P-Glycoprotein Mediates Resistance to Histidine Kinase Inhibitors

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ABSTRACT
Histidine kinase inhibitors are being developed as a new class of antimicrobial drugs. We recently demonstrated the activity of a class of histidine kinase inhibitors against a mammalian enzyme, elongation factor-2 kinase (eEF-2K), and the effect of these compounds on cancer cell viability (Arora et al., 2003). To further characterize these compounds, we studied their interaction with ATP-binding cassette transporters, which are known to mediate resistance to a variety of chemotherapeutic agents. The 24 compounds studied belong to three structural series of derivatives of 2-methylimidazolium iodide. We focused this work on a representative compound (NH125) because we found it to be most potent against both histidine kinase and eEF-2K among the series. Cell lines that expressed P-glycoprotein (P-gp) were 2- to 5-fold resistant to NH125. NH125 increased accumulation of P-gp substrates such as paclitaxel and doxorubicin but had no effect on the accumulation of non-P-gp substrates. P-gp modulators verapamil and trans-flupenthixol and MDR1-targeted siRNA increased sensitivity of multidrug-resistant cell lines to NH125. The presence of a benzyl group on the N-3 position of the 2-methylimidazolium iodide was important for the interaction with P-gp. C6-NH, an NH125-resistant cell line, markedly overexpressed P-gp compared with the parental cell line. In animal models, we found that NH125 increased by 129% the survival of sensitive P388 cells bearing mice but had no effect on mice harboring the resistant cell line. These observations indicate that certain histidine kinase inhibitors are substrates for P-gp and hence an important consideration in development of these agents as potential antimicrobial and anticancer agents.

Calmodulin-dependent protein kinases are central to many signal transduction pathways that control cell proliferation and are often deregulated in malignancy. Calmodulin-dependent kinase III, also known as elongation factor 2 kinase, is overexpressed in many cancers. eEF-2K has been previously identified in several studies from our laboratory (Bagaglio and Hait, 1993; Parmer et al., 1999) to be important for cancer cell proliferation; inhibition of this kinase decreased cancer cell viability (Parmer et al., 1997; Yang et al., 2001). We recently reported that effective targeting of eEF-2K by a novel class of histidine kinase inhibitors decreased enzyme activity and cell viability at nanomolar concentrations (Arora et al., 2003). While studying the effects of these inhibitors on cancer cell lines from various tissues of origin, we found that cells overexpressing P-gp were less sensitive to NH125. Because the 2-methylimidazolium derivatives possess structural features consistent with other P-gp–interacting agents, we investigated whether or not these histidine kinase inhibitors were transported by P-gp.

Multidrug resistance (MDR) mediated by P-gp is a deterrent to effective cancer treatment. Overexpression of P-gp is a major cause of cross-resistance of cancer cells to cytotoxic drugs with diverse mechanisms of action (Pastan and Gottesman, 1987; Murren and Hait, 1992). P-gp is a 150- to 180-kDa phosphoglycoprotein that functions as an energy-dependent drug efflux pump with broad substrate specificity (Endicott and Ling, 1989; Ford and Hait, 1990; Gottesman and Pastan, 1993). Cells expressing P-gp exhibit decreased intracellular drug accumulation because of increased drug efflux (Sirotnak et al., 1986; Hammond et al., 1989; Stein et al., 1994). As a result, tumor cells evade the cytotoxic effects of drugs by virtue of nonproductive drug-target interaction. In humans, P-gp is the product of ABCB1 gene product and is expressed in a cell- and tissue- specific manner; high levels are detectable in the kidney, liver, and intestine (Thiebaut et al., 1987). In rodents, two genes, mdr1a and mdr1b, have...
been reported to play a similar role in drug resistance. Studies in mdr1a and mdr1b knock-out mice as well as the P-gp tissue distribution studies suggested several physiological roles for P-gp including protection against toxic xenobiotics by excretion into bile, urine, or the intestinal lumen; maintenance of the blood brain barrier; and transport of steroid hormones and cholesterol (Gottesman and Pastan, 1993; Borst and Schinkel, 1996; Luker et al., 1999).

P-gp interacts with structurally unrelated compounds and cytotoxic drugs, including anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, phenothiazines, colchicines, and mitomycin (Endicott and Ling, 1989). Several studies have identified certain structural features important for interaction with P-gp. These include presence of a planar hy-
dropout ring and a positively charged amino group (Zamora et al., 1988). Our laboratory previously studied a series of substituted phenothiazines and found that hydrophobicity of the ring, length of methylene bridges connecting the phenothiazine nucleus to the amino group, and the charge on terminal amino group are directly related to their activity (Ford et al., 1989).

Bacterial histidine kinases are important enzymes that participate in two-component signaling cascade (Alex and Simon, 1994). These signal transduction systems maintain bacterial cell homeostasis and the expression of virulence factors in response to external and internal environmental stimuli (Haldimann et al., 1997; Novak et al., 1999). Histidine kinase inhibitors have potent antibacterial (Yamamoto et al., 1996) and antitumor activity (Arora et al., 2003). Bacteria and several other prokaryotes express proteins homologous to P-gp. For example, pfMDR1 is present in Plasmodium falciparum, ehPgp is present in Entamoeba histolytica, LmrA is present in Lactococcus lactis, and HlyB is present in E. coli (van Veen and Konings, 1997). The role of P-gp in development of MDR phenotype to various anticancer agents in animal models and clinical trials has been well recognized. Therefore, we investigated the effect of P-gp on the activity of a series of histidine kinase inhibitors.

**Materials and Methods**

**Drugs and Chemicals.** All NH1 compounds were derivatives of 2-methyl imidazolium iodide and were synthesized as described previously (Yamamoto et al., 2000). The 28 compounds belonged to three structural series, which differ by the substitutions on the N-3 imidazolium nitrogen: NH1-1, unsubstituted; NH1-2, benzyl group; and NH1-3, butyl group. The compounds within a series differ in the length of the alkyl chain attached to the N-1 of the imidazolium ring (Fig. 1). [3H]Paclitaxel (Taxol) and [3H]methotrexate were obtained from Moravek Biochemicals (Brea, CA). The P-glycoprotein antibody QCRL-1 were obtained from Signet Laboratories (Bedford, MA). The mouse anti-human secondary antibody was obtained from DakoCytomation (Carpentina, CA). All media and cell culture products were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

**Cell Lines.** MCF-7, MCF-7-ADR, and MCF-7-BC-19 cell lines were kindly supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE). A2780 and A2780-DX were provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY). C6 was obtained from American Type Culture Collection (Manassas, VA). P388/VMDRC.04, a MDR1-transfected mouse leukemia cell line, was characterized by our laboratory (Yang et al., 1994). All the cell lines except P388/D5 and P388/VMDRC.04 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2/95% air.

**In Vitro Sensitivity to Drugs.** The in vitro sensitivity of the cells to drugs was determined by seedling cells (5.0 × 10^4 - 1.0 × 10^5 cells/ml/well) onto 24-well plates. After 48 h, cells were washed with serum-free media and incubated with 50 nM [3H]paclitaxel or 1 μM [3H]methotrexate and various concentrations of NH125 or 25 μM of NH 12 compounds for 3 h. The reactions were stopped by adding ice-cold PBS, and the cells were lysed immediately with 1% SDS. The radioactivity in the samples was determined by scintillation counting.

The accumulation of doxorubicin was analyzed by fluorescence microscopy. Cells were incubated with 12.5 μM of doxorubicin in the absence or presence of 25 μM NH125 for 1 h. At the end of the incubation, cells were washed three times with PBS and observed under a fluorescence microscope (Nikon ECLIPSE TE200 microscope; Nikon Inc., Melville, NY). Fluorescence intensity was quantified using Image-J software.

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**In Vitro Sensitivity to Drugs.** The in vitro sensitivity of the cells to drugs was determined by an MTT cell viability assay. In brief, 5 × 10^4 cells were plated in 96-well plates and exposed to various concentrations of drug for 48 to 72 h. The formazan product
formed after a 4-h incubation with MTT was dissolved in 100% dimethyl sulfoxide and read at 550 nM using a Dynatech Microplate Reader MR5000.

**In Vivo Sensitivity to NH125.** Cells from log-phase cultures of P388/S or P388/VMDRC.04 were washed in PBS by centrifugation at 1000g for 10 min, then resuspended in sterile saline at a concentration of $1 \times 10^7$ cells/ml. One million cells in 0.1 ml of PBS were inoculated into groups of five 20- to 22-g female CD2F1 mice (Charles River Laboratories, Wilmington, MA) via the peritoneal cavities on day 0. Vehicle or NH125 (1 mg/kg) were given i.p. on days 1, 4, 6, 8, and 11. Animals were given food and water ad libitum and were checked daily for weight, signs of tumor growth, or illness. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

**Preparation of Cell Homogenates and Western Blot Analysis.** Cell monolayers were washed twice in PBS, pH 7.4, scraped into 15-ml conical tubes, and centrifuged at 1000g at 4°C for 5 min. Cell extracts were prepared by lysing cell pellets in ice-cold radioimmunoprecipitation assay buffer. The lysates were centrifuged at 15,000g for 30 min at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford (1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

 Fifty micrograms of protein was resolved by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%), followed by incubation with anti-P-gp or anti-MRP antibody (1: 500 dilution in 5% milk/PBS/Tween 20). The detection was performed using horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence detection reagent.

**Silencing of MDR1 Expression by RNA Interference.** Suppression of MDR1 gene expression by siRNA was as previously reported by our laboratory (Wu et al., 2003). siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2’-ACE protection chemistry. Cells were transfected with siRNA using oligofectamine and Opti-MEM I reduced serum medium (Invitrogen Invitrogen, Inc., Carlsbad, CA), according to the manufacturer’s protocol.

**Results**

**Effect of P-gp Overexpression on NH125 Sensitivity.** To determine whether the action of certain histidine kinase inhibitors was affected by P-gp, we compared the sensitivity of MDR cell lines to NH125 by incubating cells with the drug

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**Fig. 5.** Effect of NH125 on accumulation of $[^3]H$metothrexate in sensitive and MDR cells. MCF-7 (a), MCF-7 ADR (b), A2780 (c), A2780-DX (d), and BC-19 (e) cells were treated with different concentrations of NH125 and 1 $\mu$M $[^3]H$metothrexate for 3 h, washed thrice with ice-cold PBS, and solubilized in 1% SDS. Cellular methotrexate content was determined by scintillation counting. Each bar represents the mean ± S.D. from one of three similar experiments.
for 48 h and then measuring cell viability by the MTT assay. MDR cell lines overexpressed P-gp compared with the parental lines (Fig. 2) and were 2- to 5-fold less sensitive to NH125 compared with the parental cell lines. The IC_{50} values of MCF-7/ADR and A2780-DX were 5 and 8.1 μM, respectively, whereas the IC_{50} values of the parental lines MCF-7 and A2780 were 2.5 μM and 1.6 μM, respectively. These results were summarized in Table 1.

**Effect of NH125 on Drug Accumulation.** We next measured the effect of NH125 on the accumulation of P-gp and non-P-gp substrates using either liquid scintillation counting or fluorescence microscopy. Parental and MDR cell lines were incubated with [3H]paclitaxel and various concentrations of test compounds and 50 nM [3H]paclitaxel (Taxol) for 3 h, washed twice with ice-cold PBS, and solubilized in 1% SDS. Cellular paclitaxel content was determined by scintillation counting. Each bar represents the mean ± S.D. from one of three similar experiments. **, p < 0.01; *, p < 0.05.

![Graphs showing drug accumulation](image)

**Fig. 6.** Structure-activity relationship of histidine kinase inhibitors. MCF-7 (a), MCF-7-ADR (b), A2780 (c), and A2780-DX (d) cells were treated with 25 μM concentrations of test compounds and 50 nM [3H]paclitaxel (Taxol) for 3 h, washed twice with ice-cold PBS, and solubilized in 1% SDS. Cellular paclitaxel content was determined by scintillation counting. Each bar represents the mean ± S.D. from one of three similar experiments. **, p < 0.01; *, p < 0.05.

To further analyze the selectivity of NH125 as a P-gp substrate, we determined the effect of increasing concentrations of NH125 on the accumulation of a non-P-gp substrate, [3H]methotrexate. Figure 5 demonstrates that NH125 had no effect on the accumulation of methotrexate in any of the P-gp–overexpressing cell lines tested.

**Structure-Activity Relationship of Histidine Kinase Inhibitors to Inhibition of P-gp Function.** To analyze the structure-activity relationship of histidine kinase inhibitors to inhibition of P-gp, we studied the effect of several derivatives of 2-methylimidazolium iodide (NH1 compounds) on drug accumulation. The 28 compounds belong to three structural series that differ in the substitutions on the N-3 imidazolium nitrogen: NH1-1, unsubstituted; NH1-2, benzyl group; and NH1-3, butyl group. The compounds within a series differ in the length of the alkyl chain attached to the N-1 of the imidazolium ring (Fig. 1). Cells were incubated with 50 nM [3H]paclitaxel and 25 μM of the test compound for 3 h, and radioactivity was measured by scintillation counting. As shown in Fig. 6, the N-3 benzyl derivative of 2-methylimidazolium iodide (NH125) significantly increased the accumulation of paclitaxel. In contrast, the unsubstituted derivative (NH115) and N-3 butyl substituted derivative (NH135), with same alkyl chain on the N1-nitrogen of the imidazolium ring, did not have the similar effect on paclitaxel accumulation as NH125 (Fig. 6, b and d). Among the compounds within the NH12 (benzyl substituted ring) series that differed in length of the alkyl chain at the N-1 nitrogen of the imidazolium ring, there was no relationship between length of the alkyl chain and P-gp inhibition.

**Effect of MDR Modulators on NH125 Sensitivity.** Cells were incubated with NH125 in the presence or absence of P-gp modulators for 48 h, and cell viability was determined by MTT assay. As shown in Table 1, both verapamil and...
trans-flupenthixol increased the sensitivity of MDR cell lines to NH125 by 2- to 4-fold.

**Generation and Characterization of NH125-Resistant Cell Line.** C6 glioblastoma cells were selected for resistance to NH125 by step-wise selection with NH125 and analyzed for P-gp expression and paclitaxel accumulation. As shown in Fig. 7a, the C6-NH cell line was 10-fold resistant to NH125 as measured by MTT. Furthermore, Western blot analysis revealed a 20-fold induction of P-gp in C6-NH cells compared with parental C6 cells. (Fig. 7b, top). In contrast, MRP1 was not induced as a result of this selection (data not shown). The accumulation of [3H]paclitaxel was decreased in C6-NH compared with C6 cell line (Fig. 7c); verapamil increased the accumulation of paclitaxel in the C6-NH cell line (Fig. 7c).

**In Vivo Efficacy of NH125.** To analyze the implication of these findings on the efficacy of these drugs, we studied their effect on paired cell lines developed to analyze P-gp in vivo. One million P388/S or P388/VMDRC.04 cells were inoculated into groups of five 20- to 22-g female CD2F1 mice via the peritoneal cavities on day 0. Vehicle or NH125 (1 mg/kg) were given i.p. on days 1, 4, 6, 8, and 11. NH125 increased the survival of mice bearing sensitive P388 tumor cells by 129% (mean survival, 13.2 ± 0.9 days) compared with vehicle-treated controls (mean survival, 10.2 ± 1.0 days) (Fig. 8a). In contrast, NH125 had no effect on the survival (mean survival, 15.0 ± 1.5 days) of mice bearing P388/VMDRC.04 tumor cells versus vehicle-treated controls (mean survival, 16.4 ± 0.5 days) (Fig. 8b).

**Effect of MDR1-Targeted siRNA on NH125 Sensitivity.** To provide more direct evidence that cellular resistance to NH125 is mediated by P-gp, we tested the effect of inhibition of P-gp expression by siRNA on sensitivity of MDR cells to the compound using the method described previously by our laboratory (Wu et al., 2003). Figure 9 shows that when P-gp expression was suppressed by siRNA in MCF-7-Adr cells (Fig. 9a), the sensitivity to NH125 was increased 3- to 4-fold (Fig. 9b). Similar results were obtained with another MDR cell line, A2780DX (data not shown).

![Fig. 7.](image-url) Generation and characterization of resistant C6-NH cells. a, 4 × 10⁶/ml C6 and C6-NH cells were seeded onto 96-well plates and exposed to various concentration of the NH125 for 48 h. Cell viability was determined by MTT assay. Each point represents the mean ± S.D. from one of three similar experiments. b, C6 and C6-NH cells were collected and cell lysates were prepared and run on 7.5% SDS gel, transferred to nitrocellulose, and immunoblotted with an anti-P-gp antibody (Fig. 6b, top), and β-actin antibody (Fig. 6b, bottom). Results are representative of four similar experiments. c, cells were treated with 50 nM [3H]paclitaxel in the presence or absence of 10 μM verapamil for 3 h, washed thrice with ice-cold PBS, and solubilized in 1% SDS. Cellular paclitaxel content was determined by scintillation counting. Each bar represents the mean ± S.D. from one of three similar experiments.
Discussion

These studies indicate that P-gp mediates resistance to NH125, a novel inhibitor of eEF-2K (Arora et al., 2003). NH125 belongs to a series of histidine kinase inhibitors identified to inhibit several bacterial histidine kinases including EnvZ, PhoQ, BvgS, and EvgS, and have antibacterial activity (Yamamoto et al., 2000). We recently tested the activity of histidine kinase inhibitors versus eEF-2 kinase, a mammalian Ca²⁺/calmodulin-dependent enzyme identified as a potential target for anticancer drug development. For example, eEF-2K is increased in many malignant cell lines (Bagaglio and Hait, 1993; Hait et al., 1996) and cancer tissues (Farmer et al., 1999), is selectively activated in proliferating cells (Bagaglio and Hait, 1994), and antisense or siRNA knock-out (J. Yang, J.-M. Yang, H. Wu, W. Hait, unpublished data) decreases cell viability. NH125 was found to be the most potent eEF2K inhibitor and most effective versus malignant cell growth and viability (Arora et al., 2003). Therefore, we wished to know whether these potential therapeutic agents were subject to ABC transporter-mediated drug resistance. We found that cell lines that overexpress P-gp were less sensitive to NH125 (Table 1). Thus, we studied the role of P-gp in mediating this resistance in detail. We found that NH125 increased accumulation of P-gp substrates inside the cells (Figs. 3 and 4). Furthermore, P-gp modulators sensitize MDR cells to NH125 (Table 1). The resistant cell line developed by selection in NH125 overexpressed P-gp as shown in Fig. 7b. Finally, the efficacy of NH125 is significantly reduced in a MDR mouse model bearing P388/VMDRC.04 tumor cells, as shown in Fig. 8.

Structure-activity relationships revealed the importance of the benzyl ring at the N-3 position of the 2-methyl imidazolium iodide as an important determinant for interaction with P-gp. Whereas NH115 and NH135 compounds that lack the benzyl substitution did not increase paclitaxel accumulation in MDR cells, all compounds in the NH12 series, which contain the benzyl group on the imidazolium ring, increased the accumulation of paclitaxel (Fig. 6).

To more directly address the question of whether NH125 is a substrate for P-gp, we used RNA interference to suppress the expression of P-gp in MDR cells and then determined its effect on sensitivity to NH125. siRNA-directed suppression of P-gp increased the sensitivity of MDR cells to NH125 (Fig. 9), thereby strengthening our conclusion that P-gp mediates resistance to NH125.

These studies have implications for development of benzyl-imidazole compounds as anticancer and antimicrobial drugs. De novo and acquired expression of P-gp in various tumor tissues (e.g., renal, colorectal, and breast cancer tissues) may limit clinical utility. Because many of these or similar compounds are being used or being developed as potential antibacterial drugs, the implication of these studies needs to be considered because of the presence of P-gp like drug transporters in bacteria (Putman et al., 2000). Moreover, many normal tissues (e.g., blood-brain barrier, intestine) express P-gp and could limit absorption of these compounds, thus limiting their efficacy. Studies are in progress to determine whether structural derivatives can be developed that maintain activity against the kinases but elude P-gp and kill MDR cells.

References


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Fig. 9. Effect of MDR1-targeted siRNA on P-gp expression (a) and NH125 sensitivity (b) in MDR cells. a, MCF-7-ADR cells were treated with 200 nM of MDR1 siRNA, 200 nM scrambled siRNA, or mock. Forty-eight hours later, cell lysates were prepared from the siRNA- or mock-treated cells. Equal amounts (50 μg of proteins) of cell lysates were separated by 7.5% SDS-polyacrylamide gel electrophoresis, then transferred onto nitrocellulose membrane. The membranes were immunoblotted with an anti-P-gp (top) or anti-β-actin (bottom) antibody. Results are representative of three similar experiments. b, MDR1 siRNA-, scrambled, or mock-treated MCF-7-ADR cells were plated in 96-well tissue culture plates and treated with varying concentrations of NH125. Viability was measured using MTT assay as described under Materials and Methods. Each value represents the mean ± S.D. of quadruplet determinations. Results are representative of three similar experiments.