HMN-176, an Active Metabolite of the Synthetic Antitumor Agent HMN-214, Restores Chemosensitivity to Multidrug-Resistant Cells by Targeting the Transcription Factor NF-Y

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ABSTRACT

HMN-176 ((E)-4-[[2-N-[4-methoxybenzenesulfonyl]amino]stilbazole]-1-oxide) is an active metabolite of HMN-214 ((E)-4-[[2-(N-acetyl-N-[4-methoxybenzenesulfonyl]amino]stilbazole]-1-oxide), which has a potent antitumor activity in mouse xenograft models. In this study, we show that HMN-176 circumvents multidrug resistance in a K2 human ovarian cancer cell line selected for Adriamycin resistance (K2/ARS). Upon treatment of K2/ARS cells with 3 μM HMN-176, the GI50 of Adriamycin for the cells decreased by ~50%. To explore the molecular mechanism of this effect, we assessed the expression of the multidrug resistance gene (MDR1), which is constitutive in K2/ARS cells, at both the protein and the mRNA level. Western and reverse transcription-PCR analysis revealed that the expression of MDR1 was significantly suppressed by treatment with HMN-176. Furthermore, when administered p.o., HMN-214 suppressed the expression of MDR1 mRNA in a mouse xenograft model implanted with KB-A.1, an Adriamycin-resistant cell line. Luciferase reporter fusion gene analysis demonstrated that HMN-176 inhibited the Y-box-dependent promoter activity of the MDR1 gene in a dose-dependent manner. Moreover, we show by electrophoretic mobility shift assay that HMN-176 inhibits the binding of NF-Y, which is thought to be an essential factor for the basal expression of MDR1, to its target Y-box consensus sequence in the MDR-1 promoter. Inhibition of MDR-1 expression was achieved with pharmacological concentrations of HMN-176, suggesting that HMN-176 may act by two different mechanisms—cytotoxicity and MDR1 down-regulation—simultaneously. The data presented strongly suggest that the antitumor mechanism of HMN-176 (or its prodrug HMN-214) is quite different from those of known antitumor agents.

INTRODUCTION

Resistance to chemotherapy is a major problem in the management of malignant tumors (1). Simultaneous resistance to several types of commonly used antitumor agents is known as MDR.2 Amplification of the MDR1 gene associated with the increased expression of P-gp is frequently observed in cell lines selected in vitro by resistance to antitumor agents, although studies in humans using tumor samples and normal tissue have demonstrated increased P-gp expression without gene amplification (2–4). The MDR phenotype may also be induced by other mechanisms (5). However, transfection of the MDR1 gene into mammalian cells results in the full MDR phenotype, and the MDR phenotype consists of A, B, and C subunits (17, 18). Tax, a viral transactivator encoded by human T-cell leukemia virus type I, has also been shown to activate the transcription of MDR1 through direct interaction with NF-YB, the B subunit of NF-Y (19). This observation may have important implications for the chemotherapy of refractory virus-associated malignancies. Thus, NF-Y plays a pivotal role in the regulation of MDR1 gene expression, and it is thought to be a good molecular target for manipulating the MDR phenotype.

Trials of patients with ovarian cancer, whereas ET-743 is being developed as an antitumor agent rather than a chemosensitizer. MDR1 promoters contain putative Sp1 binding sequences and CCAAT elements (Y-boxes). The Y-box especially is absolutely required for the basal expression of human MDR1 (15, 16). This sequence is recognized by the nuclear protein NF-Y complex, which consists of A, B, and C subunits (17, 18). Tax, a viral transactivator encoded by human T-cell leukemia virus type I, has also been shown to activate the transcription of MDR1 through direct interaction with NF-YB, the B subunit of NF-Y (19). This observation may have important implications for the chemotherapy of refractory virus-associated malignancies. Thus, NF-Y plays a pivotal role in the regulation of MDR1 expression, and it is thought to be a good molecular target for manipulating the MDR phenotype.

HMN-176 ((E)-4-[[2-N-[4-methoxybenzenesulfonyl]amino]stilbazole]-1-oxide) is an active metabolite of the synthetic antitumor compound HMN-214 ((E)-4-[[2-(N-acetyl-N-[4-methoxybenzenesulfonyl]amino)stilbazole]-1-oxide). HMN-176 shows potent cytotoxicity toward various human tumor cell lines, and in mitotic cells, it causes cell cycle arrest at M phase through the destruction of spindle polar bodies, followed by the induction of DNA fragmentation. However, no direct interactions of HMN-176 with tubulin are observed (2). Moreover, in animal models, we have observed that oral administration of the prodrug HMN-214 caused no significant nerve toxicity, a severe side effect often associated with microtubule binding agents such as Taxol and VCR. In Phase I clinical trials, HMN-214 has caused sensory neuropathy and ileus in some patients. However, the grade and frequency of these adverse effects were much lower than those of typical microtubule binding agents. As expected from the mechanism of action of HMN-214 (induction of G2-M arrest in dividing cells), the main adverse effect was neutropenia.4 Although the exact molecular mechanism of HMN-176 remains to be elucidated, our recent in vitro observations have suggested the involvement of polo-like kinase in HMN-176-induced cell cycle arrest. HMN-176 does not inhibit polo-like kinase; instead, it alters its involvement of polo-like kinase in HMN-176-induced cell cycle arrest. HMN-176 shows potent cytotoxicity toward various human tumor cell lines, and in mitotic cells, it causes cell cycle arrest at M phase through the destruction of spindle polar bodies, followed by the induction of DNA fragmentation. However, no direct interactions of HMN-176 with tubulin are observed (2). Moreover, in animal models, we have observed that oral administration of the prodrug HMN-214 caused no significant nerve toxicity, a severe side effect often associated with microtubule binding agents such as Taxol and VCR. In Phase I clinical trials, HMN-214 has caused sensory neuropathy and ileus in some patients. However, the grade and frequency of these adverse effects were much lower than those of typical microtubule binding agents. As expected from the mechanism of action of HMN-214 (induction of G2-M arrest in dividing cells), the main adverse effect was neutropenia.4 Although the exact molecular mechanism of HMN-176 remains to be elucidated, our recent in vitro observations have suggested the involvement of polo-like kinase in HMN-176-induced cell cycle arrest. HMN-176 does not inhibit polo-like kinase; instead, it alters its spatial distribution. This change may keep the maturation promoting factor complex inactive, resulting in cell cycle arrest at G2-M (unpublished data).

We have previously reported that HMN-154, which is closely related to HMN176, interacts with NF-YB and thereby interrupts the binding of the NF-Y heterotrimer to DNA (20). These results strongly suggest that HMN-176 can inhibit the expression of MDR1 at the transcriptional level by inhibiting the activity of NF-Y. The main purpose of this study was to examine the inhibitory effect of HMN-176 on the expression of MDR1 in multidrug-resistant cells and the resultant restoration of sensitivity to Adriamycin.

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2 The abbreviations used are: MDR, multidrug resistance; RT-PCR, reverse transcription-PCR; EMSA, electrophoretic mobility shift assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; P-gp, P-glycoprotein; glyceraldehyde-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VCR, vincristine.

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MATERIALS AND METHODS

Drugs. The chemical structures of HMN-176 and HMN-214 are shown in Fig. 1. HMN-176 (A) was dissolved in DMSO to a concentration of 10 mM and stored at 4°C in the dark. Before each experiment, the stock solution was diluted with PBS to the appropriate concentration. Suspensions of HMN-214 (B), a prodrug of HMN-176 for oral administration, was prepared as follows: HMN-214 was placed in an agate mortar and finely ground with an agate pestle. The ground HMN-214 was suspended with an agate pestle by gradually adding 0.5% methylcellulose 4000 solution to make a 3 mg/ml suspension. This was additionally diluted with methylcellulose 4000 solution to obtain suspensions of the appropriate concentration. Adriamycin was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and dissolved in saline for injection.

Cell Culture. The K2 and K2/ARS human ovarian cancer cell lines were kindly gifted from Dr. Osamu Maeda, Kariya General Hospital (Kariya, Japan) and the KB-A1 cell line was a kind gift from Dr. Kazumitsu Ueda, Faculty of Agriculture (Kyoto University). HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). K2 human ovarian cancer cells, HeLa and KB (HeLa-origin) cells, were maintained in DMEM supplemented with 10% fetal bovine serum. For the maintenance of the multidrug-resistant K2/ARS and KB-A1 cells, 2 μM Adriamycin were also added to the medium.

Tumor Transplantation and Drug Administration. Tumor tissue was grown in advance by s.c. transplantation into nude mice. The resulting tumors were removed, cut into cubic fragments of grown in advance by s.c. transplantation into nude mice. The resulting tumors were cut into cubic fragments of 3 mm in size and suspended in PBS to a concentration of 105 cells/ml. Adriamycin was also added to the medium. Adriamycin was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

DNA Transfection and Analysis of Transient Transfections. Transient transfection of vector DNA into HeLa cells was accomplished by the lipofection method according to the manufacturer’s recommendations (Life Technologies, Inc.). The MDR-Luc construction contains 150 bp of genomic sequence from MDR1, upstream of the transcription initiation site (positions −99 to +50), in the reporter plasmid vector pGOL-3 (Promega). This vector (2 μg) was cotransfected with 40 ng of pRL-SV40, an internal control vector. The cells were allowed to recover for 24 h before being treated for 48 h with various concentrations of HMN-176. Cell extracts were prepared according to the manufacturer’s directions, and the reaction was initiated by the addition of 100 μl of luciferase assay reagent. The luciferase activity was determined for each sample by integration of the luminescence signals over a 20-s period with a Berthold Lumat LB 9507 luminometer.

Enhanced Cytotoxicity of Adriamycin in the Presence of HMN-176. K2/ARS cells were seeded into a 96-well microplate at a density of 1.5 × 104 cells/well. After incubation at 37°C and 5% CO2 for 24 h, HMN-176 was added at the indicated concentrations and incubation continued for an additional 48 h. The HMN-176 was then washed out by replacing the medium three times and Adriamycin added at the indicated concentrations. The cells were additionally incubated for 72 h at 37°C, and inhibition of growth was measured by MTT assay (22). The absorbance at 595 nm after 72-h treatment was calculated by setting the absorbance at zero time to 100%. Gln50 was calculated by the Scansoft 96 software program (Dainippon Pharmaceutical Co., Osaka, Japan).

RESULTS

Cross-Resistance Pattern of K2/ARS Cells. The Gln50, deduced from dose-response curves after a 72-h exposure are shown in Table 1. A 790-fold increase in the Adriamycin concentration was required to cause a 50% reduction in the growth of resistant cells compared with the sensitive parent K2 cells. Much higher degrees of cross-resistance were observed for Taxol and VCR (1600- and 1800-fold, respectively). A much lower degree of cross-resistance of K2/ARS cells to HMN-176 was observed (14.3-fold).

MDR1 Gene Expression in K2 and K2/ARS Cells. mRNA expression of the MDR1 gene in Adriamycin-sensitive parent K2 and Adriamycin-resistant K2/ARS cells was examined by RT-PCR. In parent K2 cells, we could hardly detect MDR1 mRNA, whereas significant levels of MDR1 mRNA were detected in K2/ARS cells (Fig. 2). Furthermore, Western blotting revealed the presence of MDR1 on K2/ARS cells but not on the parent K2 cells (Fig. 3). Thus, the multidrug-resistant phenotype of K2/ARS cells was associated with the overexpression of MDR1.

Effect of HMN-176 on Expression of MDR1 in Vivo and in Vitro. K2 and K2/ARS cells were treated with 1 or 3 μM HMN-176 for 48 h and then the amount of MDR1 mRNA was determined by RT-PCR. Treatment with 3 μM HMN-176 suppressed the expression of MDR1 mRNA by 56% (Fig. 2) and also reduced the expression of MDR1 protein as detected by Western blotting (Fig. 3). Treatment with 1 μM HMN-176 had no significant effect on the expression of MDR1 mRNA or protein.

Because K2 and K2/ARS cells failed to grow after transplantation into nude mice, we used KB and its multidrug-resistant subline KB-A1 (23) as the transplants. For in vivo experiments, we used HMN-214, an acetylated derivative HMN-176 (Fig. 1). HMN-214 is easily absorbed when p.o. administered and is readily hydrolyzed to HMN-176 in serum (data not shown). HMN-214 was p.o. administered at various doses from days 1 to 28. On day 29, tumors were isolated from animals bearing KB and KB-A1 cells in the HMN-214-treated and untreated groups. Total RNA was extracted from the...
tumor samples, and the amount of MDR1 mRNA was determined by RT-PCR. The density of the bands was assessed, and the MDR1/GAPDH ratios were calculated. The expression of MDR1 mRNA was below the detection limit in the KB strain (Fig. 4); in contrast, high levels of MDR1 mRNA were detected in KB-A.1 cells, and these levels significantly decreased upon treatment with HMN-214.

**Effect of HMN-176 on NF-Y Activity.** We examined the effect of HMN-176 on the binding of NF-Y to the MDR1 Y-box (the CCAAT core sequence) by EMSA. HMN-176 inhibited complex formation on the Y-box in a dose-dependent manner (Fig. 5B). Antibodies against both NF-YA and NF-YB produced supershifted bands, suggesting that this DNA-protein complex was NF-Y. To verify the inhibition of MDR1 promoter activity, we performed a reporter assay in the presence of HMN-176. We chose to evaluate human MDR1 promoter function in human tumor cells (HeLa), rather than drug-resistant cell lines selected in vitro because these cells conform more closely to cells from clinical isolates. Upon treatment with HMN-176, luciferase expression driven by the MDR1 promoter was suppressed in a dose-dependent manner (Fig. 5B). At a concentration of 300 nM HMN-176, ~40% inhibition of promoter activity was achieved in this system. We could not examine the effect of HMN-176 at higher concentrations because of its cytotoxicity against HeLa cells. Deletion of the Y-box (nucleotides -82 to -73) resulted in the loss of >85% of the promoter activity, and HMN-176 had no significant effect on the residual promoter activity (data not shown).

**Sensitization of K2/ARS Cells to Adriamycin by HMN-176 in Vitro.** Having demonstrated that HMN-176 inhibited the expression of MDR1 on multidrug-resistant K2/ARS cells, we next
assessed the ability of HMN-176 to sensitize these cells to Adriamycin. The addition of Adriamycin after HMN-176 pretreatment suppressed growth at all concentrations, whereas Adriamycin without HMN-176 pretreatment had no effect (Fig. 6). A ~40% reduction in the GI50 for Adriamycin was achieved by pretreatment with 3 μM HMN-176.

**DISCUSSION**

In this study, we demonstrate that HMN-176, a synthetic antitumor agent, at least in an in vitro model, circumvents the MDR phenotype by down-regulating the expression of MDR1. K2/ARS cells showed a dramatic increase in the GI50 of all antitumor agents tested compared with the sensitive parent-cell, except for HMN-176 (Table 1). Because Taxol is known to be involved in the typical multidrug phenotype associated with MDR1 overexpression (24), K2/ARS cells appear to have a classical MDR phenotype. We detected >50% reduction in MDR1 mRNA expression in K2/ARS cells after treatment with 3 μM HMN-176 for 48 h. Although a longer exposure may have a greater effect, we could not test this possibility in our experimental system because of the exposure-time-dependent cytotoxicity of HMN-176. The doses used in this study were within the range achieved in the plasma of patients undergoing Phase I clinical trials, which we know to be well tolerated. Cytotoxicity and MDR1 down-regulation are, therefore, concomitant effects at pharmacological concentrations, but the molecular targets mediating these effects seem to be different.

We have also shown that down-regulation of MDR1 can be attributed to the inhibition of NF-Y activity by HMN-176. Mutations and/or deletions that abolish NF-Y binding to the Y-box have been shown to result in a significant loss of MDR1 promoter activity (16), which indicates that NF-Y regulates the basal and inducible expression of MDR1. The Y-box, on which NF-Y acts, binds NF-Y even before induction (13, 14). In our experiments, the inhibition of DNA binding was detected when HMN-176 was preincubated with HeLa nuclear extract, which suggests that the affinity between HMN-176 and NF-Y is not so high as to displace the preexisting NF-Y complex from the high-affinity Y-boxes. Therefore we consider that in our experimental systems, NF-Y newly synthesized during physiological turnover might be a target of HMN-176. Accordingly, we could not detect a significant decrease in MDR1 mRNA or protein expression when...
K2/ARS cells were exposed to 3 \( \mu \)M HMN-176 for periods shorter than 48 h (data not shown).

Scotto et al. (25) and Abolhoda et al. (26) reported that in some metastatic sarcomas, tumor MDR1 mRNA levels increase after exposure to the DNA-damaging agent Adriamycin. They have additionally shown that the transcriptional activation of MDR-1 is mediated by NF-Y.5 The response of cancer cells to continuous or repetitive multiple stress is thought to be a crucial step in the acquisition of the MDR phenotype. However, as shown in our studies, HNM-176 treatment induced no significant up-regulation of MDR1 in either parent or drug-resistant K2 cells. Additional work is necessary to find out whether HNM-176, as well as ET-743, interferes with the acute activation of MDR1 expression.

We tested the cross-resistance of other K2 sublines selected for by cisplatin, Adriamycin, camptothecin, and Taxol. In all cases, much lower degrees of cross-resistance were observed for HNM-176 than for the other agents tested. Because it seems unlikely that resistance to these very different agents is mediated solely by MDR1, we speculate that NF-Y is a common factor involved in drug resistance mediated by multiple upstream factors, including MDR-related protein and the lung resistance protein. However, to our knowledge, the involvement of NF-Y in the regulation of the multidrug resistance protein expression has not yet been clearly established. Effects of HNM-176 on the expression of P-gp species other than MDR1 remain to be assessed. Furthermore, because MDR1 is expressed in normal tissues such as brain and kidney, the effect of HNM-176 in these tissues needs to be estimated in vivo. Our efforts to establish a cell line resistant to HNM-176 have not succeeded. Although it is not yet clear whether HNM-176 can be a substrate for MDR1, these results suggest that enhanced cytotoxicity caused by the down-regulation of MDR1 might make it difficult to establish a cell line resistant to HNM-176 itself. Therefore, we can expect HNM-176 to provide benefit by inhibiting the emergence of multidrug-resistant cells through the down-regulation of MDR1 within a tolerable dose-range. All in all, NF-Y seems to be a promising target for manipulating the MDR phenotype.

Is the inhibition of NF-Y associated with the cytotoxicity of HNM-176? After incubation of K2/ARS cells at 3 \( \mu \)M for 48 h, cell growth was significantly retarded because of a prolonged G2-M phase, but DNA fragmentation was hardly detected by the terminal deoxynucleotidyltransferase-mediated nick end labeling method at this time point (data not shown). In addition, we have demonstrated by RT-PCR that the mRNA levels of some housekeeping genes such as \( \beta \)-tubulin (data not shown) and GAPDH (Fig. 2) in treated cells are not altered so that these experimental conditions do not induce cell death, although they do retard cell growth. Therefore, it seems unlikely that the inhibitory effect of HNM-176 on NF-Y correlates with its cytotoxicity. Although HNM-176, as well as HNM-154, targets NF-Y, there may well be other targets that more directly determine the fate of the cell. We have recently obtained evidence that BRAP2, initially identified as a molecule associated with the suppressor oncogene product BRC1A (27), also binds HNM-176.6 It has been suggested that BRAP2 plays a crucial role in the nuclear-cytoplasmic shuttling of BRC1A. A defect in a molecule involved in nuclear-cytoplasmic shuttling can lead to cell death after cell cycle arrest at G2-M phase (28). In this context, BRAP2 is a possible target for HNM-176 that mediates its cytotoxic effect.

In conclusion, we have shown that the antitumor agent HNM-176 can suppress MDR1 expression by inhibiting NF-Y activity in MDR1-overexpressing multidrug-resistant tumor cells. All resistant cell lines used in our experiments were artificially established by constitutive exposure to drugs. However, in clinical settings, the emergence of such highly resistant cells seems unlikely. Therefore, we believe that the half reduction of MDR1 expression by HNM-176 may be effective in the much-less-resistant cells usually seen in clinical settings. Furthermore, a preliminary in vivo study in our laboratory revealed that the use of HNM-176 in combination with various common antitumor agents such as Adriamycin, Taxol, VCR, and etoposide showed an additive effect, suggesting that the combination of these agents is a promising approach.

On the basis of these findings, we have initiated in vitro studies with HNM-176 and in vivo studies with HNM-214 (as a prodrug for HNM-176) to test whether it enhances the antitumor activity of other antitumor agents.

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