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Investigating the Kinetic Mechanism of Inhibition of Elongation Factor 2 Kinase (eEF-2K) by NH125: Evidence for a Common *in vitro* Artifact

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Abstract

Evidence is emerging that elongation factor 2 kinase (eEF-2K) has potential as a target for anti-cancer therapy and possibly for the treatment of depression. Here the steady-state kinetic mechanism of eEF-2K is presented using a peptide substrate and is shown to conform to an ordered sequential mechanism with ATP binding first. Substrate inhibition by the peptide was observed and revealed to be competitive with ATP, explaining the observed ordered mechanism. Several small molecules are reported to inhibit eEF-2K activity with the most notable being the histidine kinase inhibitor NH125, which has been used in a number of studies to characterize eEF-2K activity in cells. While NH125 was previously reported to inhibit eEF-2K *in vitro* with an IC₅₀ of 60 nM its mechanism of action was not established. Using the same kinetic assay the ability of an authentic sample of NH125 to inhibit eEF-2K was assessed over a range of substrate and inhibitor concentrations. A typical dose-response curve for the inhibition of eEF-2K by NH125 is best fit to an IC₅₀ of $18 \pm 0.25 \mu\text{M}$ and a Hill coefficient of 3.7 ± 0.14 , suggesting that NH125 is a weak inhibitor of eEF-2K under the experimental conditions of a standard *in vitro* kinase assay. To test the possibility that NH125 is a potent inhibitor of eEF2 phosphorylation we assessed its ability to inhibit the phosphorylation of eEF2. Under standard kinase assay conditions NH125 exhibits similar weak ability to inhibit the phosphorylation of eEF2 by eEF-2K. Notably, the activity of NH125 is severely abrogated by the addition of 0.1 % triton to the kinase assay through a process that can be reversed upon dilution. These studies suggest that NH125 is as a non-specific colloidal aggregator *in vitro*, a notion further supported by the observation that NH125 inhibits other protein kinases, such as ERK2 and TRPM7 in a similar manner to eEF-2K. As NH125 is reported to inhibit eEF-2K in a cellular environment its ability to inhibit eEF2 phosphorylation was assessed in MDA-MB-231 breast cancer, A549 lung cancer and HEK-293T cell lines using a Western blot approach. No sign of decrease in the level of eEF2 phosphorylation

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Supporting Information Available – Supplementary methods include mass spectrometry and NMR data for NH125 as well as phosphorylation assays using wheat germ EF2 performed at higher concentrations of NH125. This material is available free of charge via the Internet at <http://pubs.acs.org>.

was observed up to 12 hours following addition of NH125 to the media. Furthermore, contrary to the previously reported literatures, NH125 induced the phosphorylation of eEF-2.

eEF-2K (eukaryotic elongation factor-2 kinase, also known as calcium/calmodulin dependent protein kinase III) is an atypical serine/threonine specific protein kinase whose catalytic domain has no sequence similarity to conventional protein kinases (1, 2). To date its only known substrate of physiological relevance is eEF2 (eukaryotic elongation factor-2), a ribosome binding protein that facilitates the translocation of the ribosome along mRNA during translation (3, 4). Although its structure is not yet available, mutational studies have confirmed that the eEF-2K polypeptide consists of an *N*-terminal catalytic domain connected via a linker to a *C*-terminal domain that is important for eEF2 binding (2). eEF-2K phosphorylates eEF2 mainly on Thr-56 and Thr-58 within the sequence ⁵⁰RAGETRFT*DT*RKD⁶² (5). Phosphorylation of eEF2 decreases the affinity of eEF2 for the ribosome leading to inhibition of protein synthesis (6–8).

In recent years eEF-2K has been associated with autophagy, a process favoring cancer cell survival. For example, in glioblastoma cell lines the over-expression of eEF-2K is reported to enhance autophagy, while siRNA mediated depletion of eEF-2K is reported to decrease autophagy, as measured by the formation of LC3-II, formation of acidic vesicular organelles (AVOs) and electron microscopy (9, 10). A mechanistic understanding of how eEF-2K may regulate autophagy remains to be determined. In normal cells, autophagy is considered a survival mechanism under conditions of nutrient deprivation (11, 12). Many cancer cells also induce autophagy in response to anti-cancer therapies such as radiation therapy, hormonal therapy and chemotherapy (13–28). Therefore, it has been postulated that eEF-2K may promote cancer cell survival by regulating autophagy, suggesting that eEF-2K may be a candidate for targeted cancer therapy (9, 10, 29).

The activity of eEF-2K is increased in fresh human tumor samples and several cancer cell lines (9, 10, 30–34). Its activity is also greater in proliferating cells (32, 34, 35), especially during the S phase of the cell cycle (30, 36). Recently, we discovered that eEF-2K enhances a number of processes associated with tumorigenesis through its effects on multiple signaling pathways and provided the first evidence that *in vivo* therapeutic targeting of eEF-2K expression inhibits growth of established tumors in an orthotopic xenograft model of a highly aggressive and metastatic breast cancer.

Several pharmacological inhibitors of eEF-2K have been described in the literature (Figure 1). These include the 1,3-selenazine derivative (**1**) (37), rottlerin (**2**) (34, 38) the thieno[2,3-*b*]pyridine derivative (**3**) (39) and the histidine kinase inhibitor NH125 (**4**) (40). NH125 has been used by a number of investigators to investigate eEF-2K activity in cell cultures (40–44). However, while NH125 is reported to inhibit GST-eEF-2K with an IC₅₀ of 60 nM (40) its mechanism of inhibition has not been reported.

An understanding of the mechanism and regulation of eEF-2K has been hampered by the lack of a reliable source of the kinase. However, we recently reported the expression and purification of human eEF-2K using a bacterial expression system (45). The resulting preparation is highly active and monomeric and suitable for detailed mechanistic studies. The goal of this manuscript was to first delineate the kinetic mechanism of peptide phosphorylation by eEF-2K and then to assess the mechanism by which NH125 inhibits the kinase. The kinetic analysis revealed an ordered mechanism where ATP must bind before the peptide substrate to form a productive ternary complex. Upon investigating the mechanism with NH125, we found that its ability to inhibit eEF-2K was prevented by the addition of a small amount of detergent, a hallmark of a non-specific aggregator (46–48). In a subsequent *in vitro* assay, 0–5 μM NH125 failed to inhibit the phosphorylation of wheat

germ EF2 by eEF-2K. Similar effects were observed in cellular studies using MDA-MB-231 breast cancer, A549 lung cancer and HEK-293T cell lines, where treatment with 4 μ M NH125 for 12 hours did not inhibit eEF-2 phosphorylation. Instead, NH125 treatment led to an increase in the levels of phospho-eEF2 in all of the tested cell lines. The ability of NH125 to induce phosphorylation of eEF-2 was also recently reported by *Chen et. al.* (49) using H1299 (non-small cell lung carcinoma), PC3 (prostate cancer), HeLa (cervical cancer), H460 (non-small cell lung carcinoma) and C6 (rat glioma) cell lines. Together, these data support the notion that NH125 is not a cellular inhibitor of eEF-2K.

Materials and Methods

Buffers and reagents

Competent cells used for amplification and expression were provided by Novagen (Gibbstown, NJ). Yeast extract and tryptone were purchased from US biological (Swampscott, MA). IPTG and DTT were obtained from USB (Cleveland, OH). All buffer components used in the protein expression, purification, and enzyme assays, including HEPES, Trizma base (Tris), sodium chloride, potassium chloride, EDTA, EGTA, calcium chloride, magnesium chloride, Brij-35, Triton X-100, β -mercaptoethanol, DTT, benzamidine hydrochloride, TPCK and PMSF, were ultrapure grade and were purchased from Sigma (St. Louis, MO). Ni-NTA agarose was supplied by Qiagen (Santa Clarita, CA). Amersham Biosciences (Pittsburgh, PA) provided the FPLC system and the columns for purification. P81 cellulose papers were obtained from Whatman (Piscataway, NJ). ATP was purchased from Roche (Indianapolis, IN). Radiolabelled [γ - 32 P]-ATP was obtained from Perkin Elmer (Waltham, MA). ADP was from MP Biomedicals (Solon, OH).

Enzyme expression and purification

Methods for eEF-2K and calmodulin purification will be described elsewhere (45).

Peptide synthesis

Peptide substrate (Pep-S, Acetyl-RKKYKFNEDTERRRFL-amide) and peptide inhibitor (Pep-I, Acetyl-RKKYKFNEDAERRRFL-amide) were synthesized and purified by HPLC at the Institute for Cell and Molecular Biology at the University of Texas at Austin. The peptides were raised in 25 mM HEPES, pH 7.5 and were verified by MALDI. The concentration was determined based on the absorbance at 280 nm (OD_{280}) using the extinction coefficient of $1280 \text{ cm}^{-1}\text{M}^{-1}$ and path length of 1 cm.

Two substrate kinetics assay

Assays were performed in 100 μ l volume at 30 °C in assay buffer (25 mM HEPES pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 1.5 mM CaCl_2 , 10 mM MgCl_2 , 1 μ M CaM, 40 μ g/ml BSA) containing 10 nM eEF-2K, 0–1000 μ M [γ - 32 P]-ATP (specific activity = 1000 cpm/pmol), and 0–720 μ M Pep-S (Acetyl-RKKYKFNEDTERRRFL-amide). Reaction mixtures were prepared and kept on ice until the time of assay. Reaction mixtures were incubated for 10 minutes at 30 °C and the reactions were started with the addition of [γ - 32 P]-ATP. 10 μ l aliquots were taken and spotted onto P81 cellulose papers at fixed time point intervals. The papers were washed with 50 mM phosphoric acid (5 times for 10 minutes each) and then dried following acetone wash. The amounts of radiolabelled phospho-peptides were determined by counting the associated counts/min on a scintillation counter (Packard 1500) at a σ value of 2. eEF-2K requires the binding of calmodulin and the autophosphorylation of Thr-348 to achieve full activity (Tavares, manuscript in preparation). Under the conditions of the kinetic experiments all plots of product *versus* time are linear. Observed rates were independent of the order of addition of substrates.

Product and dead-end inhibition assay

Inhibition assays with product ADP or the dead-end peptide inhibitor (Pep-I, Acetyl-RKKYKFNEDAERRRFL-amide) were conducted the same way as above. First ATP was varied (12.5–125 μ M) with Pep-S concentration fixed at 50 μ M. Then Pep-S was varied (15–90 μ M) with ATP concentration fixed at 50 μ M. The concentrations used in the inhibition assays for ADP were 0–4 mM and for Pep-I were 0–500 μ M.

Synthesis of 1-benzyl-3-cetyl-2-methylimidazolium iodide (NH125)

NH125 was synthesized according to the reported procedure (50) with some modifications. 1) *Synthesis of cetyl-2-methylimidazole*. 2-Methylimidazole (10 g, 122 mmol, 10 eq) in chloroform (150 mL) was stirred for 10 minutes at 70 °C. Bromohexadecane (7.46 mL, 12.2 mmol, 1 eq) in chloroform (15 mL) was added dropwise to the above solution and refluxed for overnight with stirring. A large excess 2-Methylimidazole was used to avoid the unfavorable doubly alkylated side product formation. The reaction mixture was dissolved with water (1 L- to remove excess 2-Methylimidazole) and organic layer was extracted with EtOAc (50 mL $\times$ 3). Combined organic layer was washed with water (50 mL $\times$ 3), dried with MgSO₄ and solvent was evaporated *in vacuum* to obtain the crude product. After purification by flash chromatography (chloroform/methanol 10:1) product was obtained as an off-white powder (2.7 g, 72%). 2) *Synthesis of 1-benzyl-3-cetyl-2-methylimidazolium iodide (NH125)*. Potassium iodide (14.18g, 10 eq) and benzyl bromide (2.92 g, 17.08 mmol, 2 eq) in chloroform (100 mL) was refluxed for 15 min. Cetyl-2-methylimidazole (2.62 g, 8.54 mmol, 1 eq) in chloroform (40 mL) was added dropwise to the above solution and refluxed overnight. Solvent was evaporated *in vacuum* to obtain the crude product. After purification by flash chromatography (chloroform/methanol 7:1) and recrystallization using ethylacetate product was obtained as a white solid (1.74 g, 52.4%). ¹H (400 MHz DMSO-d₆) δ 7.72 (s, 2H), 7.43-7.33 (m, 3H), 7.3 (d, 2H, J = 6.4 Hz), 5.39 (s, 2H), 4.1 (t, 2H, J = 7.2 Hz), 2.60 (s, 3H), 1.71 (br, 2H), 1.21 (br, 26H), 0.83 (t, 3H, J = 6.8 Hz); ¹³C (100.6 MHz DMSO-d₆) δ 144.76, 135.0, 129.44, 128.92, 128.13, 122.13, 122.03, 51.03, 48.11, 31.73, 29.49, 29.45, 29.39, 29.36, 29.31, 29.15, 26.06, 22.54, 14.41, 9.94 (some C are overlapping). ESI-MS [M+H]⁺ calculated, 397.357; observed, 397.5.

In-vitro Assays with NH125

NH125 was synthesized and its identity and purity verified by ESI-MS and NMR (figures S1 and S2 under supporting materials). Dose response inhibition assays against eEF-2K were performed using 2 nM eEF-2K, 50 μ M [γ -³²P]-ATP, 30 μ M Pep-S and various concentrations of NH125 (0–100 μ M). To test the sensitivity against detergent, the same above assays were also performed in the presence of 0.1% triton-X-100. Similarly competition assays were conducted using 2 nM eEF-2K, 30 μ M Pep-S and varying concentrations of NH125 (0–100 μ M) at different fixed concentrations of [γ -³²P]-ATP (50 μ M or 2 mM). In all cases, the enzyme was pre-incubated with [γ -³²P]-ATP for 15 minutes before initiating the reaction with NH125/Pep-S. Assay buffer for eEF-2K contained 25 mM HEPES pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 150 μ M CaCl₂, 1 μ M CaM, 40 μ g/ml BSA, 2 mM DTT, 10 mM MgCl₂ and 5% DMSO. The CaCl₂ concentration used in these assays is 10-fold lower than in the two-substrate kinetic assays described above. Under these conditions the activity of eEF-2K is slightly higher, however this has no effect on the IC₅₀ for NH125. In order to test the specificity of NH125, dose response inhibition assays were also performed against TRPM7 and ERK2. Dose response assays against ERK2 were performed using 2 nM ERK2, 500 μ M [γ -³²P]-ATP, 20 μ M Ets1 and various concentrations of NH125 (0–500 μ M). Dose response assays against TRPM7 were performed using 25 nM TRPM7, 500 μ M [γ -³²P]-ATP, 20 μ M TRPM7-peptide (sequence Ac-RKKYRIVWKSIFRRFL-NH₂) and various concentrations of NH125 (0–500 μ M).

Assay buffer for TRPM7 and ERK2 contained same components to that of eEF-2K without calcium and calmodulin.

Effect of NH125 on eEF2 phosphorylation

Purification of wheat germ EF2 is already described elsewhere (51). Assays were performed at 30 °C in assay buffer (25 mM HEPES pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 150 μ M CaCl₂, 1 μ M CaM, 40 μ g/ml BSA, 2 mM DTT, 10 mM MgCl₂ and 5% DMSO) containing 10 nM eEF-2K, 1 μ M eEF2, 7.5 μ M ATP and different concentrations of NH125 (0, 50, 500 and 5000 nM). Assay buffer containing eEF-2K (10 nM) and wheat germ EF2 (1 μ M) were pre-incubated for 30 minutes in the presence of the above mentioned concentrations of NH125. Then the assays were started with the addition of [γ -³²P]-ATP. Reactions were allowed to proceed for 10 minutes and were quenched by addition of SDS-PAGE sample loading buffer followed by heating for 10 min at 95 °C. It was made sure that the assay rate after 10 minutes corresponded to the initial rate of the reaction. The quenched samples were resolved by running in 10% SDS-PAGE and staining with Coomassie Brilliant Blue dye. Upon drying the gel, the bands corresponding to phospho-EF2 were excised and the associated counts were quantified on a scintillation counter (Packard 1500) at a σ value of 2.

Cell lines and culture conditions

HEK 293T, MDA-MB-231 (breast adenocarcinoma) and A549 (lung carcinoma) cell lines were all obtained from American Type Culture Collection (Manassas, VA). HEK 293T cell line was cultured in DMEM, while MDA-MB-231 and A549 were cultured in DMEM/F12. Both media were supplemented with 10% FBS, 50 units/mL penicillin and 50 μ g/mL streptomycin. Cell cultures were maintained at 37 °C in a humidified incubator containing 5% CO₂. All cell culture reagents were from Invitrogen.

Commercial antibodies

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-phospho-eEF2 (Thr56) antibody (#2331, 1:2000), anti-eEF2 antibody (#2332, 1:2000), anti-eEF2K antibody (#3692, 1:2000) and anti-mouse IgG, HRP-linked antibody (#7076, 1:2000). Anti-Actin, clone C4 antibody (MAB1501, 1:10000) was obtained from Millipore (Billerica, MA) and Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (#172-1019, 1:2000) was from Bio-Rad.

Treatment of cells with NH125

For analysis of effects of NH125 on phosphorylated eEF2 levels in cells, cells were seeded in 6-well plates at a density of 0.8×10^6 cells per well in 2 mL medium. After 36 h, cells were treated with 4 μ M NH125 (with a final DMSO concentration of 0.1%) for 0, 3, 6 and 12 h. Cells in the control wells were treated with DMSO (final concentration of 0.1%) for the same lengths of time.

Western blot analysis

Following treatments, cells were washed twice in PBS (pH 7.4) (Invitrogen), and lysed in ice-cold M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL) containing Halt Protease and Phosphatase Inhibitor (Thermo Fisher Scientific). Lysates were clarified by centrifugation at $15,000 \times g$ for 15 min. Total protein concentrations for each sample were determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (7.5 μ g) from cell lysates were resolved by 10% SDS-PAGE and were transferred to Amersham Hybond-P PVDF membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/

Tween 20 (TBST), and incubated with primary antibodies anti-phospho-eEF2 (Thr56), anti-eEF2 or anti-eEF2K at 4 °C overnight. The membranes were washed with TBST and incubated with the secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP Conjugate at room temperature for 2 h. To determine the total levels and phosphorylation status of specific proteins, chemiluminescent detection was performed with Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare). Anti-Actin, clone C4 and anti-mouse IgG, HRP-linked antibodies were used to monitor total actin levels as a loading control.

Data analysis

Initial velocity data for substrate and inhibition kinetics were fitted globally using a non-linear least squares approach using Scientist (Micromath). For some fitting Kaledagraph 3.5 (Synergy software) was used. Initial rate data were fitted globally to equation 1 which describes a mechanism of competitive substrate inhibition through a dead-end EB complex (see page 819, eqn. IX-384 of reference (52)). The initial rate data displaying competitive, uncompetitive and mixed-type inhibition patterns at sub-saturating concentrations of co-substrates were fitted globally to equations 2, 3 and 4 respectively. (52). A slope replot describing a mechanism of competitive substrate inhibition through a dead-end EB complex was fitted to equations 5 (see page 821, eqn. IX-386 of reference (52)). An intercept replot describing a mechanism of competitive substrate inhibition through a dead-end EB complex was fitted to equations 6 (see page 821, Fig. IX-61 of reference (52)). A slope replot at different fixed concentrations of Pep-I was fitted to equation 7 (see page 173, eqn. IV-16 of reference (52)). Dose response IC₅₀ curves were fitted using equation 8 (53).

$$V = \frac{V_{\max}[A][B]}{K_{iB}K_{mB}(1 - [A]/K_{iB}) - K_{mA}[B](1 + [B]/K_{iU}) + K_{mB}[A] + [A][B]} \quad (1)$$

$$V = \frac{V_{\max}^{\text{app}}[S]}{K_{mB}^{\text{app}}(1 + [I]/K_{iC}^{\text{app}}) - [S]} \quad (2)$$

$$V = \frac{V_{\max}^{\text{app}}[S]}{K_{mB}^{\text{app}} + [B](1 + I/K_{iU}^{\text{app}})} \quad (3)$$

$$V = \frac{V_{\max}^{\text{app}}[S]}{K_{mB}^{\text{app}}(1 + [I]/K_{iC}^{\text{app}}) - [S](1 + I/K_{iU}^{\text{app}})} \quad (4)$$

$$\text{Slope}_{1/A} = \frac{K_{mA}}{V_{\max}K_{iU}}[B] + \frac{K_{iA}K_{mB}}{V_{\max}}[B]^{-1} + \frac{K_{mA}}{V_{\max}}\left(1 + \frac{K_{iA}K_{mB}}{K_{mA}K_{iB}}\right) \quad (5)$$

$$\frac{1}{V_{\max}^{\text{app}}} = \frac{K_{mB}}{V_{\max}} \frac{1}{[B]} + \frac{1}{V_{\max}} \quad (6)$$

$$\text{Slope}_{1/A} = \frac{K_{mA}^{\text{app}}}{V_{\max}^{\text{app}}K_{iC}^{\text{app}}}[I] + \frac{K_{mA}^{\text{app}}}{V_{\max}^{\text{app}}} \quad (7)$$

$$v_i = \frac{v_0}{(1 + I/IC_{50})^h} \quad (8)$$

The kinetic parameters in deriving above equations are as follows: v , observed velocity; $V_{\max}$, maximum initial velocity; $[A]$, concentration of substrate A; $[B]$, concentration of substrate B; K_{iA} , dissociation constant for substrate A; K_{mA} , Michaelis-Menten constant for substrate A; K_{mB} , Michaelis-Menten constant for substrate B; K_{iB} , inhibition constant for EB complex; $[S]$, concentration of varied substrate S; K_{mS} , apparent Michaelis-Menten constant for substrate S; $[I]$, concentration of inhibitor I; K_{iC} , inhibition constant for EI (Enzyme-Inhibitor) complex; K_{iU} , inhibition constant for ESI complex; v_i , observed velocity in the presence of inhibitor; v_0 , observed velocity in the absence of inhibitor; IC_{50} , inhibitor concentration required to achieve 50% inhibition; h , hill coefficient. Apparent kinetic constants obtained at sub-saturating substrate concentrations are designated by 'app', for example, $V_{\max}^{app}$, K_{Ci}^{app} , K_{iC}^{app} and K_{iU}^{app} .

Results

A recent structural study of the dictyostelium protein kinase myosin heavy chain kinase suggested a possibly catalytic mechanism through a phospho-protein intermediate (54). As MHCK, is an atypical protein kinase with a predicted structural similarity to eEF-2K (33% sequence identity in the catalytic domain) we were interested in the possibility that eEF-2K catalyzes phosphoryl transfer to the peptide through a phosphoenzyme intermediate.

eEF-2K, is reported to phosphorylate the peptide RKKFGAEKT*KAKEFL, with a K_{Ci}^{app} of 580 μ M (55). Here we have utilized a similar peptide, Ac-RKKYKFNEDT*ERRRFL-NH₂ (Pep-S), to assay the activity of eEF-2K (45). Both peptides contain a basic residue at the +3 position, which is reported to be important for efficient peptide phosphorylation by eEF-2K (55) and corresponds to the Arg residue in the +3 position relative to Thr-56, the major phosphorylation site in eEF2 (5). To define the basic kinetic mechanism we examined the dependence of product formation on the concentrations of substrates by the method of initial rates (52). The appearance of product with time was linear under all experimental conditions and was highly reproducible, to within 10%.

Sequential or ping-pong kinetics

To explore the possibility of a phospho-enzyme intermediate we examined whether eEF-2K phosphorylates Pep-S through a ping-pong mechanism whose reciprocal plot exhibits a characteristic family of parallel lines (Mechanism A in Figure 2). According to this mechanism, MgATP binds the enzyme, E, and reacts to form the phospho-intermediate, E~P and MgADP. MgADP dissociates *before* B binds E~P. Subsequent phospho-transfer gives B~P which then dissociates from E. Initial rate studies were performed using ATP (0–1000 μ M) and the peptide, Ac-RKKYKFNEDTERRRFL-NH₂, (Pep-S) (0–720 μ M). Due to substrate inhibition at high peptide concentrations (described below), analysis of double reciprocal plots were performed at low peptide concentrations (0 – 90 μ M). The double reciprocal plots of $1/v$ against $1/[ATP]$ (Figure 3A) and $1/v$ against $1/[Pep-S]$ (Figure 3B) converge to the left of the $1/v$ -axis. Such patterns of convergent lines are not consistent with a ping-pong mechanism (Figure 2A), rather they are consistent with sequential mechanisms where both substrates must bind before any product dissociates (e.g. Mechanism B and C in Figure 2). While a sequential mechanism does not rule out a kinetically significant phospho-enzyme intermediate, it requires that ADP cannot dissociate from the enzyme until after Pep-S is bound. Mechanism B in Figure 2 shows two possible sequential mechanisms that conform to such a restriction.

Substrate inhibition

When analyzing the velocity dependence of eEF-2K on substrates we noted that at higher concentrations of Pep-S its velocity decreases with increasing Pep-S (Figure 3C). In contrast, MgATP exhibits a normal saturation dependence upon increasing concentration and does not inhibit the enzyme. In general, substrate inhibition occurs when substrates add to the wrong enzyme form and usually becomes evident at high substrate concentrations. As it can often provide additional mechanistic insight we decided to determine its mechanism. To evaluate substrate inhibition of eEF-2K by Pep-S the dependence of the slope ($\text{slope}_{1/\text{ATP}}$) and $1/V_{\text{max}}^{\text{app}}$ of the lines in Figure 3A were first assessed as a function of $1/[\text{Pep-S}]$. Figure 3D shows that the slope ($\text{slope}_{1/\text{ATP}}$) first decreases, passes through a minimum and then increases as $1/[\text{Pep-S}]$ decreases. In contrast, $1/V_{\text{max}}^{\text{app}}$ decreases linearly and intercepts on the ordinate at $1/V_{\text{max}}$ (Figure 3E). These data are consistent with a mechanism whereby Pep-S competitively inhibits MgATP (52). Two possible mechanisms that can account for such competitive substrate inhibition are shown in Figure 4. According to the mechanism shown in Figure 4A the binding of Pep-S (B) prevents the binding of ATP within the active site, therefore, MgATP must bind the enzyme before Pep-S (52). In the mechanism shown in Figure 4B ATP is prevented from binding the active site when Pep-S (B) binds the enzyme, E, at a second site. In both cases Pep-S is competitive with ATP binding, and inhibition can be overcome by high concentrations of MgATP. The most significant difference between the two mechanisms is that in mechanism 4B the peptide binds two sites on the enzyme, whereas in mechanism 4A there is only one. It should be noted that the data in Figure 3C are fitted globally to equation 1, which describes the mechanism in Figure 4A. The parameters for the best-fit are given in Table 1. Further support for this mechanism is presented below.

To further assess the mechanism, kinetic experiments were performed at different fixed concentrations of Pep-I and ADP. Pep-I has the same sequence Ac-RKKYKFNEDA*ERRRFL-NH₂ which is identical to Pep-S, except for a threonine to alanine substitution, thus it may be considered to be a dead-end inhibitor that binds eEF-2K in a similar manner to Pep-S. ADP is a product of the kinase reaction and is expected to bind eEF-2K in a similar manner to ATP. ADP exhibits competitive inhibition with respect to ATP (Figure 5A) and exhibits mixed-type inhibition with respect to Pep-S (Figure 5B). Pep-I exhibits competitive inhibition towards Pep-S (Figure 5C) and mixed-type inhibition towards ATP (Figure 5D). This *pattern* of inhibition (Figure 5; summarized in Table 2) is consistent with either mechanism shown in Figure 4. However, if it is assumed that Pep-S (B) and Pep-I (I) bind eEF-2K in a similar manner, and that Pep-I can be substituted for Pep-S in Figure 4 the two mechanisms may be differentiated on the basis that mechanism A involves the binding of one molecule of Pep-I to eEF-2K, while mechanism B involves the binding of two molecules of Pep-I. The linear plot of $\text{slope}_{1/\text{ATP}}$ vs $[\text{Pep-I}]$ over the range of 0–500 μM Pep-I (Figure 5E) supports the notion that eEF-2K phosphorylates Pep-S through mechanism A (52), because such a plot is expected to be non-linear for mechanism B. The lines through the data correspond to the best fit to the appropriate equations as described in the legend to Figure 5.

Inhibition of eEF-2K

1-benzyl-3-hexadecyl-2-methyl-1-*H*-imidazol-3-ium iodide (NH125) (**4** in Figure 1) is reported to specifically inhibit eEF-2K with an IC₅₀ of 60 nM in an *in vitro* kinase assay (40). It is also reported to decrease the level of eEF2 phosphorylation in cells (40–44) and to decrease viability of a number of cancer cell lines with an EC₅₀ of ~ 1–5 μM (40). However, its mechanism of inhibition has not been demonstrated. Therefore, we decided to evaluate the mechanism of inhibition of eEF-2K by NH125. We synthesized an authentic sample of NH125 (see Materials and Methods) and confirmed its structure by mass spectrometry, ¹H

NMR and ^{13}C NMR (see Figures S1 and S2 under supporting information). The ability of NH125 to inhibit eEF-2K was assessed using the same *in vitro* kinase assay as described above. Dose-response curves for NH125 were obtained at several concentrations of ATP to evaluate whether NH125 competes with the ATP binding site on the enzyme. If NH125 competed with ATP a right shift in the dose response curve would be expected as the concentration of ATP is increased. Figure 6A shows a dose-response curve exhibiting a steep dependence on the concentration of NH125 (Hill coefficient of 3.7). Dose-response curves obtained at 50 and 2000 μM are essentially superimposable (Figure 6B), suggesting that NH125 does not compete with ATP. Similar results were observed when two concentrations of peptide substrate (10 and 100 μM) were used (Figure 6C) and a 20-fold increase in the concentration of calmodulin from 0.1 – 2 μM resulted in a slight (< 2-fold) increase in the observed IC_{50} (data not shown). Notably, the IC_{50} of ~18 μM obtained in these experiments is 300-fold higher than the previously reported value of 60 nM. As the presence of small amounts of non-ionic detergents can often attenuate the effects of compounds acting as non-specific aggregators (46–48) assays were performed in the presence (0.1%) or absence of triton X-100 to test the possibility that NH125 is an aggregator. Increasing the concentration of triton X-100 from 0 to 0.1% clearly resulted in an increase in the magnitude of the observed IC_{50} from 18 μM to 452 μM (Figure 6A). When NH125 was assessed against TRPM7 (a related protein kinase) and ERK2 (an unrelated protein kinase), it was also found to inhibit both kinases with an IC_{50} of 55 and 70 μM , and Hill coefficients of 1.6 and 3.6, respectively (data not shown). Taken together, these data suggest that NH125 is a non-specific colloidal aggregator (46–48). Since NH125 is reported to potently inhibit the phosphorylation of eEF2, we tested the effect of NH125 (0–5 μM) on the phosphorylation of wheat germ eEF2 by eEF-2K *in-vitro*. As seen in Figure 6D, NH125 (0–5 μM) has no detectable effect on the rate of phosphorylation of eEF2 (1 μM) in the presence of ATP (7.5 μM) (See Materials and Methods), consistent with the kinetic analysis with the peptide. As predicted, inhibition is observed at higher concentrations of NH125 (12.5 – 50 μM) (Figure S3). In summary, a steep Hill coefficient, an absence of competition against ATP or peptide and a lack of specificity are consistent with NH125 being a promiscuous aggregator (46–48). The observed rate of decay of the autocorrelation function of a solution of 50 μM NH125 measured by dynamic light scattering provides further support for the presence of aggregate material. Comparison with a solution of tetraiodophenolphthalein, which forms aggregates of approximately 150 nm in diameter (46), suggests that NH125 forms relatively smaller particles (data not shown). Further characterization of the particulate properties of NH125, which is not the focus of this manuscript, is underway.

To examine whether the activity of NH125 was associated with the down-regulation of eEF2 phosphorylation, we examined the ability of 4 μM NH125 to affect the phosphorylation of eEF2 on Thr-56 in MDA-MB-231 breast cancer, A549 lung cancer and HEK-293T cell lines over a period of 0–12 hours (Figure 7). The expression level of eEF2, eEF-2K and actin were also assessed. Contrary to previous reports (40–44), we observed no signs of decrease in the phospho-eEF2 levels in the presence of NH125 in any of the tested cell lines (Figure 7). In fact, the phospho-eEF2 levels in all three cell lines were increased with NH125 treatment when compared to the DMSO treated cell lines at corresponding timepoints. Recently, *Chen et.al.* also reported an induction in eEF-2 levels in a variety of cancer cell lines (H1299 non-small cell lung carcinoma, PC3 prostate cancer, HeLa cervical cancer, H460 non-small cell lung carcinoma and C6 rat glioma) after treatment with NH125 for 6 hours (49). In our case, the phospho-eEF2 induction upon NH125 treatment was observed most severely in HEK293T cells and to a lower extent in MDA-MB-231 and A549 cells. In most cases, the induction of eEF2 phosphorylation was also more severe with time. HEK293T cells showed the highest amount of increase in phospho-eEF2 over the period of 12 hours. The increase in eEF-2 induction was less prominent in the other two cell lines

where the increase was observed only over a period of 6 hours. In addition to the induction of phospho-eEF2, increase in the levels of eEF-2K and eEF-2 were also observed in all cell lines, which in most cases were time dependent and correlated with the amount of phospho-eEF2 induction. Although some induction of phospho-eEF2 (in HEK293T cells), and eEF-2K and eEF-2 (more significant in HEK293T cells; lesser extent in A549 cells) were also observed in DMSO treated controls, their corresponding levels were significantly higher after treatment with NH125. Taken together, these data suggest that NH125 induces eEF-2 phosphorylation through a complex process, whose robustness appears to vary depending on cell type.

Discussion

Kinetic Mechanism of eEF-2K

The purpose of this study was to first assess the kinetic mechanism of recombinant human eEF-2K using a peptide substrate and then to determine its mechanism of inhibition by NH125. NH125 is a histidine kinase inhibitor that has been utilized in a number of recent studies as a specific pharmacological inhibitor of eEF-2K (40–44). However, despite its reported potency and specificity its mechanism of inhibition has not been determined and an activity profile against a panel of protein kinases is not available. In this study we used a form of eEF-2K (45) that exhibits some 4000-fold higher activity towards eEF2 than a previously reported preparation (55). Our kinetic characterization of eEF-2K with the peptide substrate unambiguously demonstrates a sequential kinetic mechanism. This is significant, because a recent structure of MHCK from dictyostelium provides some support for a mechanism through a phosphoenzyme intermediate. Like eEF-2K, MHCK is an atypical protein kinase with 34% sequence identity to human eEF-2K in the catalytic domain. The structure of MHCK in the presence of a peptide substrate and ATP revealed the covalent attachment of a phosphate to Asp-766 a conserved aspartate residue in the active site of MHCK, along with a bound AMP (54). MHCK was also shown to catalyze the slow exchange of ATP and ADP at the active site. The sequential mechanism favors a direct mechanism of phosphoryl transfer between ATP and the peptide hydroxyl group, rather than a reaction through a phospho-enzyme intermediate, because the latter reaction is predicted to exhibit ping-pong kinetics. It should be noted however, that a mechanism through a phospho-enzyme intermediate can be sequential if the release of ADP occurs after the peptide substrate binds (see for example Figure 2B).

Our kinetic analysis supports a mechanism of competitive substrate inhibition where Pep-S binds eEF-2K and prevents ATP from binding. The simplest explanation for this observation is that the peptide occludes the ATP binding pocket when bound to the enzyme. Whether this is related to how the enzyme is regulated by the binding of calmodulin remains to be determined. Competitive substrate inhibition while quite a rare mechanism for a protein kinase is not unprecedented (56), although the majority of protein kinases are reported to phosphorylate substrates through a random-order mechanism with little substrate inhibition (e.g. (57–60)).

NH125 is a promiscuous aggregator

NH125 was first identified as a bacterial histidine kinase inhibitor and was later reported to be a potent inhibitor of GST-eEF-2K *in vitro* (40), however the mechanism of inhibition was not determined. Recently, several laboratories have utilized NH125 to evaluate the role of eEF-2K in various biological processes. Therefore it is essential that its mechanism be understood. We prepared an authentic sample of NH125 following a previously reported procedure (50) with some modifications (See Materials & Methods). Surprisingly, we found no evidence to suggest that NH125 exhibits potent inhibition of eEF-2K as previously

reported (40). Rather, an examination of dose-response curves of observed rate *versus* the concentration of NH125 revealed an IC_{50} of 18 μM for the inhibition of peptide phosphorylation (Figure 6A). Furthermore, the dependence of the observed rate on the concentration of NH125, was best fit to a dose-response curve described by a Hill coefficient of approximately 3.7 (Figure 6A). Further analysis showed the activity of NH125 to be sensitive to added detergent (triton X-100), an observation that further suggests that NH125 inhibits eEF-2K through aggregation. Many organic compounds form colloid-like aggregates in aqueous solution and non-specific inhibition of enzymes *in vitro* due to aggregate formation is a relatively common occurrence (61). NH125 (Figure 1) has a structure consistent with detergent properties i.e it contains a polar head and long hydrophobic alkyl tail. Typically, the aggregation process is significant in the micromolar range (62). The previous report suggested that NH125 inhibited the phosphorylation of eEF2 by GST-eEF-2K with an IC_{50} of 60 nM (40). This observation is difficult to reconcile with the reported assay conditions where the enzyme was assayed at 400 nM, a concentration substantially above the reported IC_{50} . It is perhaps also significant that the eEF-2K used to originally assess NH125, is reported to be some 10^6 -fold lower than the activity of the eEF-2K used in the present study (a specific activity of $1.5 \text{ pmol min}^{-1} \text{ mg}^{-1}$ was reported, which corresponds to a k_{cat} of $2.7 \times 10^{-6} \text{ s}^{-1}$) (40). Taken together our data provides strong evidence that NH125 does not inhibit eEF-2K through a conventional mechanism of inhibition, but instead inhibits the enzyme through the reversible formation of a colloid.

Cellular effect of NH125

A number of cellular studies suggest that NH125 inhibits eEF-2K activity in cells. For example, Arora et al. first reported that a 1 μM concentration of NH125 strongly inhibits the phosphorylation of eEF2 in C6 glioma cells after incubation for 12 hours (40) and Khan et al. reported that a 2 hour pre-incubation of human microvascular endothelial cells (HMVECs) with a 200 nM concentration of NH125 resulted in the inhibition of resveratrol-induced eEF2 phosphorylation (42). NH125 has also been reported to inhibit eEF-2K *in vivo*. For example, Autry et al. reported recently that administration of a 5 mg/kg dose of NH125 to mice for 30 minutes resulted in an observable decrease in eEF2 phosphorylation in the hippocampus (43). Given that NH125 inhibits eEF-2K through a nonspecific mechanism *in vitro* the question then arises as to how NH125 inhibits eEF-2K both in cell lines and *in vivo*. When we examined the ability of 4 μM NH125 to inhibit the phosphorylation of eEF2 in MDA-MB-231 breast cancer, A549 lung cancer and HEK-293T cell lines for up to 12 hours, no inhibition was observed (Figure 7). In fact, the induction of the phospho-eEF2 was observed in all cell lines after treatment with NH125. Similar results were also reported in a recent study by *Chen et al.* using H1299 non-small cell lung carcinoma, PC3 prostate cancer, HeLa cervical cancer, H460 non-small cell lung carcinoma and C6 rat glioma cell lines after treatment with NH125 for 6 hours (49). Although our data further indicated that the induction of eEF-2 levels by NH125 may be due to the effect of NH125 on the expression of eEF-2K and eEF-2, both our data as well as the reported data from *Chen et al.* (49) suggest that the NH125 does not inhibit the phosphorylation of eEF2, and hence is not an inhibitor for eEF-2K in cells.

In summary, we have shown that eEF-2K phosphorylates a peptide substrate through a sequential mechanism. The peptide substrate inhibits the binding of ATP and must bind after ATP binds in order to form a productive ternary complex. The ability to evaluate potential inhibitors using the peptide substrate revealed that NH125 a frequently utilized ‘inhibitor’ of eEF-2K (40) does not in fact inhibit eEF-2K with high potency. In fact it most likely inhibits eEF-2K *in vitro* through a non-specific aggregation process. In addition, NH125 failed to show any inhibition of eEF-2 phosphorylation in variety of cancer cell lines, supporting the argument that NH125 is not a cellular inhibitor of eEF-2K.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

The abbreviations and definitions used are:

eEF-2K	elongation factor-2 kinase
eEF2	elongation factor-2
CaM	calmodulin
SAPK	stress activated protein kinase
p90 RSK	p90 ribosomal S6 kinase
ERK	extracellular signal-regulated protein kinase
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
Rsk-2	Mitogen activated protein kinase-activated protein kinase
PKC	protein kinase C
BSA	bovine serum albumin
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycerol-bis[2-aminoethyl ether]-N,N,N'-tetraacetic acid
PMSF	phenylmethanesulphonyl fluoride
TPCK	tosylphenylalanylchloromethane
IPTG	isopropyl- β -D-thiogalactopyranoside
ATP	adenosine triphosphate
ADP	adenosine diphosphate
ESI	electrospray ionization
MALDI	matrix-assisted laser desorption/ionization
HPLC	high-performance liquid chromatography
Pep-S	peptide substrate (Acetyl-RKKYKFNEDTERRRFL-amide)
Pep-I	peptide inhibitor (Acetyl-RKKYKFNEDAERRRFL-amide)

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I **II** **III** **IV****Figure 1. Known eEF-2K inhibitors**

The 1,3-selenazine (**1**) is a potent ATP-competitive inhibitor of eEF-2K and is reported to block the phosphorylation of eEF2 in cells at a concentration of 20 μ M but is unstable to thiols (37). Rottlerin (**2**) is a nonspecific inhibitor of multiple protein kinases (63, 64). The thieno[2,3-b]pyridine (**3**) is an ATP-competitive inhibitor of eEF-2K that lacks potency in cells (39). The mechanism of inhibition of NH125 (**4**), which was first identified as a histidine kinase inhibitor (50, 65) and later as an inhibitor of eEF-2K (40) has not been determined.

A ping-pong **B** sequential, phosphoenzyme intermediate **C** sequential, no intermediate **Figure 2. Bisubstrate kinetic mechanisms**

A. Ping-pong mechanism with a phospho-enzyme intermediate. **B.** Sequential mechanisms with a phospho-enzyme intermediate; a) random-order sequential mechanism, b) ordered sequential mechanism with ATP binding first. **C.** Sequential mechanisms with no phospho-enzyme intermediate; a) random-order sequential mechanism, b) ordered sequential mechanism with ATP binding first.

Figure 3. Dependence of reaction velocities on substrate concentration

Initial velocities were measured using 10 nM eEF-2K. **A)** $1/v$ versus $1/[ATP]$. Initial velocities were measured using several fixed concentrations of Pep-S (as indicated in μM) and varied concentrations of ATP (12.5–125 μM). Inset: $1/v$ versus $1/[ATP]$ for 360 μM and 720 μM Pep-S showing intersection at $1/v_{\text{max}}^{\text{app}}$. **B)** $1/v$ versus $1/[Pep-S]$. Initial velocities were measured using several fixed concentrations of ATP (as indicated in μM) and varied concentrations of Pep-S (15–90 μM). The lines correspond to the best fit to eqn. 1 according to the parameters in Table 1. **C)** velocity, v , versus $[Pep-S]$. Initial velocities were measured using several fixed concentrations of ATP (as indicated in μM) and varied concentrations of

Pep-S (15–720 μ M). The lines correspond to the best fit to eqn. 1 according to the parameters in Table 1. **D)** Slope replot of Figure 3A. The line corresponds to the best fit to eqn. 5 according to the parameters in Table 1. **E)** Intercept replot of Figure 3A. The line corresponds to the best fit to eqn. 6 according to the parameters in Table 1.

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Figure 5. Dependence of reaction velocities on ADP and Pep-I

A) Competitive inhibition of ADP toward ATP. Initial velocity studies at 50 μM Pep-S in the presence of several fixed concentrations of ADP (indicated above in μM) and varied concentrations of ATP (12.5–125 μM). The lines correspond to the best fit to eqn. 2 where $V_{\text{max}}^{\text{app}} = 0.024 \pm 0.001 \mu\text{M s}^{-1}$, $K_{\text{mB}}^{\text{app}} = 22 \pm 2.5 \mu\text{M}$, and $K_{\text{ic}}^{\text{app}} = 464 \pm 50 \mu\text{M}$. **B)** Mixed inhibition of ADP toward Pep-S. Initial velocity studies at 50 μM ATP in the presence of several fixed concentrations of ADP (indicated above in μM) and varied concentrations of Pep-S (30–90 μM). The lines correspond to the best fit to eqn. 4 where $V_{\text{max}}^{\text{app}} = 0.10 \pm 0.002 \mu\text{M s}^{-1}$, $K_{\text{mB}}^{\text{app}} = 300 \pm 7 \mu\text{M}$, $K_{\text{ic}}^{\text{app}} = 4300 \pm 2100 \mu\text{M}$, and $K_{\text{iU}}^{\text{app}} = 350 \pm 220 \mu\text{M}$. **C)** Competitive inhibition of Pep-I toward Pep-S. Initial velocity studies at 50 μM ATP in the presence of several fixed concentrations of Pep-I (indicated above in μM) and varied concentrations of Pep-S (15–90 μM). The lines correspond to the best fit to eqn. 2 where $V_{\text{max}}^{\text{app}} = 0.11 \pm 0.002 \mu\text{M s}^{-1}$, $K_{\text{mB}}^{\text{app}} = 300 \pm 6 \mu\text{M}$, and $K_{\text{ic}}^{\text{app}} = 294 \pm 26 \mu\text{M}$. **D)** Mixed inhibition of

Pep-I toward ATP. Initial velocity studies at 50 μM Pep-S in the presence of several fixed concentrations of Pep-I (indicated above in μM) and varied concentrations of ATP (12.5–125 μM). The lines correspond to the best fit to eqn. 4 where

$$V_{\max}^{\text{app}} = 0.04 \pm 0.001 \mu\text{M s}^{-1}, K_{\text{mB}}^{\text{app}} = 26 \pm 2.5 \mu\text{M}, K_{\text{iC}}^{\text{app}} = 455 \pm 140 \mu\text{M}, \text{ and } K_{\text{iU}}^{\text{app}} = 300 \pm 110. \text{ E)}$$

Slope replot of Figure 5D. The line corresponds to the best fit to eqn. 7 where

$$V_{\max}^{\text{app}} = 0.04 \pm 0.001 \mu\text{M}, K_{\text{mB}}^{\text{app}} = 26 \pm 2.5 \mu\text{M}, \text{ and } K_{\text{iC}}^{\text{app}} = 455 \pm 140 \mu\text{M}.$$

Figure 6. Inhibition of eEF-2K by NH125

A) Dose-response curve for NH125 in the presence (■) or absence (●) of 0.1% triton X-100. Initial rates were measured using 2 nM eEF-2K, 50 μM ATP, 30 μM Pep-S, 0 or 0.1% triton X-100 and various concentrations of NH125 (0–100 μM). The lines correspond to the best fit to eqn. 8 according to an IC_{50} of 18 ± 0.25 μM and a Hill coefficient of 3.7 ± 0.14 . In the presence of triton, the IC_{50} increases to 452 ± 243 μM with a Hill coefficient of 1.0 ± 0.28 . **B)** Sensitivity of the dose-response curve to ATP. Initial rates were measured using 50 μM (●) or 2000 μM (■) ATP in the presence of 2 nM eEF-2K, 30 μM Pep-S and various concentrations of NH125 (0–100 μM). The lines correspond to the best fit to eqn. 8 according to IC_{50} values of 19 ± 1.0 and 21 ± 1.6 μM in the presence of 50 or 2000 μM ATP respectively. **C)** Sensitivity of the dose-response curve to Pep-S. Initial rates were measured

using 10 μM (●) or 100 μM (■) Pep-S in the presence of 2 nM eEF-2K, 500 μM ATP and various concentrations of NH125 (0–100 μM). The lines correspond to the best fit to eqn. 8 according to IC_{50} values of 37 ± 2.5 and 32 ± 5.3 μM in the presence of 10 or 100 μM Pep-S respectively. **D)** Effect of NH125 on the phosphorylation of wheat germ EF2 by eEF-2K. Phosphorylation reactions were performed in the presence of different concentrations of NH125 (0, 50, 500 or 5000 nM shown in lanes 1, 2, 3 and 4 respectively) for 10 minutes. Controls with no eEF-2K (lane 5) or no eEF2 (lane 6) are also shown. Phosphorylation reactions were quenched after 10 minutes, resolved by SDS-PAGE and quantified using scintillation counter.

Figure 7. Cellular activity of NH125 by Western blot analysis

A) MDA-MB-231 breast cancer, B) A549 lung cancer and C) HEK-293T cells were treated with or without NH125 (4 μ M) for the times indicated. The phosphorylation level of eEF2 (Thr-56) and the expression levels of eEF2, eEF-2K and actin (loading control) were measured by Western blot analysis using specific antibodies (See Materials and Methods).

Table 1
Kinetic parameters for the phosphorylation of Pep-S by eEF-2K

The parameters were obtained from global fitting of the kinetic data in Figure 3C for substrate phosphorylation to equation 1.

$V_{\max}$	$0.15 \pm 0.01 \mu\text{M/s}$
K_{ia}	$16.2 \pm 6.9 \mu\text{M}$
K_{mA}	$36.5 \pm 3.5 \mu\text{M}$
K_{mB}	$393 \pm 40 \mu\text{M}$
K_{iB}	$178 \pm 16 \mu\text{M}$

Table 2
Inhibition patterns for the phosphorylation of Pep-S by eEF-2K

Varied Substrate	Fixed Substrate	Inhibitor	Mechanism	$K_{IC}^{app}, \mu M$	$K_{IU}^{app}, \mu M$
ATP ^a	Pep-S ^b	ADP ^c	Competitive ^d	464 ± 50	
Pep-S ^e	ATP ^f	ADP ^c	Mixed ^g	4300 ± 2100	350 ± 220
Pep-S ^h	ATP ^f	Pep-I ⁱ	Competitive ^d	294 ± 26	
ATP ^a	Pep-S ^b	Pep-I ⁱ	Mixed ^g	455 ± 140	300 ± 110

^a12.5 – 125 μM
^b50 μM
^c0 – 4000 μM
^dBest fit of the data according to Equation 2 for competitive inhibition
^e30 – 90 μM
^f50 μM
^gBest fit of the data according to Equation 4 for mixed inhibition
^h15 – 90 μM
ⁱ0 – 500