MS-209, a Quinoline-type Reversal Agent, Potentiates Antitumor Efficacy of Docetaxel in Multidrug-resistant Solid Tumor Xenograft Models

Mikihiko Naito, Yasuhiro Matsuba, Shigeo Sato, Hiroshi Hirata, and Takashi Tsuruo


ABSTRACT

The existence of multidrug-resistant (MDR) cells in cancer is a major obstacle to effective cancer chemotherapy. Expression of P-glycoprotein (P-gp) in cancer cells causes resistance against paclitaxel and docetaxel, as well as against vincristine and doxorubicin (ADM). MS-209 is a novel MDR-reversal agent currently under clinical evaluation, which is shown to be active against ADM and vincristine resistance in MDR cancer cells in vitro and in vivo. In this paper, we report the combined effect of MS-209 with docetaxel in various MDR cancer cell lines that express P-gp. MS-209 at 3 μM effectively overcame docetaxel resistance in MDR cancer cells, and this concentration was achieved in blood plasma for > 7 h without serious toxicity. To study the effect of MS-209 in a clinically relevant model, we compared the antitumor efficacy of docetaxel alone with that of docetaxel combined with MS-209 at equitoxic doses in established solid tumor xenograft models. Treatment with docetaxel alone at the maximal tolerated dose (MTD) showed an apparent antitumor activity to an intrinsically resistant HCT-15 tumor xenograft, and MS-209 additionally potentiated the antitumor activity of docetaxel. Against a MCF-7/ADM tumor xenograft expressing larger amounts of P-gp, docetaxel alone at the MTD showed no antitumor activity, whereas the MTD of docetaxel combined with MS-209 greatly reduced MCF-7/ADM tumor growth. These results indicate that MS-209 could be a clinically useful drug to modulate MDR in docetaxel therapy.

INTRODUCTION

Chemotherapy is indispensable for cancer treatment, as is surgical excision and radiation therapy. However, the emergence of cancer cells resistant to chemotherapy often hampers treatment results. A major mechanism is MDR, which is caused by overexpression of a drug-efflux pump, such as P-gp, on the surface of cancer cells (1–3). In MDR cancer cells, the intracellular concentration of drugs is reduced because of the drug efflux pump.

To overcome MDR, enormous efforts have been made to find an inhibitor of the drug-efflux pump, and various compounds, such as verapamil, cyclosporin, quinidine, tamoxifen, progesterone, reserpine, and others have been reported to overcome MDR in vitro (4–9). However, most of these compounds have had disappointing results in animal studies because of their dose-limiting toxicity. Hence, a new reversal agent with a potent in vivo effect has been expected to develop.

We reported previously a series of quinoline derivatives that show MDR-reversing activity in K562/ADM, a human leukemia cell line that overexpresses P-gp (10). Among them, MS-209 (Fig. 1) was one of the most potent quinoline derivatives that can reverse MDR in vitro at a clinically achievable concentration (3 μM; when 600 mg of MS-209 was p.o. administered to human, maximum plasma concentration, time to reach maximum plasma concentration, and half life were 21.0 ± 4.4 μM, 0.75 ± 0.22 h, and 2.25 ± 0.47 h, respectively; n = 6). In addition, oral administration of MS-209 enhanced antitumor activity of ADM and vincristine in vivo (11, 12) and, given alone, did not show serious toxicity at doses up to 2000 mg/kg. Thus, MS-209 is a promising MDR reversal drug, and the compound is now under clinical evaluation in Japan (Phase III) and Europe (Phase I).

Docetaxel is a newly developed anticancer drug that stabilizes the microtubule and is clinically useful to treat breast and lung cancers. It was demonstrated to be a substrate of human P-gp (13), and one of the important mechanisms of docetaxel resistance is an overexpression of P-gp in cancer cells (14, 15).

In this paper, we report the reversal activity of MS-209 to docetaxel resistance in various MDR cancer cells expressing P-gp. Moreover, we demonstrated the usefulness of MS-209 in...
doxetaxel therapy against MDR cancers by comparing the anti-tumor effect at equitoxic doses of doxetaxel alone with the combination of doxetaxel and MS-209 in established solid tumor xenograft models.

MATERIALS AND METHODS

Chemical Agents. MS-209 was synthesized by Mitsui Chemicals Inc. (Tokyo, Japan) and kindly provided by Mitsui Pharmaceuticals Inc. (Tokyo, Japan). Doxetaxel was kindly provided by Rhône-Poulenc Rorer Japan Inc. (Tokyo, Japan).

For in vitro studies, MS-209 was dissolved in DMSO before diluting with culture medium (final concentration of DMSO was 0.1–0.01%). Doxetaxel was first dissolved in ethanol at 20 mg/ml (stored at 20°C) then diluted more than 25× with sterilized, distilled water. It was additionally diluted with culture medium to the desired concentration (final concentration of ethanol was ≤0.01%).

For in vivo experiments, MS-209 was suspended in sterilized, distilled water containing 0.1% Tween 80. Doxetaxel was first dissolved in ethanol, then an equal volume of polysorbate 80 was added, and the final dilution was obtained with 5% glucose in water (ethanol/polysorbate 80/5% glucose = 5/5/90; v/v/v).

Animals and Tumor Cell Lines. Female BALB/c-nu/nu mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were maintained under specific pathogen-free conditions. Seven-week-old mice were used for this study.

Human epidermal carcinoma KB-3-1 and its colchicine-resistant subline KBCh8-5-11 were provided by Dr. Ira Pastan, Cancer Chemotherapy Center, National Cancer Institute (Tokyo, Japan). Human breast cancer cell line MCF-7, human epidermal carcinoma KB-3-1, and human ovarian cancer cell line MCF-7/ADM were provided by Dr. Robert Ozols and Thomas Hamilton, Medicine Branch, NIH. Human non-small cell lung cancer cell line PC-14 and its doxetaxel-resistant cell line PC-14/TXT were provided by Dr. Nagahiro Saijo, Pharmacology Division, National Cancer Center Research Institute (Tokyo, Japan).

For in vitro studies, these cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.25% NaHCO₃, 2 mm l-glutamine, and 86 µg/ml kanamycin sulfate at 37°C in humidified air containing 5% CO₂. For in vivo experiments, tumor lump was passaged at the axillary region of BALB/c-nu/nu mice.

Growth Inhibition Assays. Cancer cells were seeded in 96-well plates at a density of 1500–3000 cells/well in 100 µl of culture medium. After a 24-h culture, 50 µl of MS-209 solution and 50 µl of doxetaxel solution were sequentially added and incubated for an additional 72 h. Cell growth was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (16). Growth rate was expressed as the ratio of absorbance of the drug-treated wells to that of control wells, and concentrations of doxetaxel that provided IC₅₀ were determined.

Semiquantitative RT-PCR. Total cellular RNA was extracted from the above 13 cell lines with a RNeasy Mini kit (Qiagen). RT-PCR for MDR1 was conducted using the forward primer 5’-AAGCGAAGCAGTGGTCCAGG-3’ and the reverse primer 5’-ACCAACTCATCACCTGCTG-3’ with One Step RNA PCR kit (TaKaRa Shuzo, Tokyo, Japan). The primers for β-actin were 5’-GATGAGCAGCCGAGGAGG-3’ (forward) and 5’-AGGCGTACAGGGACAGCACA-3’ (reverse). The thermal cycles were as follows: (a) 1 cycle of 50°C, 30 min; 95°C, 5 min; (b) 21 cycles of 95°C, 20 s; 60°C, 1 min; and (c) hold at 4°C. The PCR products were then run at 100 V on a 2% agarose gel and visualized by ethidium bromide staining. The MDR1 bands were quantitated using Scion Image software (Scion Corp.) and normalized with that of β-actin control.

Western Blot Analysis. Cells were washed twice with ice-cold PBS(−), dissolved in PBS(−) containing 0.5% Triton (v/v), and centrifuged at 15,000 rpm (radius 6 cm) for 5 min at 4°C to remove insoluble fraction. The supernatant was used as cell lysate. Aliquots of cell lysate (30 µg of protein) were electrophoresed on an SDS-polyacrylamide gradient gel (4–20%) and then electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was probed with an anti-P-gp monoclonal antibody, C-219 (Centocor, Inc.), at a dilution of 1:50 (v/v), and horseradish peroxidase-conjugated antimouse immunoglobulin (Amersham) was used as the second antibody. Expression of human P-gp was visualized using an enhanced chemiluminescence Western Blotting kit (Amersham). The intensity of each band was quantified using Scion Image software (Scion Corp.).

Cell Cycle Analysis. K562, K562/VCR, and K562/ADM cells (20 × 10⁵ cells/20 ml) were treated with 0, 10 ng/ml of doxetaxel in the absence or presence (1, 10 µM) of MS-209. After 8 h, aliquot of the cell suspension (6 ml) was collected, washed twice with chilled PBS(−), and fixed in 70% ethanol. Then the cells were washed twice with chilled PBS(−) again, treated with RNase A (1 mg/ml) at 37°C for 30 min, and stained with propidium iodide (50 µg/ml). DNA content in the cells was analyzed using a flow cytometer FACScan (Becton Dickinson).

Determination of Plasma Level of MS-209 and Doxetaxel. MS-209 at 100 mg/kg (in 0.1% Tween 80) were p.o. administered into female SLC-CDF1 mice (7 weeks old). After 1, 4, and 7 h, 1 ml of blood was drawn from the heart (n = 3) with a heparinized syringe and centrifuged to obtain the plasma. MS-209 was extracted by 8 ml of chloroform/m-hexane (9:1, v/v) after adding 1 ml of 0.1 N NaOH to 0.5 ml of the plasma samples. The organic phase was dried with N₂ gas, and the residue dissolved in the mobile phase consisted of methanol/50 mM ammonium phosphate buffer (pH 7.0; 70:30, v/v). MS-209 was separated from its me-
Table 1  Effects of MS-209 on cytotoxicity of docetaxel in parental cells and MDR sublines overexpressing Pgp

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>IC$_{50}$ (ng/ml) of docetaxel</th>
<th>Magnitude of resistance</th>
<th>IC$_{50}$ (ng/ml) of docetaxel in the presence of MS-209</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS 1 μM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>breast</td>
<td>2.3</td>
<td>—</td>
<td>2.1 (1.1)</td>
</tr>
<tr>
<td>MCF-7/ADM</td>
<td></td>
<td>320</td>
<td>140</td>
<td>10 (33)</td>
</tr>
<tr>
<td>PC-14</td>
<td>non-small cell lung</td>
<td>6.3</td>
<td>—</td>
<td>5.0 (1.3)</td>
</tr>
<tr>
<td>PC-14/TXT</td>
<td></td>
<td>9.0</td>
<td>1.4</td>
<td>2.9 (3.1)</td>
</tr>
<tr>
<td>HCT-15</td>
<td>colon</td>
<td>51</td>
<td>—</td>
<td>2.2 (24)</td>
</tr>
<tr>
<td>A2780</td>
<td>ovarian</td>
<td>2.8</td>
<td>—</td>
<td>2.7 (1.0)</td>
</tr>
<tr>
<td>2780AD</td>
<td></td>
<td>270</td>
<td>96</td>
<td>7.7 (35)</td>
</tr>
<tr>
<td>KB-3-1</td>
<td>oral epidermoid</td>
<td>1.0</td>
<td>—</td>
<td>0.98 (1.0)</td>
</tr>
<tr>
<td>KB/MDR</td>
<td></td>
<td>5.0</td>
<td>5.0</td>
<td>0.38 (13)</td>
</tr>
<tr>
<td>KBCh8-5-11</td>
<td></td>
<td>110</td>
<td>110</td>
<td>1.7 (65)</td>
</tr>
<tr>
<td>K562</td>
<td>leukemia</td>
<td>1.6</td>
<td>—</td>
<td>1.6 (1.0)</td>
</tr>
<tr>
<td>K562/ADM</td>
<td></td>
<td>740</td>
<td>460</td>
<td>27 (27)</td>
</tr>
<tr>
<td>K562/VCR</td>
<td></td>
<td>130</td>
<td>81</td>
<td>4.5 (29)</td>
</tr>
</tbody>
</table>

Tabolites in HPLC equipped with a reversed phase column C8 (YMC-Pack A-212; 150 × 6 mm, 5 μm; YMC Inc.). The flow rate was 1 ml/min, and the effluent was monitored by UV detector at 242 nm. The concentration of MS-209 in each sample was calculated from the area ratio of the UV absorbance to that of docetaxel. Plasma samples were prepared as above. Docetaxel was extracted with acetonitrile-butylchroride (1:4, v/v) using Extrelut (MULTI RUNGE; Ref. 18). The AUC was estimated by fitting the data using a non-linear least-squares program (MULTI RUNGE; Ref. 18).

Evaluation of Antitumor Activity in Vivo. HCT-15 and MCF-7/ADM were used as representative MDR cells to evaluate reversing activity of MS-209 against docetaxel resistance in vivo. Cells were inoculated s.c. into the flank of nude mice and maintained as s.c. tumors. Tumor fragments (3 × 3 × 3 mm) were inoculated s.c., and when tumors reached 50–200 mm$^3$ in volume, animals were divided randomly into test groups consisting of six mice per group (day 0). Drugs were administered on days 0, 4, and 8. Length and width of the tumors were measured twice a week up to day 30, and the TV was calculated according to the following formula: TV = (length × width$^2$)/2. The TV was calculated as: RTV = TV$_n$/TV$_0$, where TV$_0$ is the TV at day n. Body weight of the mice was measured twice each week up to day 30 to monitor the toxic effects. Reversing activity of MS-209 was evaluated by comparing the antitumor efficacy of combination treatment of docetaxel and MS-209 with that of docetaxel treatment alone in each established tumor model. Statistical analysis was performed using Student’s t test or Aspin-Welch t test after dispersal analysis using F test, comparing the mean RTV of the combined treatment group with that of docetaxel alone group.

RESULTS

Overcoming Docetaxel Resistance by MS-209 in vitro. We first studied sensitivities to docetaxel in several human P-gp-mediated MDR cancer cells and then evaluated the reversing activities of MS-209 against docetaxel resistance in vitro. The concentrations of docetaxel required for an IC$_{50}$ of the MDR cells (5–740 ng/ml) were higher than those of the corresponding parental cell lines (1–6.3 ng/ml) when MS-209 was absent (Table 1). Cotreatment with MS-209 led to significant decrease in IC$_{50}$ of docetaxel in all of the MDR cells, in an MS-209 dose-dependent manner. When > 3 μM of MS-209 was present, the IC$_{50}$ values of docetaxel against the MDR cells became almost the same as those against the parental cells. These results indicate that P-gp-mediated MDR cancer cells show resistance to docetaxel and that 3 μM of MS-209 can completely reverse docetaxel resistance in vitro.

Correlation between P-gp Expression and Reversal of Docetaxel Resistance by MS-209. Expression levels of MDR1 gene and P-gp in MDR cells were examined using RT-PCR and Western blot analysis (Fig. 2, a and b, respectively). Then, we analyzed the correlation between the level of MDR1 mRNA expression and magnitudes of docetaxel resistance in these cells. As shown in Fig. 3a, we observed a good correlation between them. We additionally analyzed the correlation between the mRNA level and the extents of anticancer-enhancing efficacy by MS-209 and found a strong correlation (r = 0.961) between them (Fig. 3b). Consistently, P-gp level also correlated well (r = 0.900) with the efficacy of MS-209 (Fig. 3c). These results indicate that the expression of MDR1/P-gp is a crucial factor for MS-209 to potentiate...
cytotoxicity of docetaxel as evidenced in studies using ADM or vincristine (19).

**Cell Cycle Arrest by Docetaxel and MS-209 in MDR Cells.** MS-209 is considered to block P-gp-mediated docetaxel efflux and thereby increase intracellular docetaxel in the MDR cells. To confirm this hypothesis, we investigated docetaxel-induced G2-M arrest in MDR cells, K562/ADM and K562/VCR, in the presence or absence of MS-209. Without MS-209, 10 ng/ml of docetaxel could not induce G2-M arrest in these resistant cells, whereas their parental K562 cell was effectively arrested at G2-M phase (Fig. 4, third row). When 1 μM of MS-209 was cotreated, docetaxel at 10 ng/ml arrested the moderately resistant K562/VCR at G2-M but not the highly resistant K562/ADM expressing a higher amount of P-gp (Fig. 4, fourth row). MS-209 at 10 μM led to the docetaxel-induced G2-M arrest in all of the cell lines (Fig. 4, fifth row). These results indicate that MS-209 increases cellular accumulation of docetaxel by inhibiting P-gp-mediated docetaxel efflux from MDR cancer cells.

**Plasma Concentration of MS-209 after Oral Administration to Mouse.** Plasma concentration of MS-209 was determined after oral administration of 100 mg/kg of MS-209 in mouse. The highest value (18.8 ± 1.40 μM) was attained 1 h after the administration, and the concentration at 7 h after the administration was 5.91 ± 3.03 μM (Table 2). The result indicates that MS-209 at 3 μM, the effective concentration to reverse MDR in vitro, can be maintained for >7 h in plasma by oral administration at 100 mg/kg to mouse.

**Effect of MS-209 on Antitumor Activity of Docetaxel against Established Tumors Overexpressing P-gp.** To examine whether MS-209 enhances antitumor activity of docetaxel in a clinically relevant model, we evaluated antitumor activity of docetaxel alone and that of combination therapy of docetaxel and MS-209 against established human solid tumors transplanted into nude mice. We first measured the MTD of docetaxel alone and docetaxel combined with MS-209 in a dose-escalation study stepped by 1.5-fold. The MTD of docetaxel alone (three injections at 4-day intervals; q4d) was 22 mg/kg and that of docetaxel combined with MS-209 (200 mg/kg; q4d × 3) was 15 mg/kg. At these doses, mice showed a similar body weight change (Fig. 5, right panel), suggesting that these treatments were equitoxic to mice.

In an HCT-15 (colorectal adenocarcinoma)-bearing mouse model, q4d × 3 treatment of 15 mg/kg of docetaxel alone showed no apparent effect on the tumor growth (Fig. 5, top panel). In contrast, oral administration of 200 mg/kg of MS-209 30-min before injection of 15 mg/kg of docetaxel produced
significant tumor growth inhibition. The antitumor efficacy of the combination treatment at MTD was also superior to that of docetaxel alone at MTD (22/11003 mg/kg), whereas mice in both treatment groups displayed similar body weight change. The activity of oral administration of MS-209 was more clearly demonstrated in MCF-7/ADM (breast carcinoma)-bearing mice, where treatment of docetaxel alone at MTD displayed no anti-tumor efficacy (Fig. 5, bottom panel). In this model, there was statistical significance between the combination therapy and the single therapy groups at MTD.

We next examined the plasma concentration of docetaxel in mice. Although administration of docetaxel alone or docetaxel with MS-209 at MTD gave the similar body weight change (Fig. 5, right panels), docetaxel with MS-209 showed a significantly higher AUC than did docetaxel alone (Fig. 6). These findings indicate that P-gp overexpression confers docetaxel resistance to cancer cells in vivo, and coadministration of MS-209 potentiates the antitumor activity of docetaxel to MDR tumors by enhancing AUC of docetaxel and by inhibiting P-gp on the resistant tumor cells.

DISCUSSION

In this preclinical study, we evaluated MS-209, a MDR reversal agent, as a concomitant medication in docetaxel therapy using human MDR cancer cells for in vitro study and MDR tumor-bearing nude mouse models for in vivo study. Docetaxel is one of the most promising anticancer drugs in recent years. Enormous numbers of clinical trials of docetaxel-containing regimens have been conducted, and many excellent outcomes have been reported (20–22). For instance, the overall response rate was >50% of patients with advanced/recurrent breast cancer treated with docetaxel alone (23–27). Furthermore, docetaxel in combination with anthracyclines was demonstrated to be superior to a current, standard anthracycline-containing combination therapy for metastatic breast cancer (21). Thus, docetaxel could be a key drug in the next standard combination therapy. On the other hand, docetaxel is transported by P-gp, and the major mechanism of docetaxel resistance in cancer cells is the overexpression of P-gp (28). Therefore, it is considered

Table 2  Plasma concentrations of MS-209 after oral administration of 100 mg/kg to mice*  

<table>
<thead>
<tr>
<th>Time after administration (hr)</th>
<th>1</th>
<th>4</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-209 concentration in plasma (μM)</td>
<td>18.8 ± 1.40</td>
<td>7.81 ± 9.91</td>
<td>5.91 ± 3.03</td>
</tr>
</tbody>
</table>

* Mean ± SD, n = 3.
to docetaxel was observed. MS-209 at 3 μM/H9262 gene was expressed in cancer cells, the more resistance
MDR1, mediated docetaxel resistance. As shown in Fig. 3, that a potent P-gp inhibitor, such as MS-209, may improve the
clinical response of docetaxel-containing therapy in some patients.

We first studied the in vitro effect of MS-209 on P-gp-mediated docetaxel resistance. As shown in Fig. 3a, the more MDR1 gene was expressed in cancer cells, the more resistance to docetaxel was observed. MS-209 at 3 μM was shown to overcome the docetaxel resistance completely in vitro (Table 1), and this concentration could be maintained for at least 7 h when 100 mg/kg of MS-209 was administered p.o. into mice (Table 2). In the therapeutic experiments, we administered 200 mg/kg MS-209 because: (a) portions of MS-209 may bind to plasma proteins, which reduce the active MS-209 concentration in plasma; and (b) no serious toxic effect was observed by this dose of MS-209.

We used established tumor models to demonstrate potential activity of MS-209 in a clinically relevant model. MS-209 enhanced antitumor activity of docetaxel against intrinsic MDR cancer HCT-15, suggesting that MS-209 could be a useful drug against intrinsic resistant tumors in patients. In the MCF-7/ADM model, statistical significance was observed between the efficacy of the combination and that of docetaxel alone at MTD. At these doses, similar body weight change was observed, suggesting they were equitoxic, whereas stronger antitumor activity was demonstrated by the combination with MS-209. This could be explained by the enhanced AUC of docetaxel and by the inhibition of P-gp on the resistant tumor cells by MS-209. At present, we do not know why the enhanced AUC does not result in the higher toxicity in mice (at least in body weight change). Additional toxicological study on docetaxel and MS-209 combination therapy is needed.

MS-209 directly inhibits the function of P-gp and, consequently, raises the intracellular concentration of docetaxel as demonstrated by increase in mitotic arrest in MDR cells (Fig. 4). In fact, MS-209 inhibited azidopine photolabeling of P-gp efficiently and increased the accumulation of ADM and vincristine in P-gp-overexpressing cancer cells (12, 29). MS-209 can also inhibit MRP1-mediated drug efflux and exhibit significant MDR-reversing efficacy in MRPI-overexpressing cells (30, 31). It is desirable that a reversal agent inhibits both P-gp and MRPI, which are main drug-efflux pumps in MDR. Because there are some substrates that are recognized by both P-gp and MRPI (e.g., ADM and vincristine), it is no wonder that MS-209 inhibits both functions of these pumps. At present, six MRP proteins are reported to be transporters (32). We are interested in whether MS-209 inhibits the functions of other ATP-binding cassette transporters.

ACKNOWLEDGMENTS
We thank Drs. Takao Yamori and Yoshikazu Sugimoto (Japanese Foundation for Cancer Research, Tokyo, Japan) for helpful advice, and Ms. Tomoko Oh-hara (Japanese Foundation for Cancer Research, Tokyo, Japan) for assistance in pharmacokinetic analysis in this work. We also thank Drs. Nagahiro Saijo and Kazuto Nishio (National Cancer Center, Tokyo, Japan) for providing PC-14/TXT, and Rhone-Poulenc Rorer Japan Inc. (Tokyo, Japan) for providing docetaxel.

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