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Treatment of Breast and Lung Cancer Cells with a N-7 Benzyl Guanosine Monophosphate Tryptamine Phosphoramidate Pronucleotide (4Ei-1) Results in Chemosensitization to Gemcitabine and Induced eIF4E Proteasomal Degradation

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Abstract

The development of cancer and fibrotic diseases has been shown to be highly dependent on dysregulation of cap-dependent translation. Binding protein eIF4E to N⁷-methylated guanosine capped mRNA has been found to be the rate-limiting step governing translation initiation, and therefore represents an attractive target for drug discovery. Our group has found that 7-benzyl guanosine monophosphate (7Bn-GMP) is a potent antagonist of eIF4E cap binding ($K_d = 0.8 \mu\text{M}$). Recent X-ray crystallographic studies have revealed that the cap-dependent pocket undergoes a unique structural change in order to accommodate the benzyl group. Unfortunately, 7Bn-GMP is not cell permeable. Recently, we have prepared a tryptamine phosphoramidate prodrug of 7Bn-GMP, 4Ei-1, and shown that it is a substrate for human histidine triad nucleotide binding protein (hHINT1) and inhibits eIF4E initiated epithelial–mesenchymal transition (EMT) by Zebra fish embryo cells. To assess the intracellular uptake of 4Ei-1 and conversion to 7Bn-GMP by cancer cells, we developed a sensitive assay using LC-ESI-MS/MS for the intracellular quantitation of 4Ei-1 and 7Bn-GMP. When incubated with the breast cancer cell line MDA-231 or lung cancer cell lines H460, H383 and H2009, 4Ei-1 was found to be rapidly internalized and converted to 7Bn-GMP. Since oncogenic mRNAs are predicted to have the highest eIF4E requirement for translation, we carried out chemosensitization studies with 4Ei-1. The prodrug was found to chemosensitize both breast and lung cancer cells to nontoxic levels of gemcitabine. Further mechanistic studies revealed that the expressed levels of eIF4E were substantially reduced in cells treated with 4Ei-1 in a dose-dependent manner. The levels of eIF4E could be restored by treatment with the proteasome inhibitor MG-132. Taken together, our results demonstrate that 4Ei-1 is likely to inhibit translation initiation by eIF4E cap binding by both antagonizing eIF4E cap binding and initiating eIF4E proteasomal degradation.

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Notes

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Graphical abstract

Keywords

cancer; fibrotic diseases; 7-benzyl guanosine monophosphate; eIF4E; 4Ei-1

INTRODUCTION

Aberrant regulation of cap-dependent translation is essential for the development of cancer and fibrotic diseases. After transport out of the nucleus, the eukaryotic initiation factor 4E (eIF4E) binds the 5'-cap of cellular mRNAs by displacing the nuclear 5'-cap binding complex (CBC), leading to formation of the eIF4F translation initiation complex. The eIF4F complex proceeds to scan mRNAs from the 5'-3' direction, unveiling the translation initiation codon. The assembly of the eIF4F complex is the rate-limiting step for cap-dependent protein translation and depends on the availability of active eIF4E. In tumors, eIF4E concentrations are elevated by the activation of the mammalian target of the rapamycin (mTOR) pathway.¹ As a consequence, the translation of "weak mRNAs" (encoding malignancy-related proteins such as c-myc, bFGF, VEGF, cyclin D1, surviving, and ODC) are promoted disproportionately, resulting in the transformation of normal cells to tumorigenic cells.²⁻⁶ Attempts to reduce eIF4E levels in tumor tissue through methoxyethyl (MOE)-modified second-generation antisense oligonucleotides (ASOs) have been investigated by Eli Lilly and Company.⁷ Currently in phase II clinical trial, the second-generation ASO reduced the levels of eIF4E in mice human tumor xenografts as well as inhibited their growth. In addition, although the levels of eIF4E in the liver were reduced by 80%, no toxicity was found to be associated with ASO.⁷ This suggested that targeting eIF4E by reducing its cellular concentration could lead to effective cancer chemotherapies. Our group sought to develop small-molecule inhibitors of eIF4E that functioned in a similar fashion as ASOs, i.e., to reduce intracellular eIF4E concentrations without cytotoxicity.

Because of its resemblance to the initial 5'-CAP nucleotide of mRNA, cap 0, the inhibitory potency of analogues of 7-methyl guanosine (7-MeG) nucleotides have been investigated.^{8,9} Our group has found that replacement of the 7-Me group of the Me⁷-guanosine monophosphate with a benzyl group (7-Bn-GMP) increases binding affinity to eIF4E by 8-fold ($K_d = 0.8 \mu\text{M}$). Recent X-ray crystallographic studies have revealed that the cap-dependent pocket undergoes a unique structural change in order to accommodate the benzyl group.¹⁰ As mimics of capped mRNA, a diverse range of nucleotides have been designed and synthesized in order to target eIF4E and thus inhibit cap-dependent translation.¹¹⁻¹⁴ Though moderate binding affinities have been obtained for some monophosphate cap

analogues,^{14,15} the utilization of nucleotides as potential drug candidates is challenging due to the presence in the blood and on cell surfaces of enzymes such as phosphatases and 5'-nucleotidase, which rapidly convert the phosphates to the corresponding nucleosides.¹⁶ In addition, since phosphates are negatively charged at physiological pH, they are too hydrophilic to penetrate the phospholipid bilayers of membranes, thus severely limiting their cellular permeability.¹⁶

To circumvent the problems associated with using nucleotides as drugs, several pronucleotide strategies have been developed, such as phosphoramidate diesters, triesters^{17–22} and cycloSal nucleoside phosphotriesters.^{23–26} Nucleoside phosphoramidates have proven to be a promising class of compounds for this purpose, considering their high water solubility and low toxicity.¹⁶ Our group has previously synthesized a series of phosphoramidate monoesters as prodrugs of 7Bn-GMP, and confirmed that one of the synthesized phosphoramidates, 4Ei-1, was able to not only inhibit cap-dependent translation in a dose-dependent manner in cell extracts but also interdict the epithelial-to-mesenchymal transition in zebrafish embryos with no toxicity to normal embryo development.²⁷ 4Ei-1 functions as a prodrug of 7Bn-GMP and has been found to be a substrate for histidine triad nucleotide binding protein-1 (Hint1), including human Hint1, which is believed to be responsible for its intracellular bioactivation^{27,28} (Figure 1).

Previously, we have demonstrated that nucleoside phosphoramidates can undergo cellular uptake and conversion to the corresponding nucleoside monophosphate.^{13,16,29–32} Since there are currently no antagonists of eIF4E cap binding that have been found to directly inhibit eIF4E cap binding in cells or tissues, we chose to characterize the internalization and conversion of 4Ei-1 to 7Bn-GMP for both breast and lung cancer cells, as well as the effect of 4Ei-1 on the intracellular levels of eIF4E in these cells (Figure 2). In addition, because inhibition of cap-dependent translation has been shown to enhance the chemosensitivity of cancer cells to chemotherapeutics, we investigated the potential of 4Ei-1 to chemosensitize breast and lung cancer cells to gemcitabine.

MATERIALS AND METHODS

Materials

7Bn-GMP, 4Ei-1, and 7-ortho-F-Bn-GMP were synthesized according to the published procedures with some modifications.^{14,28} (Structures refer to Figure 2.) All reagents and solvents were purchased from commercial vendors without further purification. Ammonium formate and formic acid were purchased from Sigma Aldrich (St. Louis, MO). High glucose Dulbecco's modified Eagle medium (DMEM), heat-inactivated fetal bovine serum (HI-FBS), antibiotic-antimycotic (5,000 units of penicillin-5,000 μ g/mL streptomycin), trypsin (0.25% trypsin, 2.21 mM EDTA), NuPAGE 10% Bis-Tris gel, NuPAGE MES SDS running buffer (20 $\times$), PVDF membranes, HRP-Goad anti-mouse IgG + A + M (H + L), phosphate-buffered saline (PBS), and Hanks balanced salt solution were purchased from Invitrogen (Carlsbad, CA). Solvents used for final analyses are HPLC grade, filtered through a 0.22 μ m membrane filter, and degassed prior to being loaded to the column. CellTiter 96 AQueous One solution cell proliferation assay was purchased from Promega (Madison, WI). Beta-actin antibody was purchased from AbCam Inc. (Cambridge, MA). Anti-thymidylate

synthase mouse monoclonal antibody (Clone TS 106), CL-XPosure Films (5 × 7 in. clear blue X-ray film), Pierce ECL Western Blotting Substrate, and Restore Western Blot Stripping Buffer were purchased from Thermo Scientific (Waltham, MA). RNAqueous-4PCR kit was purchased from Ambion (Austin, TX). RNase-free supplies were purchased from ISC BioExpress (Kaysville, UT).

Cell Culture

Human breast and lung cancer cell lines MDA-MB-231, H460, A549, H838, and H2009 (kindly donated by Prof. Peter B. Bitterman, Dept. of Medicine, University of Minnesota) were cultured in high glucose DMEM supplemented with 10% HI-FBS and 50 units of penicillin –50 µg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Medium was changed every two or three days, and subculturing was done in the ratio of 1:4 to 1:6.

HPLC Sample Preparation

Five million MDA-MB-231, H460, H838, or H2009 cells were seeded to 12-well plates. Each well was treated with either 100 µM 4Ei-1 or fresh DMEM as controls. All samples were incubated at 37°C for the following time lengths: 0.5, 2, 4, and 29 h. Then 5 mL of ice-cold unsupplemented medium was added, and cell pellets were obtained by centrifugation. One half milliliter of a mixture of methanol and 10 mM ammonium acetate (v/v = 60%: 40%) was added to each cell pellet, followed by freezing at –20°C overnight. The cell extracts were dried by lyophilization (Labconco). One hundred microliters of 20 mM Hepes buffer (pH 7.2) was added to each dried cell extract. 2- to 50-fold dilution was carried out before final analysis. Internal standard was added to each HPLC sample at proper concentrations according to their respective response to the mass spectrometer detector. All samples were then subjected to HPLC-ESI-MS/MS analysis.

HPLC Standards Preparation

Standards were prepared as stocks at the concentrations of 50, 100, 500, 1000, 5000, 10000, 50000, 100000 ng/mL for two analytes as well as 7-ortho-F-Bn- GMP at constant concentrations. Ten microliters of standard stock and 10 µL of 7-ortho-F-Bn-GMP were added to each HPLC sample vial, and dried using lyophilization (Labconco). Then 100 µL of Hepes buffer was added to each vial. Therefore, all standards and 7-ortho-F-Bn-GMP were diluted 10-fold and the final concentrations of the eight standards are 5, 10, 50, 100, 500, 1000, 5000, 10000 ng/mL.

HPLC-ESI-MS/MS Instrumentation

HPLC methods used an autosampler with a cooled sample storage compartment at 4°C and a ternary pump system (Acquity UPLC). All HPLC-ESI-MS/MS analyses were carried out in the positive mode and multiple reaction monitoring (MRM) mode using an electrospray triple-quadrupole mass spectrometer (Waters TQ Detector). Chromatographic separation was achieved with a capillary Acquity UPLC HSST3-C18RP column 2.1 mm × 100 mm, 1.8 µm (Acquity UPLC). The column temperature was maintained at 35°C. The flow rate is 400 µL/min, and the sample injection volume is 5 µL.

Effect of Ion Pairing Reagent

Acquiring an efficient separation profile of each analyte while maintaining the ionic strength of eluting system at a minimum level is important in terms of generating the maximum mass spectrometer signal for the analytes.^{33–36} The effect of ion-pairing reagents on the separation was investigated by carrying out HPLC-ESI-MS/MS analyses with standard samples containing fixed concentrations of 4Ei-1, 7Bn-GMP, and 7-ortho-F-Bn-GMP. The gradient LC conditions are as follows: (1) 0–6 min, 3% B to 97% B; (2) 6–8 min, 97% B; (3) 8–9 min, 97% B to 3% B; (4) 9–12 min, 3% B. The gradient eluting profile yielded desired separation profile with good separation and narrow peaks. Therefore this LC condition was adopted for the following studies. The ion pairing reagents studied in this paper included 0.1% formic acid, and 25 mM ammonium formate or 25 mM ammonium acetate. The response was measured by comparing the peak area of each analyte in the presence of various ion pairing reagents. Solvent A is 0.1% formic acid in water, and solvent B is 25 mM ammonium formate or 25 mM ammonium acetate in 80: 20 (water/acetonitrile).

HPLC Method Development

The HPLC eluting profile was optimized for separation and sensitivity as discussed above. The mass spectrometer was operated in positive mode, with nitrogen as a nebulizing and drying gas. Both analytes and internal standard were tuned by direct infusion to the mass spec detector before loading to the column. The spray voltage was set to 3.95 kV, and the capillary temperature was 350°C. Ion source parameters and MS/MS parameters were optimized using standard procedures. (Detailed compound tuning profile refers to the Supporting Information, and the proposed fragmentation pathways of all four compounds refer to the Supporting Information.)

Matrix Effect

The analyte was spiked either into an actual blank sample (sample extracted from H460, H838, or H2009 cells) or into pure solvent (70% A and 30% B) at a known concentration. Then the relative peak area ratio of the analytes spiked into the actual blank sample was compared to being spiked into pure solvent.

Measurement of Intracellular Concentrations of the Metabolite and the Phosphoramidate

A standard curve was generated to correlate the peak area ratio of target analyte to its internal standard with the analyte's concentration in the standard stocks. Quantitation of prodrugs and metabolites was carried out using MassLynx (Waters, Milford, MA). Quantification of 4Ei-1 was carried out as follows: the peak area ratio of 4Ei-1 to internal standard in real samples was determined by comparing it to the 4Ei-1 standard curve determined as described above. Quantification of 7Bn-GMP was carried out in a similar way except that the internal standard concentration was adjusted according to its response to the mass spectrometer.

Western Blot Analysis

One half million MDA-MB-231, H460, H838, and H2009 cells were seeded in 10 mm culture plates overnight. Old media were replaced with either unsupplemented, 50, 100, 200,

or 500 μ M 4Ei-1 in media before incubating at 37°C for 24, 48, or 72 h. For the proteasomal degradation studies, 10 μ M MG-132 was combined with 500 μ M 4Ei-1 in the treatment of H2009 cells. The cells were allowed to grow in the presence of both 4Ei-1 and MG-132 for 24, 48, and 72 h. Media were replaced every 12 h. Cell cultures were harvested immediately after prodrug treatment. To harvest, cells were trypsinized, washed, and pelleted. Numbers of living cells were counted for individual plates to compare viability. To extract cell lysates, lysis buffer (150 mM NaCl, 50 mM Tris, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor) was added to pellets before three cycles of freeze (−80°C, 12 min)/thaw (37°C, 2 min). For each cell lysate, triplicates were loaded onto SDS–PAGE, transferred to nitrocellulose (Invitrogen, Grand Island, NY), blotted with mouse anti-eIF4E and anti-actin polyclonal antibodies (LifeSpan BioSciences, Seattle, WA), and imaged with SuperSignal West Pico Kit (Thermo Scientific, Rockford, IL).

Densitometry

Film negatives were scanned using BioRad (BioRad, Hercules, CA). Intensities for both control (β -actin) and experimental (eIF4E) bands were quantified by Molecular Analyst (BioRad, Hercules, CA) densitometer. The pixels in each band were summed to yield the raw reading. Baseline was selected as the background across the film and subtracted from the raw reading. The eIF4E level used for comparison was the ratio of β -actin/eIF4E.

Colony Forming Assay

MDA-MB-231, H460, A549, and H838 cells were seeded as triplicate sets into 6 well plates with 500 cells per well. After 6 h cells were left untreated or treated with gemcitabine or 4Ei-1 alone or in combination. When (9 day) colonies were of appropriate size, cells were fixed for 10 min in 10% formalin, washed with water, and stained with coomassie blue, images were collected, and colonies were manually counted. The colony number was expressed as the mean $\pm$ SD normalized to untreated cells.

RESULTS

Effect of Ion Pairing Reagents and LC Eluting Profile

The use of ion pairing reagents has been a commonly used method for tuning the retention time of ionic analytes.^{37,38} Formic acid and ammonium formate were employed respectively as the ion-pairing reagent. The corresponding UV chromatograms suggested that 10 mM ammonium acetate at pH 6.65 resulted in efficient separation of analytes and ISTDs as well as reasonable signals (data not shown). DMHA gave a similarly efficient separation, but the mass spectrometer response was suppressed compared to ammonium acetate. TBAA was able to give a better mass spectrometer response. However, it was less volatile than ammonium acetate and caused a residual buildup at the ion source chamber after a few sample injections, which greatly impaired reproducibility. Therefore, the gradient eluting profile with 10 mM ammonium acetate as the ion-pairing reagent was identified as the most efficient separating condition for the following HPLC-ESI-MS/MS analyses.

Matrix Effect

Since matrix can potentially suppress ionization efficiency and therefore reduce sensitivity, we evaluated the matrix effect by comparing the relative peak area ratio of the analytes spiked into an actual blank sample to being spiked into pure solvent at a known concentration. No significant difference was observed between the two samples (data not shown), suggesting that our sample preparation procedures may have removed most cellular components that could potentially cause substantial MS signal suppression. Therefore, the matrix effect could be neglected in this study and the following standards and quality control samples were prepared in HEPES buffer.

Standard Curves and Quality Controls

The concentration of 7-ortho-F-Bn-GMP (Figure 2) was adjusted according to their responses relative to their respective target analyte. The standard curve with high correlation coefficients was derived as illustrated in the Supporting Information with one representative chromatogram shown in Figure S1. In order to ensure that a test run is valid and the results are reliable, quality control samples are treated in exactly the same manner as the test samples and used to validate the test run. Quality control samples were prepared and analyzed in the same way as experimental samples with standard deviations within 4–8% and the determined values all within 20% of the expected target concentration.

Bioactivation of 4Ei-1 Is Time-Dependent

The 7Bn-GMP phosphoramidate pronucleotide 4Ei-1 (100 μ M) was incubated with MDA-MB-231, H460, H838, and H2009 cells at 37°C for various time lengths. Within 5 min, 7Bn-GMP was detectable intracellularly. The detectable amount of 7Bn-GMP increased with an extended incubation time for up to 4 h. At the 4 h time point, 56.5 ± 11.4 pmol of 7Bn-GMP per 5 million MDA-MB-231 cells was observed. Similar amounts of 7Bn-GMP were detectable (56.4 ± 10.3 pmol) when the incubation time was further extended to 24 h (Figure 3A). Comparably, five million lung cancer cells contained 157 ± 2 , 195 ± 24.5 , or 31.8 ± 6.1 pmol of 7Bn-GMP (Figure, 3B) after 2 h, which was maintained over the next 24 h. In all cases, the amount of 7Bn-GMP was 3- to 4-fold higher than that of 4Ei-1 (Figure 3B,C). Given that the volume of a typical single cell has been calculated to be 1.8 pL,^{39,40} the intracellular concentration of 7Bn-GMP reached 6.0 ± 1.0 μ M by 4 h for MDA-MB-231 cells, and 17.4 ± 3.0 , 21.7 ± 2.7 , or 3.5 ± 0.7 μ M for all three lung cancer cells.

Compound 4Ei-1 Reduces the Intracellular Levels of eIF4E in a Dose-Dependent Manner

To characterize the effect of 4Ei-1 on intracellular eIF4E, we treated the representative cell line, H2009, with 4Ei-1 for various time periods and determined the amount of eIF4E by Western blot analysis. The cells were treated with variable concentrations of 4Ei-1 and the media exchanged with fresh media every 12 h to maintain an approximately constant 4Ei-1 concentration. As can be seen from the Western blot analysis in Figure 4A, while no loss of eIF4E was observed over time for nontreated cells, over the course of 24 h a dose-dependent decrease in the levels of eIF4E was clearly evident for 4Ei-1 treated cells, with complete loss observed at a concentration of 500 μ M. No higher molecular weight eIF4E positive proteins were observable. This effect was more pronounced for incubations of 48 and 72 h, with

nearly 50% loss of eIF4E observed when H2009 cells treated with 4Ei-1 concentrations of 100 μ M and 50 μ M, respectively (Figure 4B). Complete loss of eIF4E was observed for H2009 cells treated with 4Ei-1 concentrations of 500 μ M and 200 μ M and incubated for 48 and 72 h, respectively. A similar dose-dependent loss of eIF4E was observed for MDA-MB-231 cells treated with 4Ei-1 (Figure 4C). Thus, the degree to which treatment with 4Ei-1 contributes to the loss of eIF4E is dependent on the concentration of the prodrug and the incubation time period.

Compound 4Ei-1 Induced Diminution of eIF4E through Proteasome Degradation

To address the mechanism contributing to the loss of eIF4E from cells treated with 4Ei-1, H2009 cells were treated with the proteasome inhibitor MG 132 (10 μ M) in the presence of 500 μ M 4Ei-1 for 0, 24, 48, and 72 h. To ensure that the results would not be affected by the stability of the two compounds, the cell culture medium containing fresh MG 132 and 4Ei-1 was replaced every 12 h and the amount of eIF4E determined by Western blot analysis (*vide supra*). As shown in Figure 4d, MG 132 blocked the ability of 4Ei-1 to reduce the levels of eIF4E, suggesting that proteosomal degradation is at least partially responsible for the observed decrease in eIF4E protein levels after treatment of cells with 4Ei-1. Interestingly, little cytotoxicity was observed with an MTS assay for H2009 cells grown in culture and treated with 4Ei-1, with nearly identical rates of division observed between the control and treated cells (data not shown).

Treatment of Lung and Breast Cancer Cells with 4Ei-1 Increased the Cytotoxicity of Gemcitabine

To assess the ability of 7Bn-GMP to chemosensitize cancer cells to a chemotherapeutic, we carried out colony forming assays with lung cancer cells with nontoxic levels of both 4Ei-1 (25–75 μ M) and gemcitabine (0.075–0.5 nM)—which is clinically used for the treatment of lung cancer—for 9 days. As represented in Figure 5A, neither gemcitabine nor 4Ei-1 had an effect on colony formation by H460 cells. However, when combined, a 40% reduction in colony formation was observed (Figure 5A,B). At a higher concentration of 4Ei-1 (50 μ M), colony formation was reduced by 40%, and when combined with gemcitabine, a reduction of 75% in colony formation was observed. In breast cancer MDA-MB-231 cells, cell viability dropped to 50% of control, compared to 85% and 87% for gemcitabine and 4Ei-1, respectively (Figure 5B). For both H460 and A549 cells chemosensitized with 25 μ M 4Ei-1, 0.5 nM gemcitabine reduced colony numbers by 40% (Figure 5C,D). For H838 cells this effect was even more significant (Figure 5E), resulting in a nearly 70% reduction in colony numbers.

DISCUSSION

Compound 4Ei-1, which is a substrate of the ubiquitous intracellular phosphoramidase, Hint1, is a prodrug of 7Bn-GMP, a known antagonist of eIF4E.^{27,41,42} Recent studies have demonstrated dose-dependent inhibition of cap-dependent translation when 4Ei-1 was injected into zebra fish embryos.¹³ When tissue lysates were treated with 4Ei-1, the compound was found to be converted to 7Bn-GMP. Nevertheless, the amount of intracellular

conversion of 4Ei-1 to 7Bn-GMP was not determined, nor was the ability of 4Ei-1 to cross the cellular membrane determined.

Previously, we observed that amino acid phosphoramidates of AZT were able to be taken up by lymphocytic cells and converted to substantial amounts of AZT-MP.^{15,43} Although suggestive, our results with AZT phosphoramidates do not necessarily predict the intracellular uptake of 4Ei-1. Consequently, we developed an analytical method using LC-ESI-MS/MS to determine the intracellular levels of both 4Ei-1 and 7Bn-GMP in MDA-MB-231, H460, H838, and H2009 cells that had been treated with 4Ei-1. Our data demonstrated that the intracellular uptake of 4Ei-1 and conversion to 7Bn-GMP by breast and lung cancer cells could be observed live minutes after treatment with 4Ei-1, with a plateau reached at approximately 4 h. Lung cancer cell lines retained as much as 4-fold more 7Bn-GMP and 4Ei-1 than MDA-MB-231 cells (Figure 3), with a spike in the concentration of the prodrug observed for the lung cancer cells during the first few minutes. The difference between the two types of mammalian tissues suggests that the mechanism of internalization is not due to a membrane fluid process, but a specific transporter that may be differentially expressed. In addition, the levels of the putative activating phosphoramidase may vary between the tissues, further contributing to differences in the intracellular levels of prodrug. The results of ongoing studies of 4Ei-1 transport and Hint activity by these cells should offer a rationale for the variation in prodrug uptake and metabolism by these tissues.

Inhibition of cap-dependent translation either by targeting eIF4E by ASO or RNAi or by inhibition of eIF4A has been shown to chemosensitize cancer cells to cisplatin, gemcitabine, and doxorubicin.^{44–46} Gemcitabine is the first line treatment for a number of cancers, including lung and pancreatic cancer, and as salvage therapy for metastatic breast cancer.^{47–49} Consequently, since 4Ei-1 was shown to be cell permeable and to deliver 7Bn-GMP, we chose to evaluate the ability of 4Ei-1 to chemosensitize both lung and breast cancer cells to gemcitabine (Figure 5). Results from colony forming assays demonstrated that nontoxic doses of 4Ei-1 significantly reduced (>30%) the viability of MDA-MB-231, H460, H549, and H838 cells when treated with nontoxic doses of gemcitabine.

To probe the mechanism of 4Ei-1 on chemosensitization, we examined the prodrug's effect on the intracellular levels of eIF4E in both lung and cancer cells. For H2009 cells, longer incubation times (24–72 h) resulted in a more pronounced decrease in eIF4E levels (Figure 4A,B). However, at the highest concentration (500 μ M), complete loss of eIF4E, regardless of the time of incubation, was observed. A similar trend was observed for MDA-MB-231 cells, although when the results for the 72 h time point were compared with those for the H2009 cells, significantly more eIF4E appeared to be lost from H2009 cells than from MDA-MB-231 cells when treated with the lower doses of 4Ei-1 (Figure 4B,C). This difference may reflect our observation that, upon treatment with 4Ei-1, greater amounts of 7Bn-GMP accumulated in H2009 cells than MDA-MB-231 cells (Figure 4A,B).

Since eIF4E expression is dependent on cap-dependent translation, the loss of eIF4E from cells treated with 4Ei-1 could be attributed to inhibition of eIF4E translation. However, when cells were treated with 4Ei-1 and the proteasome inhibitor, MG-132, the levels of eIF4E remained unaffected. These results strongly suggest that the binding of 7Bn-GMP to eIF4E

results in targeted proteasomal degradation, without significantly affecting eIF4E expression. The mechanism of eIF4E reduction induced by 7Bn-GMP remains to be determined. Ubiquitination of eIF4E has been observed for cells subjected to heat shock conditions.⁵⁰ Nevertheless, our inability to observe the reported higher molecular weight species by Western blot analysis suggests that eIF4E ubiquitination is not a prerequisite for proteasomal degradation.

Interestingly, while incubation of the cells with 100 μ M 4Ei-1 resulted in intracellular concentrations of 7Bn-GMP approximately 10-fold greater than the K_d of 7Bn-GMP for eIF4E, only 50% loss of eIF4E was observed at higher concentrations. These results are consistent with our previous observation that the IC_{50} value for inhibition of *in vitro* translation by 7Bn-GMP is 20-fold higher than the eIF4E K_d .²⁷ Thus, although the intracellular accessibility of the cap-binding site of eIF4E is significantly restricted, at higher 4Ei-1 extracellular concentrations, the intracellular 7Bn-GMP levels likely rise to levels capable of thermodynamically shifting the intracellular eIF4E concentration to greater amounts of the 7Bn-GMP bound form, which may be more susceptible to proteasomal degradation.

Because of the importance of eIF4E in cap-dependent translation the development of small molecule cell permeable antagonists, which may be used as chemical biological tools or drug leads, has been pursued.⁵¹ Despite these efforts, oligonucleotide based gene knock-down approaches have been the only method for examining the role of eIF4E in cells.⁵² Taken together, our results demonstrate that 4Ei-1 is cell permeable and bioactivated, presumably by Hints, to the eIF4E antagonist, 7Bn-GMP. In addition, consistent with previous reports of the effect of eIF4E knock-down on cancer cell chemosensitization, both lung and breast cancer cells treated with nontoxic concentrations of 4Ei-1 were chemosensitized to nontoxic concentrations of the anticancer drug, gemcitabine. The mechanism of intracellular eIF4E inhibition may involve two mechanisms, depending on the concentration of 4Ei-1: direct binding to the eIF4E cap-binding site and induction of eIF4E proteasomal degradation. The results of ongoing studies should clarify the role of eIF4E regulation on cap-dependent translation.

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ABBREVIATIONS

eIF4E	eukaryotic initiation factor 4E
7Bn-GMP	N ⁷ -benzylated guanosine monophosphate
Bn⁷G	N ⁷ -benzyl guanosine
HPLC-ESI-MS/MS	high performance liquid chromatography coupled to electrospray tandem mass spectrometry
SRM	selected reaction monitoring

ISTD	internal standard
HINT	histidine triad nucleotide binding protein

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Figure 1.
Proposed rationale for the intracellular uptake and conversion of 4Ei-1 to 7Bn-GMP.

Figure 2.
Structures of 7Bn-GMP, 4Ei-1 and the internal standard *o*-F-7Bn-GMP.

Figure 3.

Intracellular levels of 7Bn-GMP and 4Ei-1 after treatment with 4Ei-1 for variable time periods. (A) Intracellular levels of 7Bn-GMP and 4Ei-1 in MDA-MB-231 cells treated with 100 μ M 4Ei-1 at 37°C. (B) Intracellular amounts of 7Bn-GMP in H460, H838, and H2009 cells treated with 100 μ M 4Ei-1 at 37°C. (C) Intracellular amounts of 4Ei-1 in H460, H838, and H2009 cells treated with 100 μ M 4Ei-1 at 37°C. All data shown are the average $\pm$ SD of three separate experiments.

Figure 4.

Expression of eIF4E by H2009 and MDA-MB-231 cells treated with 4Ei-1. (A) Representative Western blot film for H2009 cells treated with variable concentrations of 4Ei-1 for 24 h. (B) Normalized eIF4E/ β -actin values in H2009 cells treated with various concentrations of 4Ei-1 for 24, 48, and 72 h. The eIF4E/ β -actin values in nontreated H2009 cells were set to 100%. (C) Normalized eIF4E/ β -actin values in MDA-MB-231 cells treated with various concentrations of 4Ei-1 for 72 h. (D) Representative Western blot film for H2009 cells treated with 4Ei-1 (500 μ M) and the proteosomal inhibitor MG 132 for variable time periods.

Figure 5. Enhanced cytotoxicity of MDA-MB-231, H460, A549, and H838 cells by combined treatment with gemcitabine and 4Ei-1. (A) Representative colony forming assay image of lung cancer H460 cells in response to treatment with gemcitabine, 4Ei-1 or in combination (coomassie blue stain). (B–E) Histograms of the normalized, H460, A549, H838, and MDA-MB-231 colony numbers following 4Ei-1 treatments with and without gemcitabine. There was a considerable decrease in colony formation in the combined treatment as compared with either single agent alone. Columns: mean of three determinations of colony number. Bars: SD.