Identification and Characterization of an Inhibitor of Eukaryotic Elongation Factor 2 Kinase against Human Cancer Cell Lines

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ABSTRACT
Recent evidence suggests that the machinery of protein synthesis may provide novel targets for anticancer drugs. For example, aberrations in protein synthesis are commonly encountered in established cancers, and disruption by mutation or overexpression of translation factors can cause cellular transformation. We previously demonstrated that the activity of eukaryotic elongation factor 2 (eEF-2) kinase was markedly increased in several forms of malignancy and that nonspecific inhibitors of this enzyme promoted cell death. On the basis of the predicted amino acid sequence of eEF-2 kinase deduced from the cloned cDNA, we hypothesized that inhibitors of prokaryotic histidine kinases might also inhibit the activity of eEF-2 kinase. We describe herein the screening of a series of imidazolium histidine kinase inhibitors and the identification of an active lead compound, NH125. NH125 inhibited eEF-2 kinase activity (IC50 = 60 nM) in vitro, blocked the phosphorylation of eEF-2 in intact cells, and showed relative selectivity over other protein kinases: protein kinase C (IC50 = 7.5 μM), protein kinase A (IC50 = 80 μM), and calmodulin-dependent kinase II (IC50 > 100 μM). NH125 decreased the viability of 10 cancer cell lines with IC50 ranging from 0.7 to 4.7 μM. Forced overexpression of eEF-2 kinase in a glioma cell line produced 10-fold resistance to NH125. In conclusion, these results suggest that identification of potent inhibitors of eEF-2 kinase may lead to the development of new types of anticancer drugs.

INTRODUCTION
eEF-22 kinase is a Ca2+/calmodulin-dependent protein kinase that phosphorylates and inactivates eEF-2, an elongation factor that facilitates translocation of peptidyl t-RNA from the ribosomal A site to P site (1–3). We previously identified the activity of eEF-2 kinase as a Ca2+/calmodulin-dependent enzymatic activity that was increased in several cancer cell lines and fresh human specimens (4–6). In addition, the activity was greater in proliferating cells (4, 5, 7) and during the S phase of the cell cycle (8). Changes in expression of eEF-2 kinase was also associated with cell cycle progression (9), cellular differentiation (10), and oogenesis (11). We previously demonstrated that inhibition of eEF-2 kinase by a nonspecific inhibitor, rottlerin (12), or degradation of the kinase after disruption of its complex with heat shock protein 90 by geldanamycin (13) blocked glioma cells at that inhibition of eEF-2 kinase. We describe herein the screening of a series of imidazolium derivatives for relative selectivity over other protein kinases: protein kinase C (IC50 < 100 nM), and calmodulin-dependent kinase II (IC50 > 100 μM). NH125 decreased the viability of 10 cancer cell lines with IC50 ranging from 0.7 to 4.7 μM. Forced overexpression of eEF-2 kinase in a glioma cell line produced 10-fold resistance to NH125. In conclusion, these results suggest that identification of potent inhibitors of eEF-2 kinase may lead to the development of new types of anticancer drugs.

MATERIALS AND METHODS

Drugs. All compounds were derivatives of 2-methyl imidazolium iodide and were synthesized as previously described (17). The 28 compounds belonged to three structural series, which differ by the substitutions on the N-3 imidazolium nitrogen: NH-1, unsubstituted; NH-2, benzyl group and NH-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). All of the compounds were dissolved in 70% ethanol.

Materials. His-tagged eEF-2 kinase was purified from Saccharomyces cerevisiae as described previously (18). GST-tagged eEF-2 kinase (pGEX-6P) was a gift of Dr. Ann Stock, (University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School), eEF-2 kinase was expressed and purified as described below. [γ-32P]ATP, donkey antirabbit IgG, and enhanced chemiluminescence Western blot reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other enzymes and their substrates were purchased from Calbiochem (San Diego, CA). The anti-eEF-2 and antiphospho-eEF-2 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). All media and cell culture products were purchased from Life Technologies, Inc. (Grand Island, NY). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture. C6, T98G, U-138 MG, U-87 MG, A172, A2780, HeLa, PC3, OVCAR-3, and MCF-7 cell lines were obtained from American Type Culture Collection (Manassas, VA). T98G, U87, and U138MG were grown in 10:1, Ham’s F-10 media:DMEM, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All other cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell cultures were maintained in a humidified incubator at 37°C with 5% CO2.

Partial Purification of eEF-2 Kinase. Escherichia coli strain BL21 containing the expression plasmid pGEX-6P were grown overnight at 37°C in 100 ml of Luria Bertani plus 100 μg/ml ampicillin. Two liters of Luria Bertani containing 100 μg/ml ampicillin were inoculated with 100 ml of the overnight culture and incubated at 37°C to an A600 of 0.8. IPTG was added to a final concentration of 1.0 mM, and the culture was additionally incubated at 16°C overnight. Cells were then centrifuged at 400 × g for 15 min, resuspended (5 ml/g cell pellet), and incubated on ice for 20 min in BugBuster protein extraction reagent (Novagen, Inc., Madison, WI) containing protease inhibitor mixture III (Calbiochem). All additional steps were carried out at 4°C and as per manufacturer’s protocol (Amersham Pharmacia Biotech). Briefly, cell lysates were centrifuged at 12,000 × g for 20 min. The supernatants were recovered and applied to glutathione-Sepharose 4B column according to manufacturer’s protocol. The column was washed three times with 10 bed volumes of 1× PBS and eluted with 10 mM glutathione elution buffer/ml of bed volume. Eluate containing GST-tagged eEF-2 kinase was additionally analyzed by SDS-PAGE and staining with Coomassie brilliant blue. The final kinase

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3 The abbreviations used are: eEF-2, elongation factor 2; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; PKA, protein kinase A; PKC, protein kinase C; CaMK-II, calmodulin-dependent kinase II; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].
preparation had a specific activity of 1.5 pmol phosphate transferred/mg protein/min.

**eEF-2 Kinase Assay.** eEF-2 kinase activity was measured by two methods: (a) a filter-based assay; and (b) by immunoblotting using antiphospho-eEF2 antibody. For both of these, reactions were carried out in 20 μl of total volume containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1.5 mM CaCl₂, 100 μg/ml calmodulin, 2 μM His-tagged eEF-2 and 400 nM GST-eEF-2 kinase, and ATP mixture [50 μM ATP with 1 μCi (γ-33P)ATP]. The kinase mixture without ATP was prepared on ice and then preincubated for 15 min at room temperature. Kinase reactions were started by adding ATP and allowed to progress at 30°C for 30 min. For the filter-based assay, the reaction was terminated by adding 20 μl of cold 1.5% phosphoric acid, and 5 μl of the reaction were applied to P81 Whatman phosphocellulose paper. The paper was washed three times in 500 ml of 0.5% phosphoric acid and once with 200 ml of acetone. The paper was then air-dried and immersed in 10 ml of scintillation mixture. Radioactivity was counted using a Beckton-Dickinson liquid scintillation counter (Model LS-6000). For immunoblotting, the reactions were stopped by addition of 20 μl of 3× Lamelli buffer [190 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, and 0.003% bromphenol blue dye]. Samples were boiled for 5 min and resolved by 7% SDS-PAGE and processed for Western blotting as described below. Conditions for both assays were chosen to ensure linearity of the reaction with respect to time of incubation and concentration of enzyme.

**Assays for CaMK II, PKA, and PKC.** CaMK-II activity was measured according to the manufacturer’s instruction (Calbiochem) with minor modifications. The reaction mixture contained in a total volume of 20 μl of 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1.5 mM CaCl₂, 100 μg/ml calmodulin, 10 μM autocamtide, 5 ng of purified rat brain CaMK-II, and ATP [50 μM ATP with 1 μCi (γ-33P)ATP]. The reaction was initiated by adding ATP and allowed to progress at 30°C for 2 min. The reaction product was measured as described above for the filter-based assay of eEF-2 kinase.

The activity of PKA was measured in a total assay volume of 20 μl containing 25 mM MES (pH 6.7), 5.5 mM magnesium acetate, 0.5 mg/ml histone H1, 20 ng of purified PKA catalytic subunit, ATP [50 μM ATP with 2 μCi (γ-33P)ATP]. The reaction was initiated by adding ATP and allowed to progress at 30°C for 5 min. The reaction product was measured as described above for the filter-based assay of eEF-2 kinase.

The activity of PKC assay was measured as per manufacturer’s (Calbiochem) protocol. The assay mixture (20 μl) consisted of 20 mM Tris (pH 7.5), 5 mM MgCl₂, 1 mM EGTA, 200 μM selectide, 2.5 ng of purified PKC catalytic...
subunit, and ATP [100 μM ATP with 2μCi (γ-32P)ATP]. The reaction was initiated by adding ATP and allowed to progress at 30°C for 10 min. The reaction product was measured as described above for the filter-based assay of eEF-2 kinase.

Preparation of Cell Homogenates for Detection of Cellular eEF-2 and Phospho-eEF-2. Cell monolayers were washed twice in PBS (pH 7.4), scraped into 15-ml conical tubes, and centrifuged at 1000 × g at 4°C for 5 min. Cell extracts were prepared by homogenizing cell pellets in ice-cold homogenization buffer [25 mM HEPES (pH 7.4), 100 mM sodium chloride, 20 mM NaPPi, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 0.1 mM sodium orthovanadate] using a Dounce Homogenizer. The homogenates were centrifuged at 15,000 × g for 30 min at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Western Blot Analysis. Fifty μg of protein or 20 μl of the in vitro eEF-2 phosphorylation reaction (described above) were resolved by 7% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS/Tween-20 (0.05%), followed by incubation with antiphospho-eEF-2 antibody (1:500 dilution in 10% milk/PBS-T) or anti-eEF2 antibody (1:1000 dilution). The detection was performed using horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence detection reagent. Blots were scanned by Hewlett Packard Scan Jet and intensity of protein bands were quantified by Quantity One software (Bio-Rad Laboratories).

Cellular Viability Assay. The viability of cells was measured using an MTT assay. Briefly, 5 × 104 cells were plated in 96-well plates and exposed to various concentrations of drug for 48–72 h. The formazan product formed after 4 h incubation with MTT was dissolved in 100% DMSO and read at 550 nm using a Dynatech Microplate Reader MR5000.

Generation of Stable Cell Lines Expressing Different Levels of eEF-2 Kinase. The preparation of T98G eEF-2 kinase transfectants was described previously (13). Briefly, T98G human glioblastoma cells were transfected with a pSTAR vector expressing a full-length eEF-2 kinase cDNA. Transfectants were selected in 500 μg/ml G418, and resistant colonies were expanded and maintained in 200 μg/ml G418. The cell lines were analyzed for the expression of eEF-2K by Western blotting.

Flow Cytometry. The effect of NH125 on the cell cycle distribution was analyzed by measuring the DNA content of C6 cells in presence or absence of various concentrations of drug. Briefly, the cells were treated for 18 h, harvested by trypsinization, washed with PBS, and fixed by dropwise addition of cold 70% ethanol for 30 min on ice. Approximately 1 × 106 cells were washed and resuspended in 1 ml of PBS containing 0.1 mg/ml RNase A and 5 μg/ml propidium iodide. The cells were stained with propidium iodide for 30 min at room temperature. Fluorescence intensities were determined by quantitative flow cytometry, and profiles were generated on a Coulter Epics FACScan Analyzer.

RESULTS

Effect of NH1 Compounds on the Activity of eEF-2 Kinase. The activity of eEF-2 kinase was measured by two different assays as described in “Materials and Methods.” For the purpose of quantification and screening, we developed a filter-based liquid scintillation assay using the purified recombinant enzyme. Because the kinase can autophosphorylate, all results were confirmed by Western analysis of phosphorylated substrate.

The structures of NH1 compounds are shown in Fig. 1. Among the compounds tested, only NH1 2 series inhibited eEF-2 kinase (Fig. 2, A and B). NH125 was the most potent derivative, with an IC50 of 60 nm (Fig. 2, C and D).

Effect of NH125 on the Activity of Other Kinases. To determine the relative selectivity of NH125 against eEF-2 kinase, we examined the effect of this compound on other kinases. NH125 was 1000- to >100,000-fold more potent against eEF-2 kinase (IC50 = 60 nm) than
against PKC (IC$_{50}$ = 80 µM), PKA (IC$_{50}$ = 7.5 µM), and CaMK-II (IC$_{50}$ > 100 µM).

**Effect of NH125 on eEF-2 Kinase in Intact Cells.** We next determined the effect of NH125 on the phosphorylation of eEF-2 in whole cells. C6 glioma cells were treated with various concentrations of NH125 for 12 h, then cellular extracts were prepared and analyzed by Western blot using antiphospho-eEF-2 antibody. As shown in Fig. 3, NH125 decreased the cellular content of phospho-eEF-2 without affecting total content eEF-2 content.

**Effect of NH125 on Cell Viability of Cancer Cells.** To determine the effect of eEF-2 kinase inhibition on the viability of cancer cells, a panel of 10 cell lines was incubated with various concentrations of NH125 for 48–72 h and assayed for viability with MTT. As shown in Table 1, NH125 was a potent inhibitor of cell viability with IC$_{50}$s between 0.7 and 4.8 µM. Other compounds in the NH12 series did not inhibit cell viability at concentrations up to 100 µM (data not shown).

**Effect of Overexpression of eEF-2 Kinase on Drug Sensitivity.** To further analyze whether the effects of NH125 on cell viability were attributable to effects on eEF-2 kinase activity, we tested if the effect of NH125 on cell viability could be altered by overexpression of the enzyme. In these experiments, we compared the sensitivity to NH125 in two isogenic cell lines that differed in expression of eEF-2 kinase by virtue of stable transfection of T98G cells with an expression vector containing a full-length eEF-2 kinase cDNA (13). As shown in Fig. 4, the activity of NH125 was decreased 10-fold in the eEF-2 kinase transfectants.

**Effect of NH125 on Cell Cycle Distribution.** Several groups have studied the activity of eEF-2 kinase during the cell cycle and found that its activity is increased during S phase (8, 9). Rottlerin, a less potent and less specific inhibitor, decreased the number of cells entering S phase (12). Therefore, we determined the effect of NH125 on the cell cycle distribution of C6 glioma cells. As shown in Fig. 5, NH125 significantly reduced the number of cells in S phase and produced an accumulation of cells in G$_1$-G$_2$. Thus, NH125 appears to block cell cycle transit at the G$_1$-S boundary. In contrast, NH124 was one-third (12 versus 26% G$_1$) as effective in delaying G$_1$-S transition than NH125 (data not shown).

**DISCUSSION**

We identified a potent and relatively specific inhibitor of eEF-2 kinase that blocked the growth of a variety of malignant cell lines. Protein kinases are attractive targets for drug discovery because of their critical role in signal transduction pathways that are critical to normal cell growth and differentiation. In addition, these enzymes provide a rich platform for medicinal chemists because they often require unique coactivators or have distinct catalytic domains as defined by both biochemical and structural analyses. The rationale for targeting eEF-2 kinase was based on data from our laboratory and others linking aberrations in protein synthesis, including the activation of this enzyme, to the growth and viability of transformed cells (4–7, 19, 20).

The benzyl imidazolium series was selected for investigation because of their activity against EnvZ, a prokaryotic histidine kinase (17). We initially suspected that histidine kinase inhibitors might be active against eEF-2 kinase because of potential structural similarities in the ATP folds of the enzymes that were predicted based on analyses of amino acid sequence (14–16). However, a recent study (21) of the crystal structure of a similar enzyme, ChaK, revealed that the structural features of the ATP-fold may more likely resemble conventional protein kinases.

Nonetheless, our studies of the benzyl imidazolium series uncovered a potent and relatively specific inhibitor of eEF-2 kinase. The structure activity analysis of these data suggested that the presence of a benzyl ring at position N3 of the imidazolium ring is critical for inhibitory activity because the NHI-1 and NHI-3 series, which lack
the benzyl group, did not inhibit kinase activity. The length of the alkyl chain attached to the N1 imidazolium nitrogen did not give a clear correlation with the activity of the compounds. However, it appears that an optimal chain length is required because compounds with longer than C16H33 chain (NH126, NH127, and NH128) do not inhibit eEF-2 kinase effectively.

NH125 appears to be one of the most potent inhibitors of eEF-2 kinase yet to be described. For example, Gschwendt et al. (22) described the activity of rottlerin, a natural product purified from Mallotus philippinensis, as having an IC50 of 2.5 μM against eEF-2 kinase but had similar potency against PKC. Cho et al. (23) described a series of 1,3 selenazine derivatives as specific inhibitors of eEF-2 kinase with IC50 of 0.3 μM. NH125 is more potent against eEF-2 kinase than against PKC, PKA, or CaMK-II. We chose to study three kinases (PKC, PKA, and CaMKII) that represent the two major classes of serine/threonine protein kinases and play important roles in cell growth. Also, CaMK II was chosen to rule out an effect of the inhibitor on CaM as opposed to a direct effect on the kinase.

Several lines of evidence suggest that NH125 targets eEF-2 kinase in cancer cells and that the effects on cell viability are likely related to this activity. First, derivatives that lacked significant activity against the kinase had no effects on cell viability (data not shown). Second, NH125 decreased the phosphorylation of eEF-2 when incubated with intact cells (Fig. 3). Third, overexpression of the target enzyme decreased the activity of the drug (Fig. 4).

In conclusion, we identified a potent and relatively specific inhibitor of eEF-2 kinase with significant activity against several human cancer cell lines. Future studies designed to determine the activity of this and other derivatives in vivo would allow us to define whether eEF-2 kinase is a viable target for future drug development.

REFERENCES

Fig. 5. Effect of NH1 25 on cell cycle distribution of C6 glioblastoma cells. C6 cells were treated with 0.5–2 μM NH125 for 24 h, trypsinized, fixed, and stained with propidium iodide as described in “Materials and Methods.” Data shown are the FACScan profile of one of three representative experiments. The percentage of cells in G0–G1, S and G2–M phases are shown in the inset.


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