-resistant cancers.

Cytochrome c release from mitochondria and caspase activation are often used as hallmarks of apoptosis induction by various chemotherapeutic agents (Panvichian et al., 1998; Oyaizu et al., 1999). Our results show that caspase-3, -8, and -9 were activated by MMPT in H460, H460/TaxR, and H460/VinR cells, indicating that MMPT induces cell death via caspase activation. However, the mechanism by which MMPT achieves these effects remains to be characterized. Although p53 plays an important role in induction of apoptosis, the results of our cytotoxicity assay demonstrated that MMPT was effective in p53-null H1299 cells, p53 mutant H322 cells, and p53 wild-type H460 cells, suggesting that MMPT-induced cell death of cancer cells is not correlated with p53 status, and a functional p53 is not required for this process.

Our data showed that MMPT-mediated JNK activation played an important role for MMPT-induced apoptosis. Recent reports have focused on the roles of JNK activation in the regulation of apoptosis when cells are exposed to DNA damage, chemotherapeutic agents, or cytokines (Kryriakis and Avruch, 1996; Verheij et al., 1996; Stadheim and Kucera, 2002). However, the molecular events involved in the death signal transduction via JNK activation are yet to be characterized. Our current data showed that MMPT-induced apoptosis, including cytochrome c release and caspase activation, was abrogated when cells were pretreated with the specific JNK inhibitor SP600125. This result is consistent with previous reports that activated JNK regulates phosphorylation of mitochondrial proteins during apoptosis and induces apoptosis through a mitochondrial pathway (Kroemer and Reed, 2000; Tournier et al., 2000; Chauhan et al., 2003) but is not sufficient to account for MMPT-mediated cytotoxic effect. Other cellular events or apoptosis signaling must be present in addition to JNK activation.

Our data also showed that MMPT suppressed the growth of subcutaneously inoculated H460 tumors, indicating that this compound is effective in vivo. Although IC50 for H460 in cultured cells was about 1000-fold different between MMPT and paclitaxel, the doses required for in vivo suppression of H460 tumors might be much closer for these two agents. At least, 40 mg/kg MMPT induced similar activity of 4 mg/kg paclitaxel. Moreover, no obvious acute toxicity was observed in animals treated with 40 mg/kg MMPT, suggesting that MMPT could be relatively safe. Due to limited supply of the compound, we are not able to determine long-term toxicity and maximum tolerable dose in vivo at this time. Nevertheless, the in vitro study on fibroblasts and mesenchymal stem cells suggested that this compound might have some selectivity against cancer cells, although the underlying mechanism is not yet clear. It is also possible that differences in uptake of the agent, metabolism, or cellular response in signal transductions may lead to selective cell killing observed in vitro. Additional studies on its molecular mechanism and on pharmacokinetic properties in vivo, including absorption, distribution, metabolism, excretion, and toxicity, will be required to before exploring its potential clinical applications.

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References


Address correspondence to: Dr. Bingliang Fang, Department of Thoracic and Cardiovascular Surgery, Unit 445, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. E-mail: bfang@mdanderson.org