

Evaluation of Eukaryotic Initiation Factor 4E (eIF4E) Antagonist 4Ei-1 in Mammalian
Breast Cancer and Lung Cancer Cells : Chemosensitization with Low Cytotoxicity

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Thank you, Dr. Wagner, for all your support and guidance.

Thank you, mom, for everything.

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Abbreviations Used

EIF4E: Eukaryotic Initiation Factor 4E
Me⁷GTP: 7-Methyl Guanosine Triphosphate
HINT: Histidine Triad Nucleotide Binding Protein
4Ei-1: 4E Inhibitor 1
Bn⁷GMP: 7-Benzyl Guanosine Monophosphate
SBDD: Structural Based Drug Design
3D-QSAR: Three-dimensional Quantitative-activity Relationship
DHFR: Dihydrofolate Reductase
MTX: Methotrexate
AZT: Azidothymidine
D4T: 2'-3'-didehydro-2'-3'-dideoxythymidine
D4A: 2'-3'-didehydro-2'-3'-dideoxyadenine
IsoddA: Iso-dideoxyadenine
FLT: 3'-fluoro-3'-deoxy-L-thymidine
DdC: 2'-3'-dideoxycytidine
DdA: 2'-3'-dideoxyadenine
3TC: 2'-3'-dideoxy-3'-thiacytidine
HHint1: Human HINT 1
EIFx: Eukaryotic Initiation Factor x (e.g. 4F, 4G, etc.)
GTP: Guanosine Triphosphate
Met: Methionine
ODC: Ornithine Decarboxylase
VEGF: Vascular Endothelial Growth Factor
MAP-K: Mitogen-activated Protein Kinase
Me⁷G: 7-Methyl Guanosine

GF: Growth Factor
PI3K: Phosphoinositide 3 Kinase
MTOR: Mammalian Target of Rapamycin
4E-BP: 4E Binding Protein
GMP: Guanosine Monophosphate
IC₅₀: Half Maximal Inhibitory Concentration
RLUC: *Renilla reniformis* Luciferase
FLUC: Firefly Luciferase
IRES: Internal Ribosomal Entry Site
P_{T7}: T7 Promoter
Pol: Polymerase
Amp^R: Ampicillin Resistance
CHD: Cycloheximide
HEK: Human Embryonic Kidney
HA: Hemagglutinin
Ub: Ubiquitin
PML: Promyelocytic Leukemia Protein
PRH: Proline-rich Homeodomain Protein
VPg: Viral Protein Genome-linked
EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Im3P: Triimidazolylphosphine
SCP: Salicylchlorophosphine
PvCl: Pivaloyl Chloride
DMSO: Dimethyl Sulfoxide
NMR: Nuclear Magnetic Resonance
DEAE: Diethylaminoethyl Cellulose
TEAB: Triethyl Ammonium Bicarbonate
UV: Ultra Violet
HR-MS: High Resolution Mass Spectrometry
DMF: Dimethyl Formamide
THF: Tetrahydrofolate

dCK: Deoxycytidine Kinase
RR: Ribonucleotide Reductase
HENT1: Human Equilibrative Nucleotide Transporter-1
HPLC: High Performance Liquid Chromatography
ESI: Electrospray Ionization
SRM: Selected Reaction Monitoring
^oF-Bn⁷GMP: *ortho*-Floro 7-Benzyl Guanosine Monophosphate
ISTD: Internal Standard
QC: Quality Control
EDTA: Ehtylene Diamine Tretraacetic Acid
EGTA: Ethylene Glycol Tetraacetic Acid
DTT: Dithiothreitol
DMEM: Dulbecco's Modified Eagle Medium
HI-FBS: Heat-inactivated Fetal Bovine Serum
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
DMHA: N, N-dimethylhexylamine
TBAA: Tetrabutyl Ammonium Acetate
Hsp27: Heat Shock Protein 27
MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MRM: Multiple Reaction Monitoring
HCV: Hepatitis C Virus
NMP: N-methylpyrrolidone

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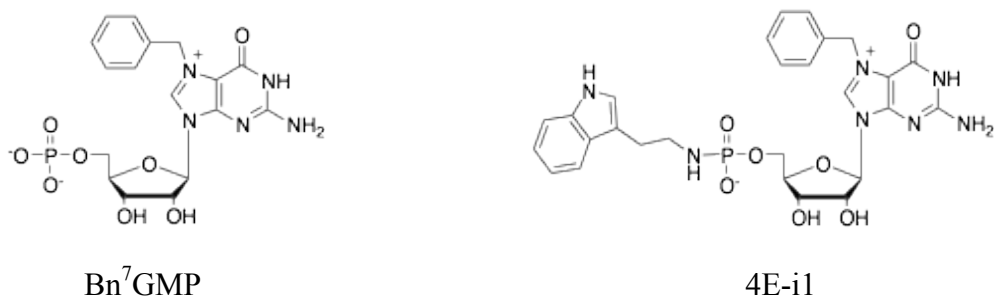
Chapter 1

Introduction

1.1 Introduction

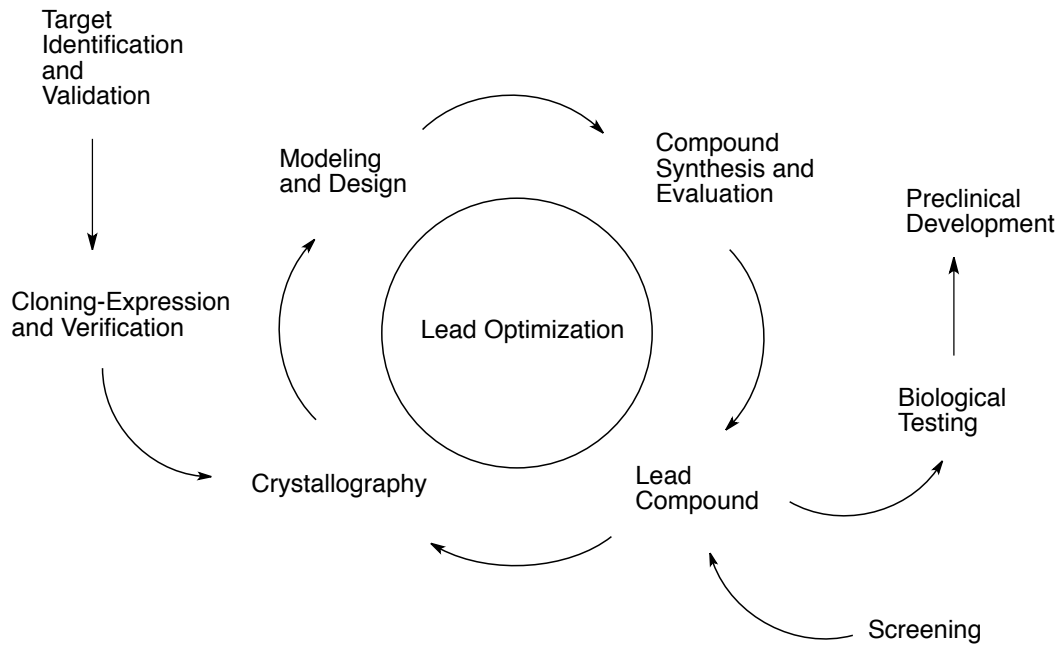
Overexpression of eukaryotic initiation factor 4E (eIF4E) induces cell malignancy by undermining the selective translation of housekeeping proteins over growth factors [1]. Pro-nucleotide phosphoramidates are a class of water soluble eIF4E inhibitors with low toxicity toward mammalian tissue cells, and our library of phosphoramidates negatively regulates eIF4E-mRNA cap (7-methylGTP) binding [2-7]. Here we present (**Figure 1**) a novel histidine triad nucleotide binding protein (HINT)-dependent pro-nucleotide eIF4E-inhibitor 1 (4E-i1, $IC_{50} = 16.7 \pm 3.2 \mu\text{M}$) [8]. Metabolism studies have shown rapid conversion of 4E-i1 to its active parent compound, 7-benzylGMP, in zebrafish eggs, MCF-7, H460, and H2009 cell lines. Compound 4E-i1 inhibited cap-dependent luciferase translation in cell-free assays, and also showed synergistic effects in breast and lung cancers with gemcitabine. Our work provides strong motivation for the continued development of compounds designed to normalize cap-dependent translation as novel chemo-preventive agents and therapeutics for cancer.

Figure 1. Structures of active eIF4E antagonist Bn⁷GMP and pro-nucleotide 4Ei-1. 4Ei-1, the phosphoramidate nucleotide pro-drug of Bn⁷GMP, is designed to have better cell permeability and *in vivo* activity than its parent.



We started our search for suitable small-molecule inhibitors by following Structural Based Drug Design (SBDD). SBDD is an iterative process (**Figure 2**), in which x-ray crystallography has been the predominate technique used to elucidate the three-dimensional structure of drug targets. Given that many proteins undergo considerable conformational change upon ligand binding, it is important to design mRNA Me⁷-GTP analogues based on the crystallographic structures of protein-ligand complexes, not the unliganded structure.

Figure 2. Iterative Process of Drug Design. The drug-discovery process usually takes at least three cycles of optimization before a satisfactory lead is identified. The lead optimization is fueled by protein crystallography, biological testing, and other screening methods.



Adapted from “Structure-based drug design: computational advances” [9]

Three-dimensional quantitative structure-activity relationship (3D-QSAR) calculation directed us to focus on only a few modifiable functional groups on Me⁷-GTP. A library of 20 compounds were designed and synthesized, and top candidates were identified [8, 10, 11]. Murine eIF4E-DHFR fusion protein was expressed in BL21 *Escherichia coli* cells and purified via a methotrexate (MTX) cellulose affinity column. Pure eIF4E was obtained after incubating the fusion protein with human thrombin [12]. Crystallography images of the *apo* and *holo* eIF4E showed strengthened π - π interaction of the analog guanine region after binding, suggesting additional π systems be implemented [13]. Following this lead and binding affinity assays, we chose Bn⁷-GMP as a platform.

The negative charges on Bn⁷-GMP hindered its cell permeability. To neutralize the monophosphate group, several masking groups were proposed. Lipophilic phosphotriesters were extensively studied, but they can be rapidly converted to the monophosphate before reaching target tissues [14]. Chapter 2 has a representative list of these pro-nucleotides. We sought to construct phosphoramidates as pro-drug delivery systems. Phosphoramidates were known for their water solubility, low toxicity, fair stability in biological systems, and simple intracellular activation. A few examples of phosphoramidate prodrugs are phosphoramidate diesters of AZT [15, 16], d4T [17], 2'-deoxyuridine [18], d4A [19], isoddA [20], FLT [21], ddC [22], ddA [18], hypoxallene [23], and 3TC [24]. A separate ongoing project in our lab on human Hint1 revealed Hint1 substrate specificity [10]. According to the phosphoramidate activity assays we found attaching an amino acid, tryptophan, at the P-N bond would significantly increase the release of the monophosphate from the pro-nucleotide [10].

Tryptophan-containing phosphoramidates were shown to penetrate cell membranes through diffusion. In this dissertation the proposed mechanism and biological effects of phosphoramidate 4E-i1 in cancer progression will be addressed.

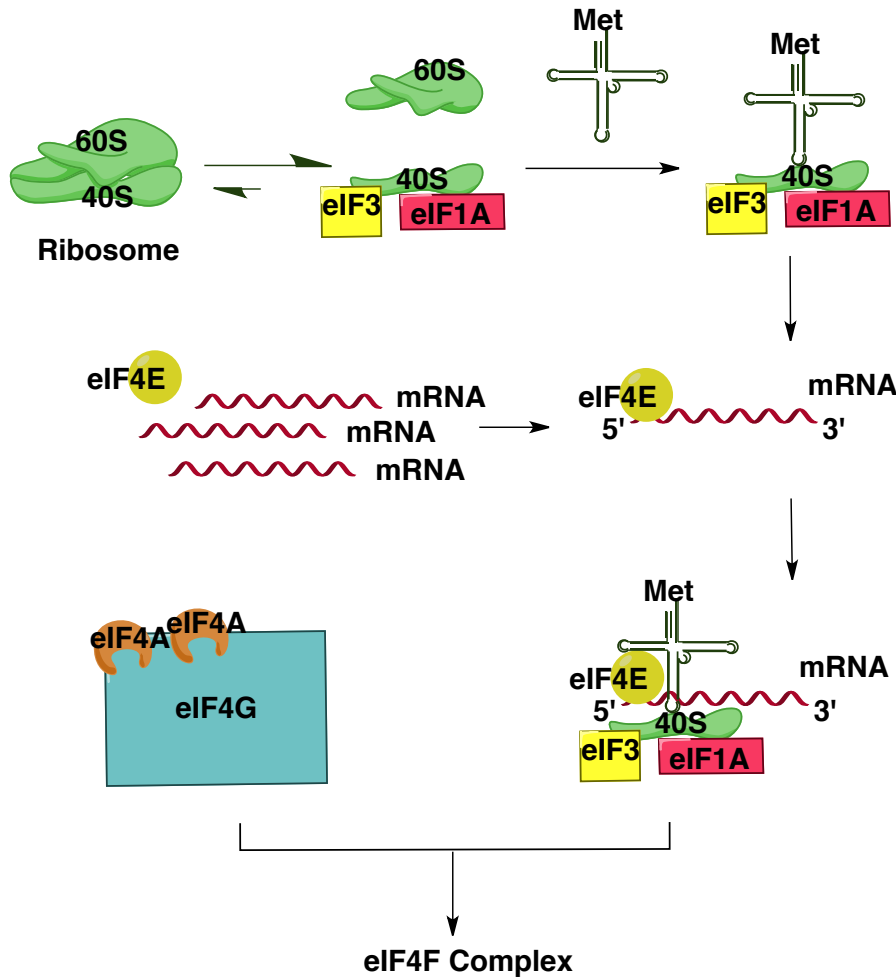
1.2 eIF4E as an anti-cancer target

Our understanding of the origins of cancer has changed dramatically over the past three decades. The discovery of mutant, tumor-associated oncogenes in human tumors led to a simple model of cancer formation. Normal cells carry certain growth-normalizing genes, the presence of which is essential to maintain normal proliferation. Disruptions in cellular communication contribute to the loss of such regulation. Cancer cells thrive without external growth signals and ignore anti-growth signals [25].

Translation initiation on the majority of cellular mRNAs is mediated by a cap-dependent mechanism. Cellular mRNAs are modified at the 5' end by the addition of a 7-methylguanosine residue, known as the “cap” structure. The cap structure found at the 5' end of all cytoplasmic mRNAs is recognized by the cap-binding complex (eIF4F) of eIF4E, together with the RNA helicase eIF4A and eIF4G, which acts as a scaffold to bridge the mRNA to the 40S ribosomal subunit via its interaction with eIF3. This results in formation of the 43S pre-initiation complex on the mRNA. Scanning of the mRNA then occurs, and recognition of the initiation codon requires other initiation factors, eIF1, eIF1A, and the ternary complex eIF2.GTP.Met-rRNAi. Once the initiation codon is located, the 48S initiation complex is formed. Subsequent binding of the 48S complex to the 60S ribosomal subunit (with the involvement of eIF5 and 5B) results in formation of the 80S complex and the release of initiation factors from the ribosome [26, 27]. The

scheme is shown on the next page in **Figure 3**.

Figure 3. Formation of the eIF4F Initiation Complex. The eukaryotic translation initiation complex (eIF4F complex) is required for the majority of eukaryotic protein synthesis. The assembly of eIF4F involves the recruitment of methionine t-RNA, eIF3, and eIF1A by the ribosome 40 subunit; recognition of mRNA 5' cap to eIF4E; and the delivery of the 40S-mRNA group to the eIF4G docking protein by eIF4E.



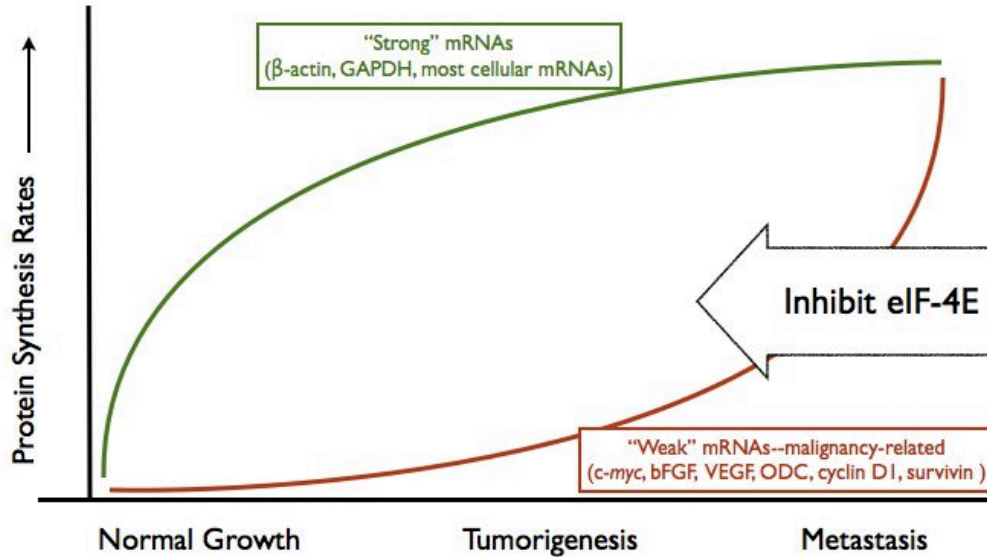
The connection between eIF4E and tumorigenesis was first discovered in mammalian tissue cultures [28, 29]. Overexpression of eIF4E was almost universal across cancers of the lung, breast, prostate, bladder, cervix, and colon [30-36]. In addition, experimental models have shown overexpression of eIF4E dramatically altered cellular morphology and induced cellular transformation leading to cancer [28, 29, 37-39]. Inquiries into this connection have cast useful insights on eIF4E's role in tumorigenesis (mechanism: see section 1.3).

Due to its critical role in translation initiation, eIF4E is an ideal target for control of the rate of protein synthesis. Much is known about the biological function of eIF4E and its importance in processes such as growth and tumourigenesis, yet we know little about how its expression is regulated. Serum, growth factors or immunological activation in T cells have all been shown to lead to increased transcription of the eIF4E gene [40]. The eIF4E promoter contains binding sites for both c-Myc and hnRNPK, which have both been implicated in transcriptional control of the protein [1].

Perhaps surprisingly, overexpression of eIF4E does not lead to a global increase in translation levels. Only a subset of mRNAs, known as 4E-sensitive mRNAs, is translationally upregulated. Examples are mRNA's encoding genes involved in growth, such as cyclin D1, ornithine decarboxylase (ODC) and Vascular Endothelial growth factor (VEGF) [41-44]. Overexpression of eIF4E induces transformation of cells, and although high levels of eIF4E have been observed in several cancers, the significance of the levels and the role in tumor progression is still not resolved. It is believed that overexpression of eIF4E may promote the increased translation or export of a subset of mRNAs that encode proteins involved in cell proliferation and tumourigenesis. Indeed,

eIF4E-dependent mRNA transport has been shown to be upregulated in a small number of cancers, such as acute myeloid leukemia [28]. Within cap-dependent mRNA pools there are two distinct groups of mRNAs, the weak and strong. Weak mRNAs usually originate from oncogenes and have weak affinity to the initiation complex, especially eIF4E, and are therefore rarely translated; strong mRNAs encode housekeeping proteins necessary to survival (**Figure 4**). Under cellular stress, however, eIF4E becomes overexpressed and undermines the selectivity designed to separate weak mRNAs from strong mRNAs. Evidently, cellular transformation occurs and leads to cancer. Because of its role in cap-dependent protein synthesis, eIF4E has been identified as an anti-cancer target.

Figure 4. Reducing translation initiation factor eIF4E suppresses malignancy. Normal cell growth differs from tumorigenesis and metastasis on the expression levels of certain survival proteins such as c-myc, bFGF, VEGF, ODC, cyclin D1, and survivin. Inhibition of eIF4E can reduce the expression of mRNAs encoding the discussed proteins, thus restores normal cellular activities in cancer cells.



Adapted from “eIF4E expression and its role in malignancies and metastases” [28]

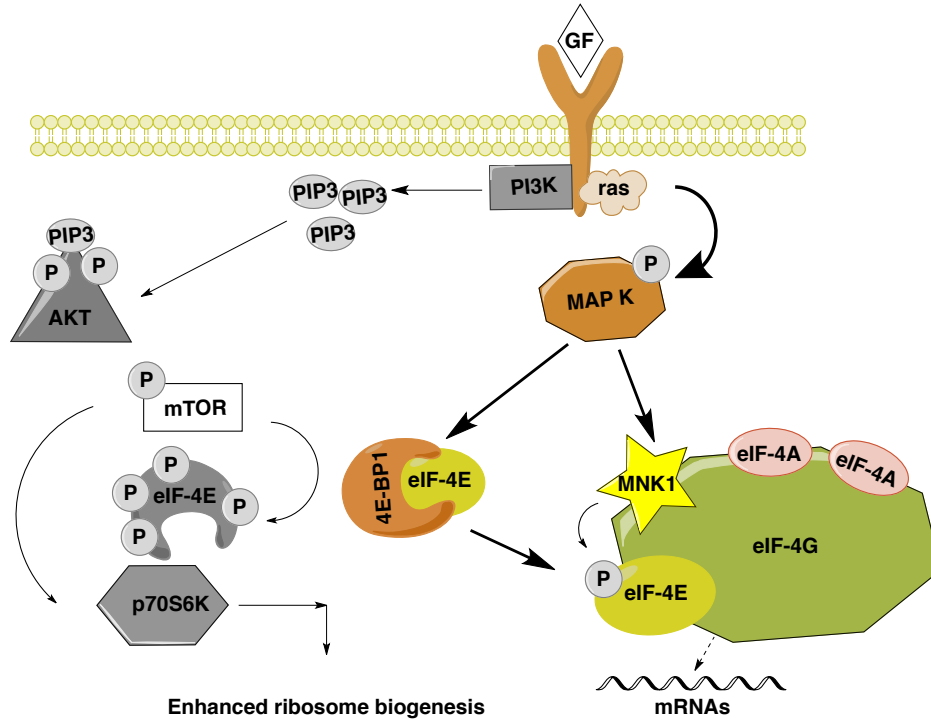
1.3 Targeting the interaction between eIF4E and 7-methyl capped mRNA

1.3.1 Introduction

The regulation of cap-dependent translation can be traced back to the *ras* pathway, as well as other intracellular signaling pathways (**Figure 5**). Many enzymes could be targeted to disrupt the weak mRNA translation in tumor cells, but inhibition of the upstream enzymes such as MAP-K would result in undesirable side effects. However, the targeted enzyme also has to be involved in the early stage of translation for an efficient therapy.

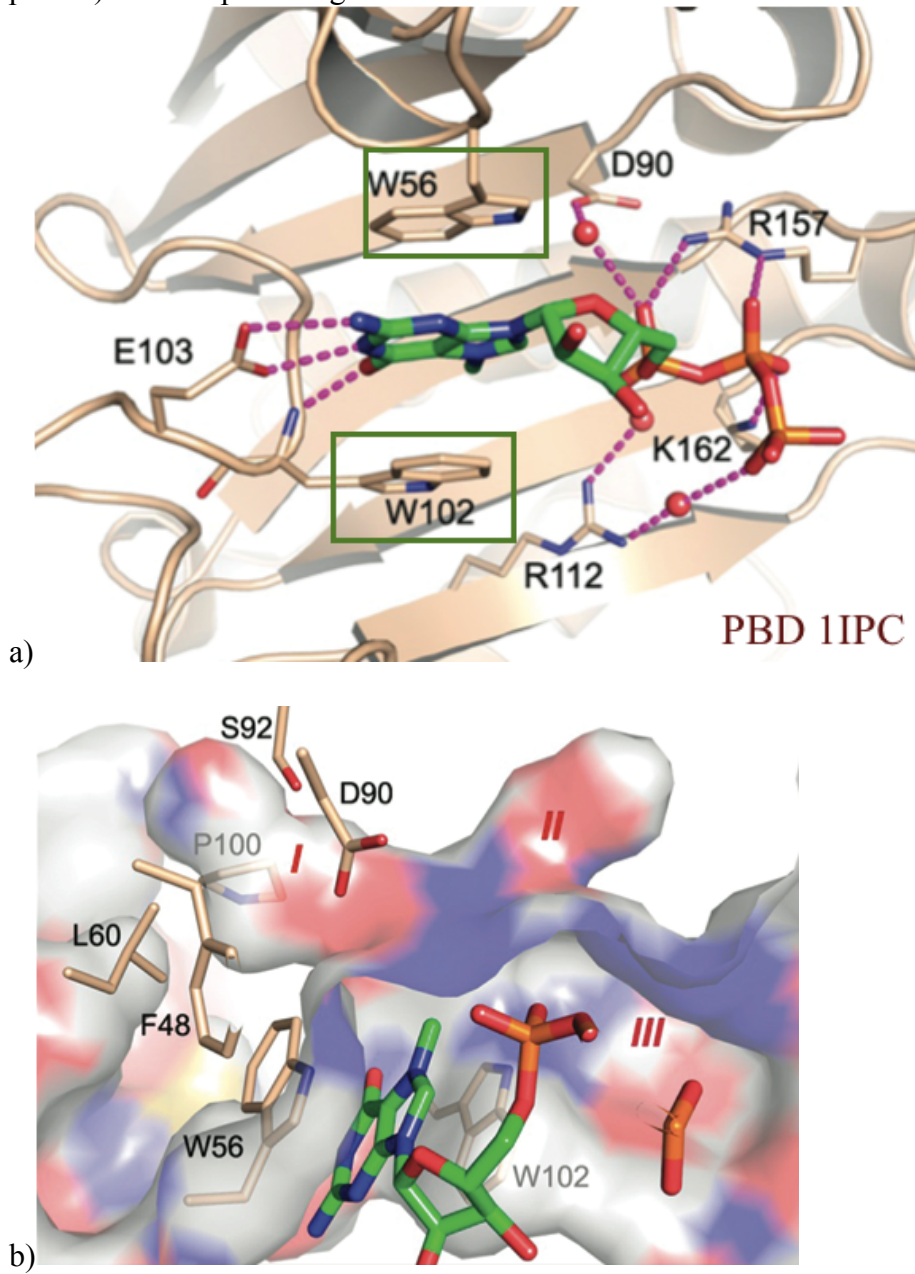
Inhibition methods of the eIF4E pathway include small-inhibitors of 4E-Me⁷G capped mRNA [43], 4E-4G interaction, 4A helicase function, and antisense oligonucleotides [45]. The most common and extensively studied way of inhibition is through nucleic acid derivatives that mimic the 5'-cap structure of mRNAs. The rationale for this is that the binding of 4E to mRNAs is the rate-limiting step of protein synthesis. The inhibition at this stage would significantly reduce the translation of weak mRNAs.

Figure 5. The mTOR Signaling Pathway and Its Downstream Cap-Dependent Translation. One mechanism for the overexpression of eIF4E in cancer cells is the releasing of growth factors. When activated by a growth factor, phosphorylation of protein kinase (AKT) releases de-phosphorylated eIF4E from the 4E binding protein (4E-BP). The growth factor also triggers the *ras* protein pathway that leads to an abundance of phosphorylated 4E-BP, which frees excess amount of eIF4E to bind mRNAs.



Wagner and group have studied the effects of several cap-derived small molecules. A library of twenty analogs was constructed [8, 11]. In this library, modifications were made to guanosine monophosphate (GMP), the ribose nucleic acid unit of the 5'-cap. Based on the IC₅₀ values determined by immunoblotting, no significant reduction in efficacy was found with 2'-deoxyribose analogs. The addition of N-7 electrophilic groups increased binding affinity while the phosphoramidates lost all affinity to eIF4E. To further explore the N-7 substituents, eif4E crystal structure and 7-alkyl guanosine analogs were compared *in silico*. A close examination of the active site revealed the binding events in such sequence: negatively charged phosphate forms ionic bonds with positively charged amino acid residues R112, R157, K159, and K162; guanosine base slides between W56 and W102 to establish π - π electron stacking; naturally the N-7 bulky substituent points into a hydrophobic pocket deep inside of the binding site, hitherto increasing the observed binding affinity (**Figure 6**). Both *in vitro* and *in vivo* data supported these findings [8].

Figure 6. Crystal structure of eIF4E cap-binding site with Bn^7GTP . a) Ribbon representation of the 7-MeGTP binding in the cap-binding slot of eIF4E; b) Molecular surface representation of pockets I (lipophilic pocket), II, and III (phosphate binding pocket) in the cap-binding site.



N-7 methylated or benzylated guanosine analogs are poor agents in cell diffusion because of the negative charges on the phosphates, despite their great affinity to eIF4E. This concern was addressed from the beginning when the library of analogs was constructed. Attempts to neutralize the charged phosphate by introducing amino acid conjugated phosphoramidates only reduced the efficacy of these compounds *in vitro* [8]. When another *in vitro* study indicated equivalent or higher potency for phosphoramidates in relation to 7-benzyl GMP, the mechanism for such discrepancy was under investigation right away. The merits of the finding are discussed in section 1.3.3. On the other hand, phosphoramidates became plausible eIF4E antagonists and the center of this work.

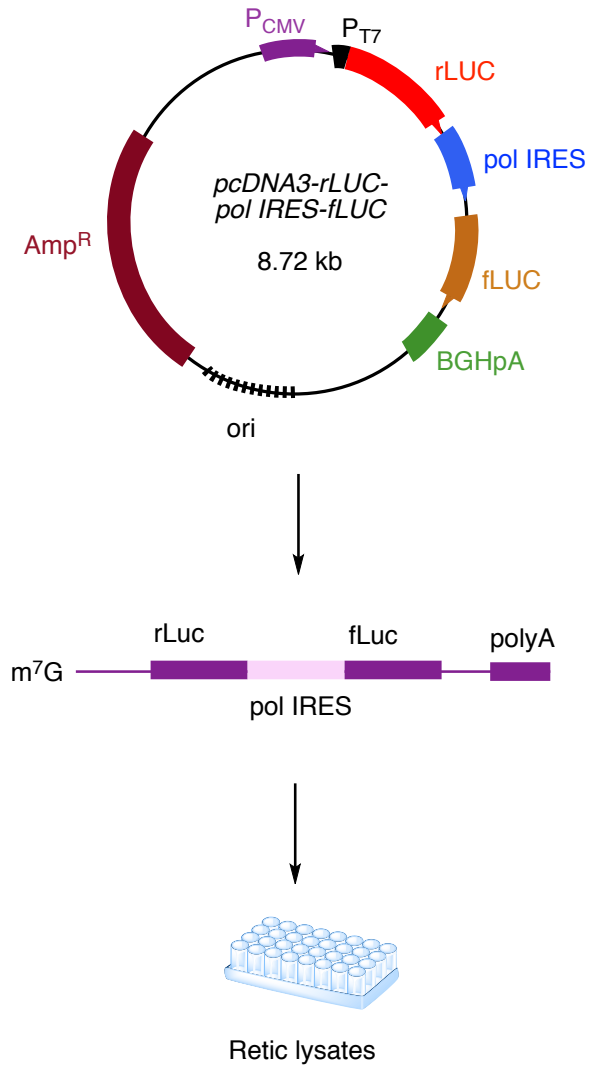
Relevant studies showed that free eIF4E is more prone to ubiquitin-induced proteasome degradation [46]. When an antagonist occupies binding site of eIF4E, it will most likely be recognized as “free enzyme” because the enzyme lacks the usual association to 4EBP or the eIF4F complex. Section 1.4 will go into this in more detail.

1.3.2 Inhibition of Cap-Dependent Translation by Cap Analogues *in Vitro*

Despite the reduced efficacy in fluorescent quenching experiments [8], it became necessary to evaluate propitious cap analogues *in vitro* for their inhibitory effects on cap-dependent translation. To achieve this, Wagner and group employed a cell-free translation assay that uses the dual-luciferase mRNA, RLUC-POLIRES-FLUC, as a reporter (**Figure 7**). In this system, translation of *Renilla reniformis* luciferase (RLUC) is strictly cap-dependent, whereas translation of firefly luciferase (FLUC) proceeds in a cap-independent manner via an internal ribosomal entry site (IRES). The activities of

firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding a luciferase assay reagent to generate a luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the Renilla luciferase reaction is initiated by simultaneously adding a different reagent to the same tube. The signal generated from this latter reaction decays slowly over the course of the measurement. The integrated format of the dual luciferase reporter assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions. The vectors may be used to co-transfect mammalian cells with any experimental and control reporter genes.

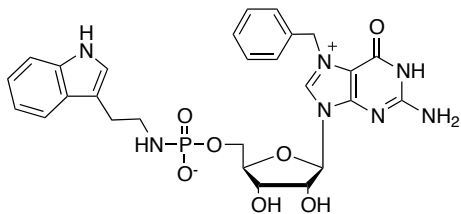
Figure 7. The Dual-luciferase Translational Activity Assay. Two distinct luciferase genes are embedded in the pcDNA3-rLUC-pol IRES-fLUC plasmid. Based on their different bio-luminescence wavelength, the detector will separate the signal intensity of cap-dependent translation (rLUC) from that of the cap-independent translation (fLUC).



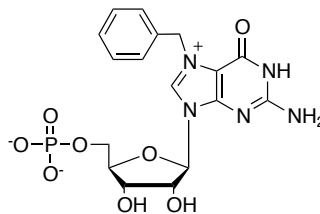
Adapted from Promega Dual-luciferase Translational Activity Assay Kit.

While most of the 7-BnGMP phosphoramidates revealed only marginal inhibitory potency compared to that of their parent compounds 7-BnGMP and 7-MeGTP, alteration of 7-BnGMP with D-phenylalanine (4Ei-2) or D-alanine (4Ei-3) resulted in retention of more than 60% of the inhibitory activity, and 7-BnGMP tryptamine-phosphoramidate (4Ei-1) retained more than 80% of the inhibitory activity of the parent compounds, with an IC_{50} comparable to that of 7-BnGMP (16.7 ± 3.2 vs 15.9 ± 2.0 μ M) (**Figure 8**, [8]).

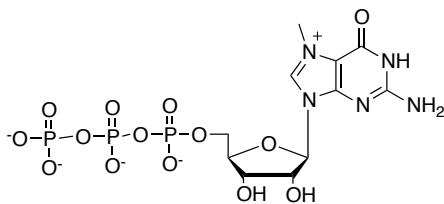
Figure 8. Evaluation of Phosphoramidates and Their Parent Compounds



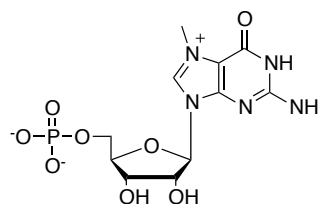
4E-i1, $IC_{50} = 16.7 \pm 3.2 \mu M$
 $K_d = 31.0 \pm 1.0 \mu M$



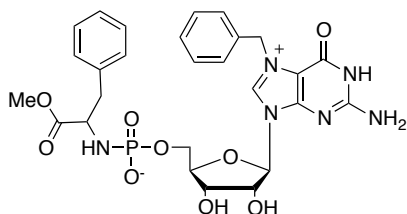
Bn⁷GMP, $IC_{50} = 15.9 \pm 2.0 \mu M$
 $K_d = 0.800 \pm 0.060 \mu M$



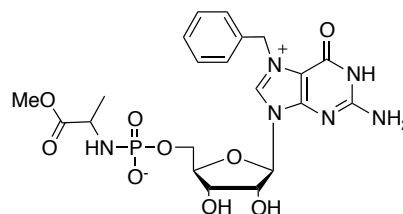
Me⁷GTP, $IC_{50} = 5.0 \pm 0.6 \mu M$
 $K_d = 0.010 \pm 0.003 \mu M$



Me⁷GMP, $IC_{50} = 200 \pm 30 \mu M$
 $K_d = 7.5 \pm 0.4 \mu M$



4E-i2, $K_d = 81.0 \pm 12.8 \mu M$



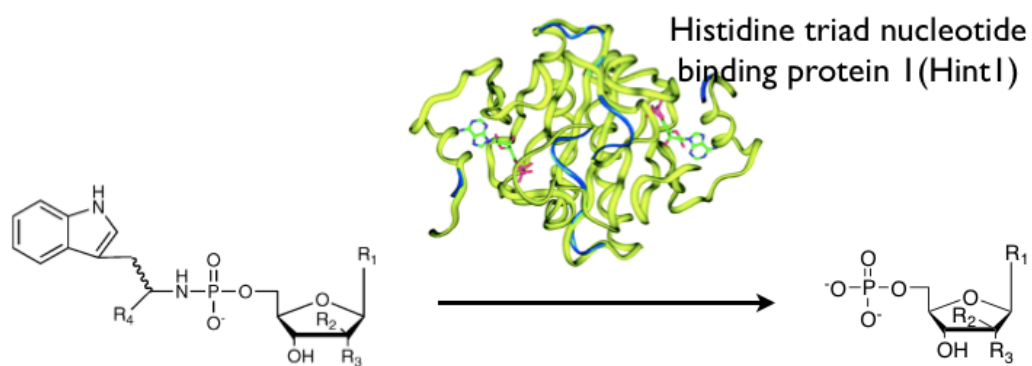
4E-i3, $K_d = 55.02 \pm 7.4 \mu M$

1.3.3 Human Hint1 and phosphoramidates

It has been shown that 4Ei-1 is a substrate for human Hint1, and rabbit tissues express the highly homologous (95%) rabbit Hint1. The high activity demonstrated by 4E-i1 in retic lysates and low affinity to eIF4E could not be explained unless there was enzyme activation. Examination of the rabbit reticulocyte lysates for HINT1 activity on the basis of endogenous phosphoramidase showed the lysates possess 23 ng of active Hint per 20 μ L of lysate. Thus, while 4Ei-1 has a low affinity for eIF4E, its ability to serve as a substrate for endogenous rabbit Hint1 enables it to be rapidly converted to the active species, 7-BnGMP [8].

The *in vivo* efficacy of phosphoramidates, as it turned out, was the result of intracellular enzyme activation. Mammalian cells produce histidine triad protein (HinT), a member of the phosphoramidase family, which hydrolyzes phosphoramidates to release the protecting group and the active monophosphate (**Figure 9**). Substrate specificity for Hint1 is somewhat strict. Chou et al. (2007) synthesized and tested various nucleotide phosphoramidates and discovered that the tryptamine group is necessary for efficient Hint1 activation [10].

Figure 9. Rationale for Using Tryptamine Phosphoramidate as Pro-drug



1.3.4 Inhibition of Cap-Dependent Translation by Cap Analogues *in Vivo*

Once 4E-i1 had been selected as a potential pro-drug for translation-induced abnormality in cancer cells, its pharmacological parameters such as efficacy, toxicity, and biological activity were examined in teleost zebrafish (*Danio rerio*), a popular model organism for drug discovery [47]. The dual-luciferase reporter mRNA designed for the *in vitro* studies was microinjected with 4E-i1 into freshly fertilized zebrafish eggs. Cycloheximide (CHD), a toxin that inhibits both cap-dependent and IRES-mediated reporter translation, was co-injected as control. Consistent with the cell-free experiments, injections of 4Ei-1 (and other phosphoramidate test compounds, not discussed here) in doses ranging from 5 to 25 pmol per egg inhibited up to 30% of cap-dependent translation without adverse effects on cell division or development. As seen in the cell-free system, introducing active compounds led to a dose-dependent increase in IRES-reporter translation, thereby excluding a nonspecific toxic effect of the compounds on the translational machinery or the organism itself. These findings indicate that 7-BnGMP and its phosphoramidated derivative 4E-i1 can inhibit cap-dependent translation by up to 30% in the physiological context of living vertebrate cells without cytotoxicity [47].

1.4 EIF4E Ubiquitination

Murata and Shimotohno had reasons to believe translation initiation factor 4E (eIF4E) is subject to ubiquitination and proteasome-dependent degradation [46, 48]. In their work in 2006, human embryonic kidney (HEK) 293T cells were used as eIF4E-expressing models. Immunoprecipitation and immunoblotting of FLAG-tagged eIF4E and/or HA-tagged ubiquitin demonstrated the specific linkage of Ub and 4E, in mono-

and poly-conjugated forms. Single mutations in phosphorylating and cap-binding residues did not affect 4E ubiquitination significantly. In contrast, mutation of the eIF4G-binding residue (W73A) resulted in dramatic increase of ubiquitinated 4E and shortened protein half-life. Accumulation of poly-ubiquitinated 4E was also observed in the presence of MG132, a proteasomal protease inhibitor. Treatment with MG132 also elevated levels of both WT and W73A mutant eIF4E. Although 4E ubiquitination did not seem to disrupt its cap-binding abilities, it impeded phosphorylation (at Ser-209, in which case a phospho-eIF4E antibody was used) and eIF4G-recruiting. Interestingly, the binding of 4E-BP contributed negatively to the extent of 4E ubiquitination, and only non-ubiquitinated eIF4E was associated with 4E-BP. Immunoassays might suggest ubiquitination through Chip (a E3 ligase), specifically Hsc70. Endogenous eIF4E levels were also investigated through Western Blot analysis, and similar results prevailed. It was noted that levels of endogenous eIF4E ubiquitination was very low. Two years later, Clemens and group [30] confirmed the hypothesis that 4E-BP phosphorylation reduced its affinity for eIF4E.

It is important for us to address whether 4E-11 inhibits or enhances proteasome degradation of eIF4E using Western Blot analysis. Experimental details and future objectives will be elucidated in Chapter 3.

1.5 Discussion

In addition to the traditional roles of eIF4E, recent investigations have discovered new eIF4E interactions with proteins involved in development, cell cycle control and viral translation initiation [49-51].

The majority (~68%) of eIF4E is located in nuclear bodies inside the nucleus through a transporter protein 4E-T [52]. Once inside the nucleus, it interacts with a number of homeodomain proteins, which bind to eIF4E and regulate cap binding and inhibit mRNA export. One of the best-studied proteins was promyelocytic leukemia protein (PML). The majority of nuclear eIF4E co-localizes with PML in complexes referred to as PML nuclear bodies [53], changes in which arise as a result of stress e.g. virus infection or treatment with interferons. It is thought that PML regulates eIF4E mRNA transport in response to such stresses. PML and the proline-rich homeodomain protein (PRH) both bind to a site on eIF4E that is distal to the cap-binding site, but binding inhibits the cap-4E interaction. Around 200 such homeodomain proteins contain potential eIF4E-binding sites and it has been suggested that they may play a role in regulating eIF4E. Hence, such interactions may be involved in cell growth, differentiation and development [54].

Positive-stranded RNA viruses have evolved a number of novel mechanisms for translation initiation on their mRNAs. For example, the gastroenteritis-causing calciviruses use a novel mechanism for translation initiation; their mRNAs are covalently attached to a viral “cap-substitute” protein to recruit eIF4E [49]. Calicivirus VPg appears to bind eIF4E at a site distinct from both the cap and the 4E-BP1 binding sites as a VPg.eIF4E.4E-BP1 complex [50]. This interaction is unique amongst mammalian RNA viruses. Hence, although the general function of VPg-directed translation is shared, the mechanisms by which the VPg proteins interact with eIF4E are distinct.

These discoveries raised more questions for eIF4E-targeting therapy. The inhibitor 4E-i1 might lead to inactivation of eIF4E and ultimately reducing intracellular eIF4E

level, but the mechanism in which eIF4E is reduced is still unknown. There might be a conformational change in the apo-eIF4E enough for poly-ubiquitination, supported by the research on the phosphorylation of 4E-BP. The conformational change could also lead to the activation of eIF4E export in the nucleus, followed by degradation. The first matter to address, perhaps, is the method of degradation. We plan to study effects of 4E-i1 coupled with a proteasome inhibitor such as MG132.

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Chapter 2

Chemistry

2.1 Introduction

Phosphoramidate prodrugs have been shown to enhance biological activity of parent nucleosides by increasing the intracellular nucleoside 5'-triphosphate levels via improved intracellular transport and/or bypassing the rate-limiting monophosphorylation step [1-8]. In most cases, the bottleneck in the metabolic activation of nucleosides is the enzymatic phosphorylation of the modified nucleoside by cellular kinases [9-16]. Therefore, a number of prodrug strategies have been developed to improve anti-cancer efficacy and reduce toxicity by delivering the modified nucleoside-monophosphate into the cell [6, 7, 10, 11, 17-24]. Our group has conducted experiments to screen a library of compounds for activity against eIF4E [3, 8, 25, 26]. We have decided to focus on a few phosphoramidate analogues and study their biological effects extensively (**Figure 1**). To date there are two most commonly employed methods of making phosphoramidates [27]. The first is to combine the monophosphate and the masking group (usually amino acid derivatives) by an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) - mediated coupling, followed by column chromatography. The second is the H-phosphonate method described by Todd and later developed by Stawinski et al [28]. In this approach, H-monophosphonates were prepared by various phosphorylating reagents such as triimidazolylphosphine (Im3P, [29]) and salicylchlorophosphine (SCP, [30]). A mixed anhydride was obtained from the H-monophosphonate by the activation of a condensing reagent such as pivaloyl chloride (PvCl) and then attacked with an alcohol in situ [31, 32]. While phosphoramidates are known to be somewhat stable to strongly acidic conditions, our synthetic works have shown phosphoramidates to have little to no stability to strongly basic and nucleophilic conditions. We have chosen the EDC-

mediated coupling for our phosphoramidates to avoid such complications.

Another structure we are interested in is the phosphoramidite diester proposed by McGuigan [11, 19-23]. The diester could be prepared by treatment of nucleoside with POCl₃ as described before [27]. We hope to compare activities and other pharmacokinetic properties between these two classes of compounds.

2.2 Experimental

2.2.1 7-benzyl GMP

With slight modification to a published synthesis [3, 26, 33], guanosine monophosphate disodium salt (0.81 g, 2 mmol) was suspended in anhydrous DMSO (5 mL) under Argon. Benzyl bromide (1.71 g, 5 equiv.) was added drop-wise with a syringe. The reaction mixture contained a white precipitate and turned clear after 20 hours. The progress of the reaction was checked by ³¹P NMR. Upon completion, excess benzyl bromide was extracted with anhydrous ethyl ether and discarded. The aqueous layer containing 7-benzyl guanosine monophosphate (7-BnGMP) and DMSO was loaded onto DEAE Sephadex A25 HCO₃⁻ anion exchange column and eluted with gradient triethylammonium bicarbonate (TEAB, 0 - 0.6 M). UV active fractions were pooled and lyophilized overnight to yield a white powder. The white powder was dissolved in water and passed through Dowex-50WX8-200 (Na⁺ form) cation exchange column. UV active fractions were pooled and lyophilized to give the desired 7-BnGMP sodium salts as white amorphous solids in 90 - 95 % yield.

¹H NMR (D₂O, 300 MHz): δ 9.42 (s, 1H), δ 6.92-7.21 (m, 5H), δ 5.92 (s, 1H), δ 5.41 (s, 2H), δ 3.92-4.63 (m, 6H, ribose). ³¹P NMR (D₂O, 300 MHz): δ 4.81. HR-MS (ESI neg)

$C_{17}H_{19}N_5O_8P$, m/z: calcd 452.10, found 452.10.

2.2.2 7-methyl GMP

Guanosine monophosphate disodium salt (0.41 g, 1 mmol) was suspended in anhydrous DMSO (4 mL) under Argon. Iodomethane (0.14 g, 5 equiv.) dissolved in anhydrous DMSO (1 mL) was added drop-wise with a syringe. The reaction mixture contained a white precipitate and turned clear after 20 hours. The progress of the reaction was checked by ^{31}P NMR and thin-layer chromatography. Upon completion, excess iodomethane was extracted with anhydrous ethyl ether and discarded. The aqueous layer containing 7-methyl guanosine monophosphate (7-MeGMP) and DMSO was loaded onto DEAE Sephadex A25 HCO_3^- anion exchange column and eluted with gradient triethylammonium bicarbonate (TEAB, 0 - 0.6 M). UV active fractions were pooled and lyophilized overnight to yield a white powder. The white powder was dissolved in water and passed through Dowex-50WX8-200 (Na^+ form) cation exchange column. UV active fractions were pooled and lyophilized to give the desired 7-MeGMP sodium salts as white amorphous solids in 90 - 95 % yield.

1H NMR (D_2O , 300 MHz): δ 8.92 (s, 1H), δ 8.56 (s, 1H), δ 6.16 (d, 1H, ribose), δ 4.75 (t, 1H), δ 4.51 (t, 1H), δ 4.03-4.75 (m, 5H, ribose), δ 3.58 (s, 2H). ^{31}P NMR (D_2O , 300 MHz): δ 4.98. HR-MS (ESI neg) $C_{11}H_{15}N_5O_8P$, m/z: calcd 376.07, found 376.07.

2.2.3 Tryptamine 7-Benzyl Guanosine Phosphoramidate (4Ei-1)

With slight modification to a published synthesis [26], 7-benzyl-guanosine monophosphate disodium salt (0.25 g, 0.50 mmol) and tryptamine hydrochloride salt

(2.00 mmol, 4 equiv.) were dissolved in H₂O (200 μL), and the pH of the solution was adjusted to 6.5 by drop-wise addition of 1N aqueous HCl. To the foregoing was added EDC (0.48 g, 2.50 mmol, 5 equiv.), and the resultant solution was allowed to stir at 55°C for 2 hours. The product mixture was concentrated by lyophilization, and the residue was chromatographed on silica gel, eluting with CHCl₃/MeOH/H₂O (5:2:0.25, containing 0.5% NH₄OH). The solid obtained, EDC salt of the phosphoramidate, after evaporation of the solvent was passed through an ion-exchange column (Dowex-50WX8-200, Na⁺ form), and the relevant fractions were pooled and lyophilized. Further purification was carried out with CombiFlash chromatography utilizing a silica gel system as described above to give the desired phosphoramidate sodium salts as white amorphous solids in 35-40% yield.

¹H NMR (D₂O, 300 MHz): δ 8.82 (s, 1H), δ 8.49 (s, 1H), δ 7.11-7.60 (m, 10H), δ 6.16 (d, 1H, ribose), δ 5.96 (s, 2H), δ 4.78 (dt, 1H, ribose), δ 4.51 (t, 1H, ribose), δ 4.05-4.45 (m, 3H, ribose), δ 3.63 (s, 2H), δ 2.92 (dd, 2H), δ 2.07 (dd, 2H). ³¹P NMR (D₂O, 300 MHz): δ 8.87. HR-MS (ESI pos) C₂₇H₃₀N₇O₇P, m/z: calcd 595.19, found 595.19.

2.2.4 Tryptamine 7-Methyl Guanosine Phosphoramidate

7-Methyl-guanosine monophosphate disodium salt (0.10 g, 0.27 mmol) and tryptamine hydrochloride salt (0.17 g, 4 equiv.) were dissolved in H₂O (80 μL), and the pH of the solution was adjusted to 6.5 by drop-wise addition of 2N aqueous HCl. To the foregoing was added EDC (0.26 g, 1.35 mmol, 5 equiv.), and the resultant solution was allowed to stir at 55°C for 2 hours. The product mixture was concentrated by lyophilization, and the residue was chromatographed on silica gel, eluting with CHCl₃/MeOH/H₂O (5:2:0.25,

containing 0.5% NH₄OH). The solid obtained, EDC salt of the phosphoramidate, after evaporation of the solvent was passed through an ion-exchange column (Dowex-50WX8-200, Na⁺ form), and the relevant fractions were pooled and lyophilized. Further purification was carried out with CombiFlash chromatography utilizing silica gel as described above to give the desired phosphoramidate sodium salts as white amorphous solids in 50 - 55% yield.

¹H NMR (D₂O, 300 MHz): δ 8.92 (s, 1H), δ 8.36 (s, 1H), δ 7.60 (dt, 1H), δ 7.47 (s, 1H), δ 7.32 (m, 1H), δ 7.05-7.21 (m, 2H), δ 6.16 (d, 1H, ribose), δ 4.78 (dt, 1H, ribose), δ 4.51 (t, 1H, ribose), δ 4.05-4.45 (m, 3H, ribose), δ 3.92 (s, 3H), δ 3.63 (s, 2H), δ 2.92 (dd, 2H), δ 2.07 (dd, 2H). ³¹P NMR (D₂O, 300 MHz): δ 7.91. HR-MS (ESI pos) C₂₁H₂₆N₇O₇P, m/z: calcd 519.16, found 519.16.

2.2.5 7-benzyl guanosine

To a dried round-bottom flask was added anhydrous guanosine (0.6 g, 2.1 mmol) and 30ml of anhydrous DMF. The flask was purged under vacuum and refilled with Argon several times to eliminate moisture. Benzylbromide (2 g, 5 equiv.) was injected into the stirring mixture with a syringe. The reaction was stirred for 48 hours and the cloudy mixture became clear. Most of the solvent DMF was removed with a high-vacuum rotary evaporator to yield a dry powder. Anhydrous ethyl ether (~200ml) extraction removed unreacted benzylbromide and additional DMF. Proton NMR was taken and showed DMF as impurity (1 : 1 as product : DMF, 83.7% product w/w). Taking the impurity into account the final yield was 635.7mg of white solid (80.2%).

¹H NMR (MeOD, 300 MHz): δ 8.62 (s, 1H), δ 8.41 (s, 1H), δ 7.49 (dt, 2H), δ 7.26-7.33

(m, 3H), δ 6.03 (d, 1H, ribose), δ 5.65 (s, 2H), δ 4.55 (t, 1H, ribose), δ 4.51 (t, 1H, ribose), δ 4.38 (m, 1H, ribose), δ 3.78 (m, 1H, ribose), δ 3.66 (m, 1H), δ 3.59 (m, 1H, ribose), δ 3.58 (m, 2H). HR-MS (ESI pos) $C_{17}H_{20}N_5O_5P$, m/z: calcd 374.15, found 374.15.

2.2.6 Phenylalanyl Phosphorchloridate (methyl 2-chlorophenoxy phosphorylamino propanoate)

A solution of distilled triethylamine (0.88 mL, 2.1 equiv.) in anhydrous CH_2Cl_2 (18 mL) was added dropwise via an addition funnel into a vigorously stirred solution of phenyl phosphorodichloridate (0.87 g, 3.00 mmol) and L-alanine methyl ester hydrochloride (0.42 g, 3.00 mmol) in anhydrous CH_2Cl_2 (25 mL) at -70 °C over a period of 3 hours under argon atmosphere. Subsequently, the resultant mixture was allowed to gradually warm up to room temperature and stirred overnight at room temperature. The solvent was removed on a rotary evaporator. Anhydrous ethyl ether (30 mL) was added to dissolve the product and then filtered under aspirator pressure to remove the remaining white solid. The white solid was rinsed with anhydrous ethyl ether (2×6 mL). The ether layers were combined and evaporated to dryness to afford a viscous oil. This oil was further purified via silica CombiFlash chromatography and eluted with 2.5% methanol in chloroform. Phenylalanyl phosphorchloridate (0.9 g, 80 %) was a pale pink-yellow viscous oil.

1H NMR (CD_2Cl_2 , 300 MHz): δ 7.89 (s, 1H, DMF), δ 7.01-7.21 (m, 5H), δ 3.68 (s, 3H), δ 3.63 (dd, 1H), δ 2.88 (s, 3H, DMF), δ 2.80 (s, 3H, DMF), δ 1.28 (d, 3H). ^{31}P NMR (D_2O , 300 MHz): δ 7.77-7.79 (d, 1P). HR-MS (ESI pos) $C_{10}H_{13}ClNO_4P$, m/z: calcd 277.03,

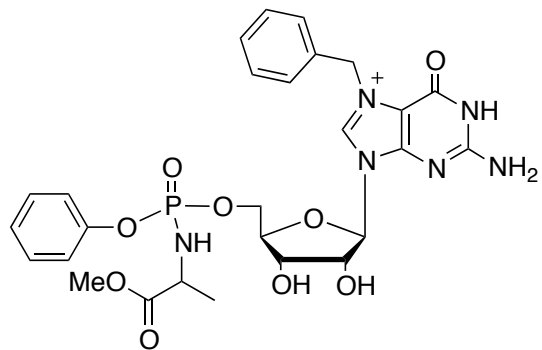
found 277.03.

2.2.7 7-Benzyl methoxyalanylphenyl-phosphoryl guanosine (SL-II-67, Figure 1)

To a solution of 7-benzyl guanosine (0.28 g, 1 mmol) in anhydrous THF (5 mL) was added N-methylimidazole (0.49 g, 6 equiv.), then phenylalanyl phosphorochloridate (0.9 g, 3 equiv.) in anhydrous THF (5 mL). The resultant reaction mixture was stirred vigorously at room temperature under argon atmosphere in the dark. Progress of the reaction was monitored by TLC (silica gel, 5 % methanol in chloroform). The reaction was complete after 48 hours of stirring and the THF was removed on a rotary evaporator. Chloroform (25 mL) was added to dissolve the residue. Then the chloroform solution was washed with 1.0 M aqueous HCl solution (2 × 20 ml), saturated aqueous NaHCO₃ solution (2 × 20 ml), water (2 × 20 ml), and brine (1 × 20 ml). The organic layer was dried over anhydrous Na₂SO₄ and evaporated to dryness to afford the crude product as a pale-yellow viscous oil. Purification was conducted by CombiFlash chromatography (silica gel, 1.2% to 2.5% methanol in chloroform) to give 12% pure product as a nearly colorless and viscous oil.

¹H NMR (D₂O, 300 MHz): δ 8.52 (s, 1H), δ 7.21-7.60 (m, 10H), δ 6.46 (d, 1H, ribose), δ 6.06 (s, 2H), δ 4.69 (t, 1H, ribose), δ 4.59 (t, 1H, ribose), δ 4.15-4.58 (m, 3H, ribose), δ 3.92 (s, 3H), δ 3.63 (m, 1H), δ 3.42 (dd, 2H), δ 1.27 (d, 3H). ³¹P NMR (D₂O, 300 MHz): δ 5.57-5.60 (d, 1P). HR-MS (ESI pos) C₂₇H₃₂N₆O₉P⁺, m/z: calcd 615.20, found 615.20.

Figure 1. Chemical structure of SL-II-67



2.3 Discussion

The syntheses of phosphoramidates presented in this chapter follow the EDC - mediated coupling of nucleoside monophosphate and amino acid derived protecting group. The general yield of this reaction according to literature is usually in the single digits [26, 27], but we have improved it to 30 - 40%. EDC is very moisture sensitive and could form the EDC - tryptamine byproduct in water rapidly. To obtain optimum yield, EDC should be added in small portions over the reaction period. The other critical parameter is the pH of solution. EDC requires an acidic environment to be activated through protonation on the diazo group, but too much acidity would reduce the effectiveness of tryptamine as a nucleophile. Furthermore, basic condition triggers de-alkylation at the guanosine N-7 position as well as decomposition of phosphoramidates [18, 34, 35]. Therefore the pH of the solution should never exceed 7 and be kept around 6.5 for best results. Purification of the final product should also be handled with great care. An excess application of tryptamine complicates the elution profile from silica gel chromatography, and it is advised to perform at least two such separations before ion-exchange chromatography.

4Ei-1 has greater IC_{50} than its 7-methyl analog [4], and the better affinity could be explained through the extra π interaction offered by the 7-benzyl group of 4Ei-1. Except for the difference in efficacy, the two compounds share identical uptake and metabolism pathways and this makes the 7-methyl analog a suitable control compound for 4Ei-1. Experiments testing 4Ei-1 should include the 7-methyl analog as a comparison.

2.4 References

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Chapter 3

Chemosensitization of Lung and Breast Cancer Cells to Gemcitabine Treatment with a Pronucleotide of eIF4E Antagonist 4Ei-1 and Insights into Its Mechanism

3.1 Introduction

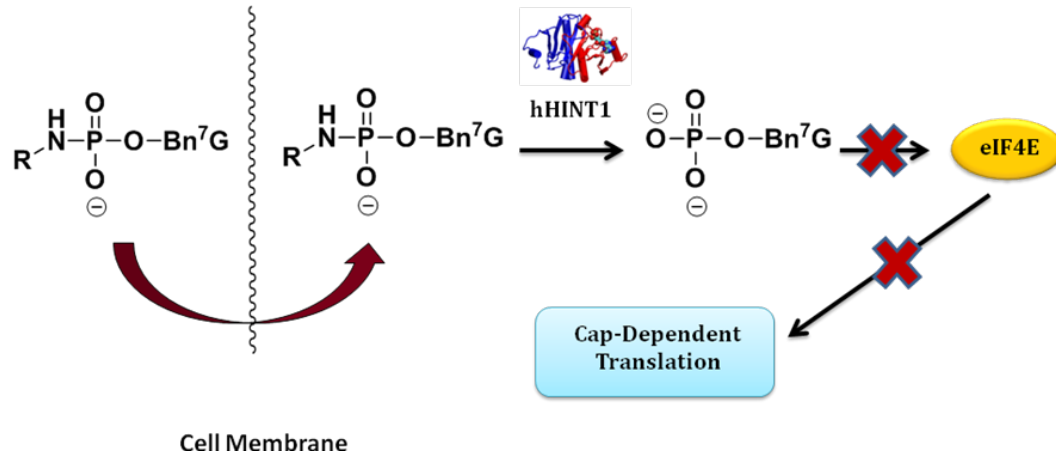
Aberrant regulation of cap-dependent translation appears to be essential for the development of cancer and fibrotic diseases [1]. After transport out of the nucleus, the eukaryotic initiation factor 4E (eIF4E) binds the 5'-cap of cellular mRNAs by displacing nuclear 5'-cap binding complex (CBC), leading to formation of the eIF4F translation initiation complex. The eIF4F complex then scans 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 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) is promoted disproportionately resulting in the transformation of normal cells to tumorigenicity [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 analogs of 7-methyl guanosine (7-MeG) nucleotides has 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 has been developed to target eIF4E for inhibiting 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 enzymes such as phosphatases and 5'-nucleotidase present in blood and on cell surface, which rapidly convert the phosphates to the corresponding nucleosides [16]. In addition, since phosphatases are negatively charged under 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 group of phosphoramidate monoesters as prodrugs of 7Bn-GMP, and has 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-

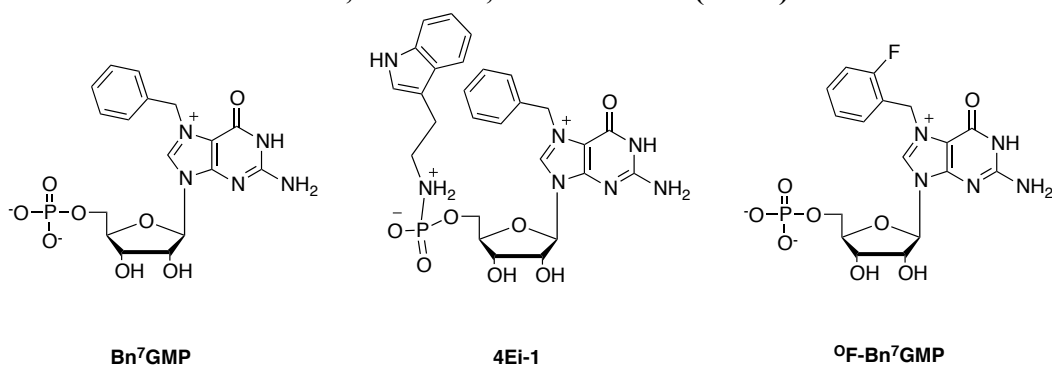
mesenchymal transition in zebrafish embryos with no toxicity to the normal embryo development [27]. 4Ei-1 functions as a prodrug of 7Bn-GMP and has been found to be a substrate for histidine triad nucleotide binding protein-1 (HINT1), including HINT1, which is believed to be responsible for its intracellular bioactivation [28, 29] (**Figure 1**).

Figure 1. Rationale of phosphoramidate as a prodrug



In the United States the leading causes of cancer death in men and women are lung cancer and breast cancer, respectively [30]. Once the patient has a tissue diagnosis and has been accurately staged, the treatment plan can be determined. The patient's ability to tolerate treatment is of utmost importance, whether the planned therapy is surgery, radiation therapy, chemotherapy, or multi modality therapy [31]. This became our rationale to first examine pronucleotide 4Ei-1's cellular metabolism, and efficacy in breast and lung cancer cells. Previously, we have demonstrated that nucleoside phosphoramidates can undergo cellular uptake and conversion to the corresponding nucleoside monophosphate. 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.

Figure 2. Structures of 4Ei-1, Bn⁷GMP, and Bn⁷GMP (ISTD).



In this report we have validated that 4Ei-1 was converted to Bn⁷GMP by human HINT1 (hHINT-1) in both breast cancer MDA-MB-231 and lung cancer H460, H838, and H2009 cells. We developed a capillary HPLC-ESI-MS/MS method for this efficient and rapid analysis of the intracellular metabolism of the pronucleotide 4Ei-1. The question whether 4Ei-1 reduced cap-dependent translations both *in vivo* and *in vitro* through the same mechanism as eIF4E-specific ASOs' was also addressed. Western blot analysis showed reduction of eIF4E concentrations in MDA-MB-231, H460, H838, and H2009 cells in the presence of 4Ei-1, and the effect was reversed when 4Ei-1 was absent. The same observation was reported with second-generation eIF4E-specific ASOs. Furthermore, the reduction of eIF4E concentrations resulting from 4Ei-1 was reversed when a proteosomal inhibitor MG132 was added to these cell cultures. Several other groups have supported that inactive eIF4E is modified by polyubiquitination and degraded by the proteasome [32-35]. We propose one of the possible pathways of eIF4E diminution as proteasome degradation.

We intended to combine 4Ei-1's ability to inhibit cap-dependent translation with existing cancer chemotherapy to achieve a synergistic effect. Nucleoside analogue gemcitabine is a very powerful drug for both breast and lung cancers, but chemoresistance and cytotoxicity remain problematic. Gemcitabine is taken up by human equilibrative nucleoside transporter-1 (hENT1) and activated inside the cell by a series of kinase-facilitated events: the nucleoside is first phosphorylated to gemcitabine monophosphate by deoxycytidine kinase (dCK), and further by nucleoside monophosphate kinases and nucleoside diphosphate kinases to gemcitabine triphosphate. Both diphosphate and triphosphate contribute to cytotoxic effect on carcinoma cells, but

through different mechanisms. Gemcitabine diphosphate inhibits ribonucleotide reductase (RR) and decreases deoxycytidine concentration, a process that enhances gemcitabine phosphorylation. Gemcitabine triphosphate is incorporated into DNA and causes DNA replication to terminate. Cancer cells develop resistance by either down-regulating enzymes in the gemcitabine activation pathway, or activation of the protein kinase (Akt) pathway. We predicted that reduction of eIF4E concentrations by 4Ei-1 would not affect the gemcitabine activation pathway: enzymes involved in this pathway are encoded on strong mRNAs that are translated more frequently than weak mRNAs. Meanwhile, 4Ei-1 may sensitize cancer cells to gemcitabine by inhibiting translation of those weak mRNAs encoding proteins in the Akt pathway.

Our colony forming assays with MDA-MB-231, H460, H549, and H838 cells showed 50% decrease in colony numbers when non-toxic levels of 4Ei-1 and gemcitabine were added together. Although the mechanism of this synergistic phenomenon is still unknown, our report had provided for the first time a small-molecule antagonist of eIF4E could do so by reducing intracellular eIF4E concentrations.

3.2 Material and Methods

3.2.1 Materials

Bn⁷GMP, 4Ei-1, ¹⁸F-Bn⁷GMP 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 and were used 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 penicillin – 5,000 µg/ml streptomycin), Trypsine (0.25% trypsin, 2.21 mM EDTA), NuPAGE[®] 10% Bis-Tris gel, NuPAGE[®] MES SDS running buffer (20X), 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 µm 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 X 7 inches 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).

3.2.2 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 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.

3.2.3 HPLC Sample Preparation

5 million MDA-MB-231, H460, H838, or H2009 cells were seeded to 12-well plates. Each well was treated with either 100 μ M 4Ei-1 or fresh DMEM as controls. All samples were incubated at 37 °C for the following time lengths: 0.5, 2, 4, 29 hours. Then 5 ml ice-cold unsupplemented medium was added and cell pellets were obtained by centrifugation. 0.5 ml 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 (57). The cell extracts were dried by lyophilization (Labconco). 100 μ L 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 standards were 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.

3.2.4 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 $^{\text{O}}\text{F-Bn}^{\text{7}}\text{GMP}$ at constant concentrations. 10 μ L of standard stock and 10 μ L of $^{\text{O}}\text{F-Bn}^{\text{7}}\text{GMP}$ were added to each HPLC sample vial, and dried using lyophilization (Labconco). Then 100 μ L HEPES buffer was added to each vial. Therefore, all standards and $^{\text{O}}\text{F-Bn}^{\text{7}}\text{GMP}$ were diluted 10-fold and the final concentrations of the eight standards are: 5, 10, 50, 100, 500, 1000, 5000, 10000 ng/ml.

3.2.5 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 selected reaction monitoring (SRM) 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 X 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.

3.2.6 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 [36-39]. 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, Bn⁷GMP, and ^oF-Bn⁷GMP. The gradient LC condition is 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. 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 in 80 : 20 (water : acetonitrile).

3.2.7 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. The optimal retention times for the analytes and internal standards were determined 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.

3.2.8 Matrix Effect

The analytes were spiked into either 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 those spiked into pure solvent.

3.2.9 Measurement of Intracellular Concentrations of the Metabolite and the Phosphoramidate

A standard curve was generated for both 4Ei-1 and Bn⁷GMP. Quantitation of prodrugs and metabolites were 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 Bn⁷GMP was carried out in a similar way except that the internal standard concentration was adjusted according to its response

to the mass spectrometer.

3.2.10 Colony forming assay

H460 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 d) colonies were of appropriate size, cells were fixed for 10 min in 10% formalin, washed with water, stained with coomassie blue, images collected and colonies manually counted. The colony number was expressed as the mean +/- SD normalized to untreated cells.

3.2.11 Western Blot Analysis

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 degrees for 24, 48, or 72 hours. Media were replaced every 12 hours. 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 hours. Cell cultures were harvested immediately after pro-drug 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 (150mM NaCl, 50mM Tris, 50mM NaF, 10mM Sodium Pyrophosphate, 1mM EDTA, 1mM EGTA, 1mM DTT, and protease inhibitor) was added to pellets before three cycles of freeze (-80 degrees, 12 minutes)/thaw (37 degrees, 2 minutes). 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).

3.2.12 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.

3.3 Results

3.3.1 Effect of Ion Pairing Reagents and LC Eluting Profile

The use of ion pairing reagents has been a commonly used method for optimizing the retention time of ionic analytes [40, 41]. 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 sufficiently strong 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 the 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.

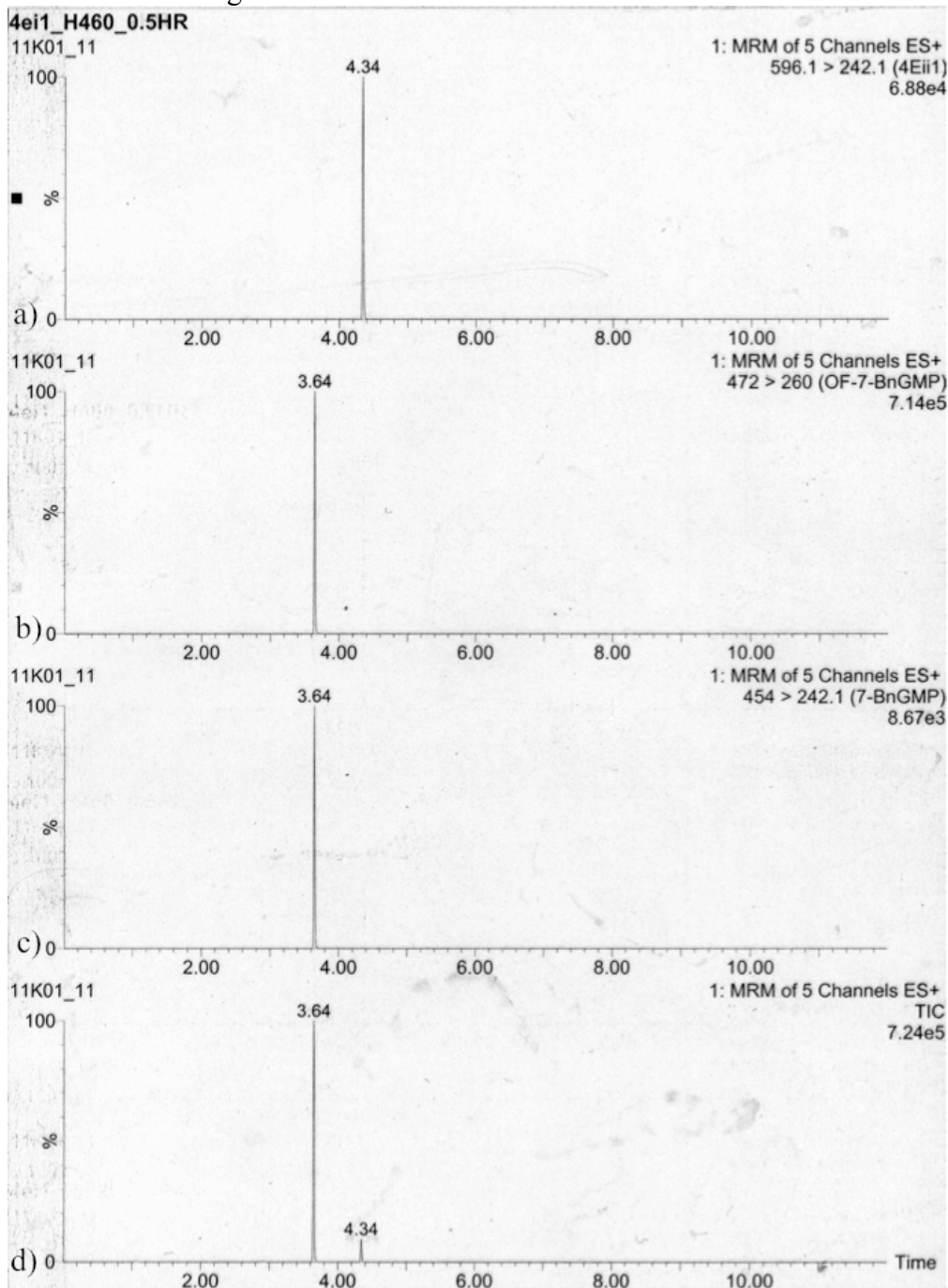
3.3.2 Matrix Effect

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 that spiked into pure solvent at a known concentration. No significant difference was observed between the two samples (data not shown). It suggested that our sample preparation procedures may have removed most cellular components which could potentially cause substantial MS signal suppression. So the matrix effect could be neglected in this study. The following standards and quality control samples were therefore prepared in Hepes buffer.

3.3.3 Standard Curves and Quality Controls

The concentrations of $^9\text{F-Bn}^7\text{GMP}$ were adjusted according to their responses relative to their respective target analyte. One representative chromatogram was shown in **Figure 3**. In order to assure 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 are 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.

Figure 3. Representative chromatograms and structures of 4Ei-1, Bn⁷GMP, and Bn⁷GMP (ISTD). (a) Chromatogram of 4Ei-1 channel in MRM mode. (b) Chromatogram of ISTD channel in SRM mode. (c) Chromatogram of Bn⁷GMP channel in MRM mode. (d) Chromatogram of total ion current (TIC). The X axis is the elution time in minute, and the Y axis is the relative abundance of the ion. The most intense ion is assigned an abundance of 100. The retention times are annotated near target peaks. The standards are always run along with the samples to be analyzed. Shown below is one representative chromatogram.

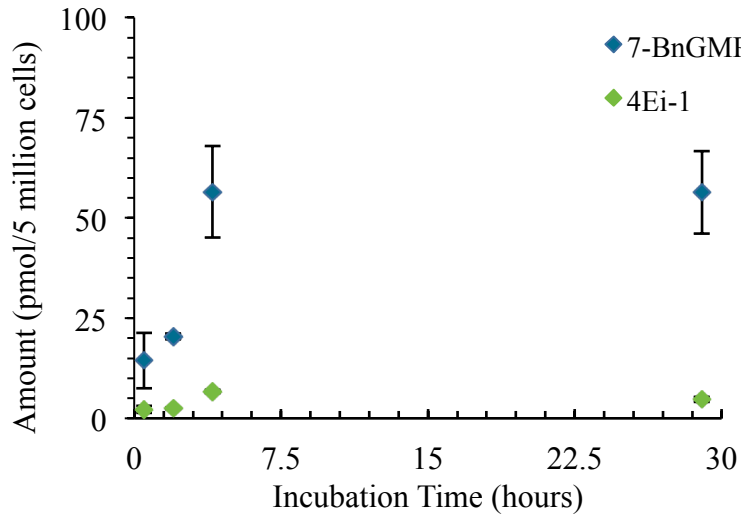


3.3.4 Bioactivation of 4Ei-1 is Time-dependent

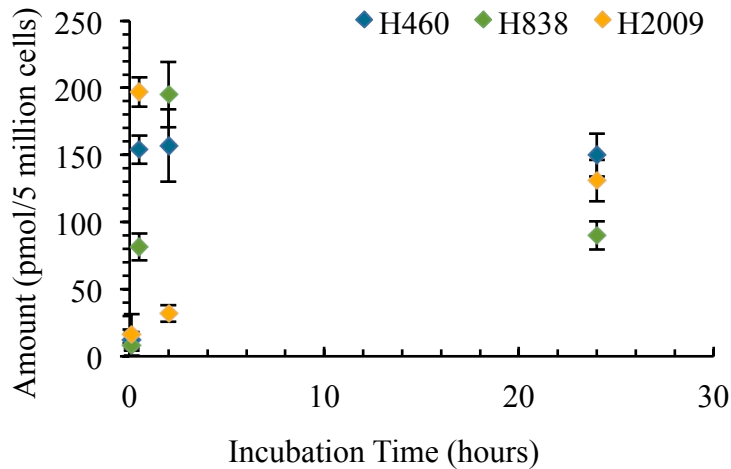
The Bn⁷GMP phosphoramidate pronucleotide 4Ei-1 (100 μM) was incubated with MDA-MB-231, H460, H838, and H2009 cells at 37 °C for various time lengths. Within 5 minutes, Bn⁷GMP was detectable intracellularly. The detectable amount of Bn⁷GMP increased with an extended incubation time for up to 4 hours. At the 4-hour time point, 56.5±11.4 pmol of Bn⁷GMP in 5 million MDA-MB-231 cells was observed. Similar amounts of Bn⁷GMP were detectable (56.4±10.3 pmol) when the incubation time was further extended to 24 hours (**Figure 4a**). Comparably, one million lung cancer cells contained around 37 pmol of Bn⁷GMP (**Figure 4b**) after 4 hours and stayed stable at 24 hours. In all cases, the amount of Bn⁷GMP was 3- to 4- fold higher than that of 4Ei-1 (**Figure 4a and 4c**). Taken the volume of cells into consideration (single cell volume was calculate as 1.8 pL according to the studies of Kim and colleagues [37]), the intracellular concentration of Bn⁷GMP reached 6.26±1.14 μM by 4 hours in MDA-MB-231 cells, and about 20 μM in all three lung cancer cells.

Figure 4. Intracellular amounts of Bn⁷GMP and 4Ei-1 in MDA-MB-231 (a), H460, H838, and H2009 (b: Bn⁷GMP, c: 4Ei-1) cells. Cells were incubated for various times at 37 °C with 100 μM 4Ei-1. Results of data derived from three different experiments are expressed as average values from triplicate wells. All data shown are the average ± SD of three separate experiments.

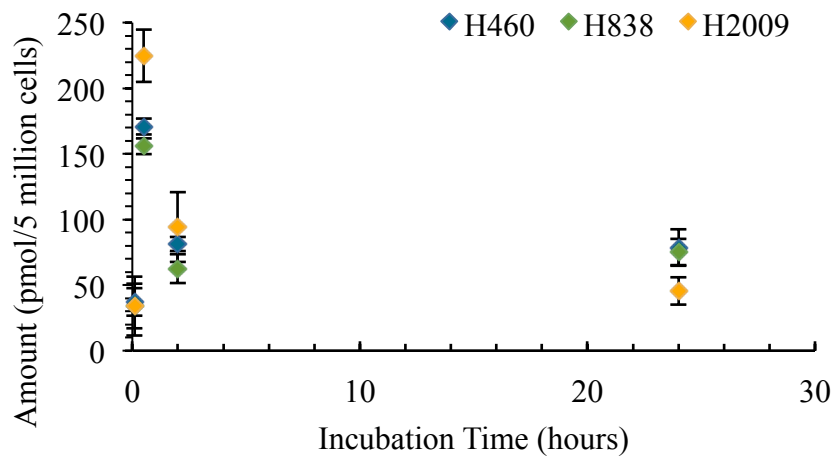
a)



b)



c)



3.3.5 Compound 4Ei-1 Inhibited Cellular eIF4E in a Dose-Dependent Manner

Cellular eIF4E levels were detected using mouse anti-eIF4E primary and anti-mouse secondary antibodies. At first we examined eIF4E levels during a 24-hour period without replacing media containing 100 μM 4Ei-1. The purpose of this short time-course experiment is to find out how long it took 4Ei-1 to reduce intracellular eIF4E level. H2009 cells were treated with 100 μM 4Ei-1 for 2 hours and analyzed at 0, 30 minutes, 2, 4, and 24 hours. The amount of intracellular eIF4E started to decrease at 30 minutes and continued to do so for the next four hours, and increased back up at 24 hours (**Figure 5a-b**). In the presence of 500 μM 4Ei-1, eIF4E almost completely reduced to zero (**Figure 5c-d**) as soon as 24 hours after treatment. The intracellular levels of eIF4E were receding in a dose-dependent manner across all three time-points. The effect of the lower 4Ei-1 concentration (50 μM) was not seen until the 72-hour continuous incubation was completed (**Figure 5d**). In general, similar phenomenon was observed with all concentrations of pro-drug: 4Ei-1 became more effective in limiting cellular eIF4E levels when higher dosage was used, or with extended incubation time (up to 72 hours).

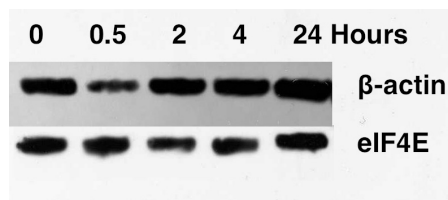
3.3.6 Compound 4Ei-1 Induced Diminution of eIF4E through Proteasome Degradation

Proteasome inhibitor MG132 (10 μM) was added to H2009 cell cultures in the presence of 500 μM 4Ei-1. To ensure stability of the two compounds in solution the media was replaced with fresh MG132 and 4Ei-1 every 12 hours. Lysates from H2009 cells were analyzed using western blot with mouse anti-eIF4E antibody. As shown in **Figure 5e**, MG132 treatment revived the half-life of eIF4E and reversed the 4Ei-1-

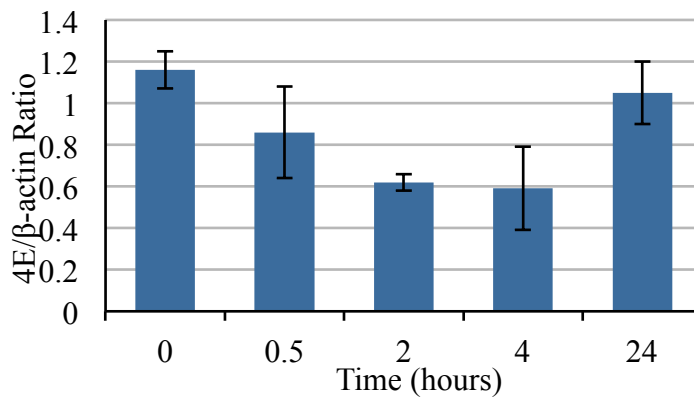
induced knockdown of eIF4E, indicating that decreases in eIF4E protein levels after 4Ei-1 treatments occur through proteosomal degradation.

Figure 5. Western blot analysis. a) Representative western blot film from the time-dependent experiment. Top: mouse anti-eIF4E blotted with anti-mouse antibodies, 60-second exposure. Bottom: mouse anti-actin blotted with anti-mouse antibodies, 30-second exposure. b) Intracellular eIF4E / β -actin ratios in H2009 cells treated with 100 μ M 4Ei-1. c) Representative western blot film from the dose-dependent experiment. d) Normalized eIF4E / β -actin values in H2009 cells treated with various concentrations of 4Ei-1 for 24, 48, and 72 hours. The eIF4E / β -actin values in non-treated H2009 cells were set to 100%. e) Representative western blot film from the proteosomal inhibitor MG132 experiment. f) Normalized eIF4E / β -actin values in MDA-MB-231 cells treated with various concentrations of 4Ei-1 for 72 hours.

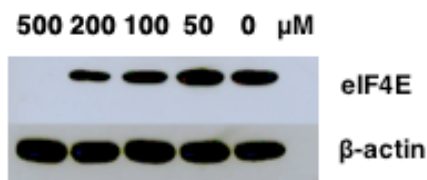
a)



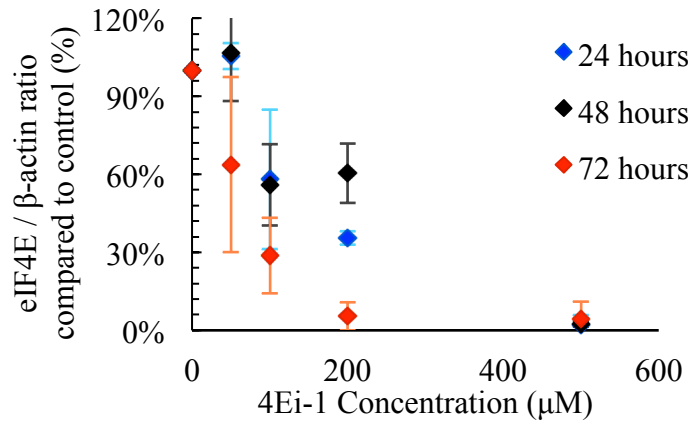
b)



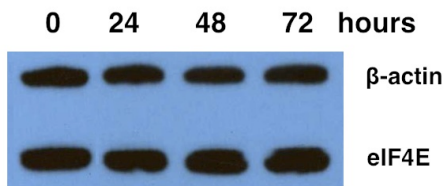
c)



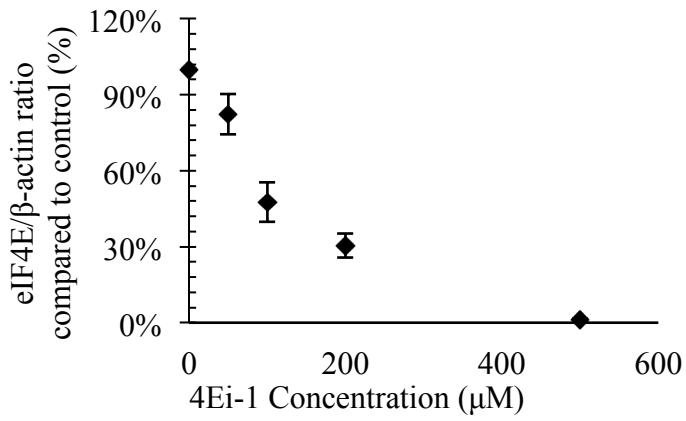
d)



e)



f)

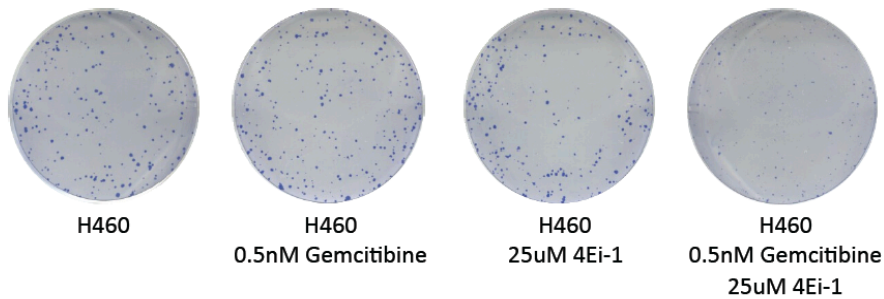


3.3.7 Treatment of Gemcitabine Combined with 4Ei-1 Increased Cytotoxicity by as much as 50%

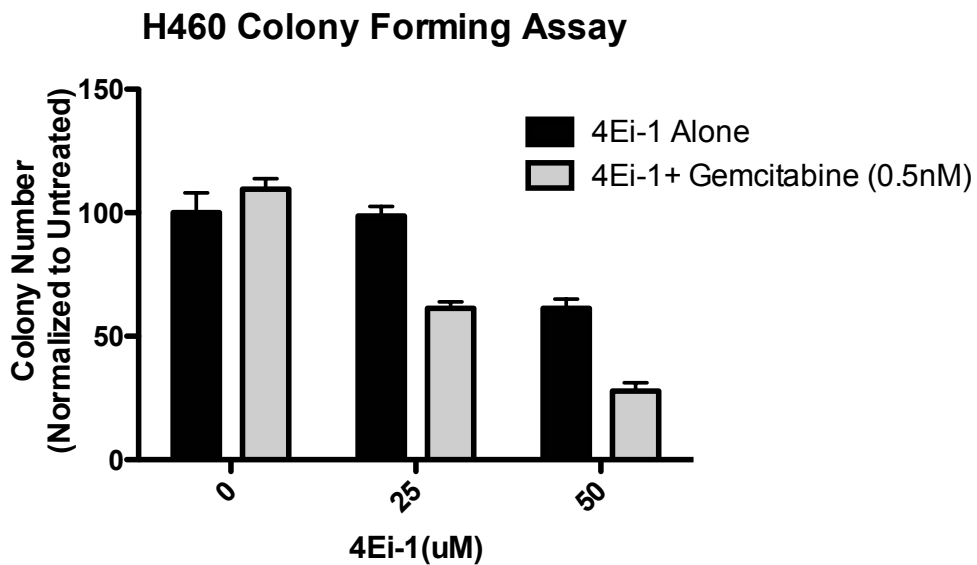
At non-toxic levels of gemcitabine (0.075 nM - 0.5 nM) or 4Ei-1 (25-75 μ M) the colony-forming assay showed similar effect on breast and lung cancer cell survival (**Figure 6**). As represented in **Figure 6a**, the reduction of colony numbers was very obvious after combined treatments of 4Ei-1 and gemcitabine. 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 6b**). In both H460 and A549 cells chemosensitized with 25 μ M 4Ei-1, 0.5 nM of gemcitabine reduced colony numbers by 40% (**Figure 6c and 6d**). In H838 cells this effect was even more significant (**Figure 6e**), resulting in a nearly 70% reduction of colony numbers.

Figure 6. 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) Graphic depiction of the normalized MDA-MB-231, H460, A549, and H838 colony numbers following 4Ei-1 treatments with and without gemcitabine. There was 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.

a)

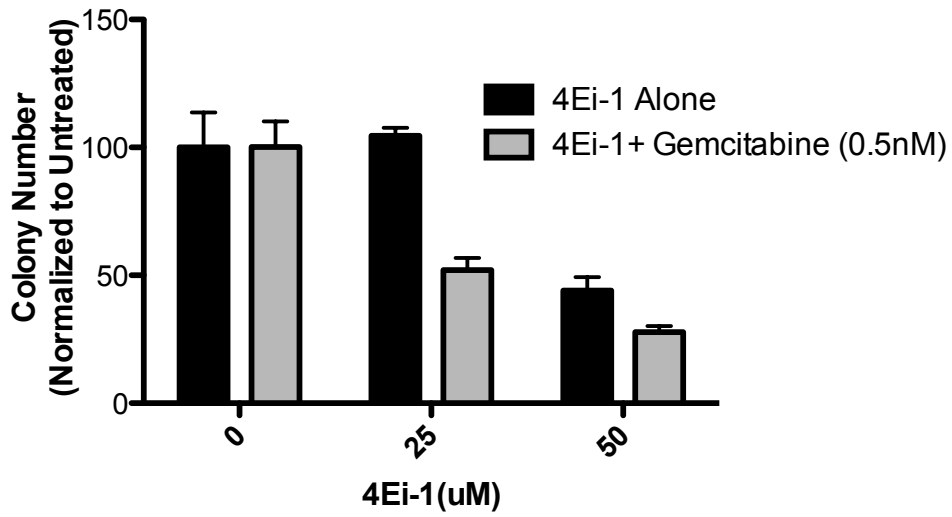


b)



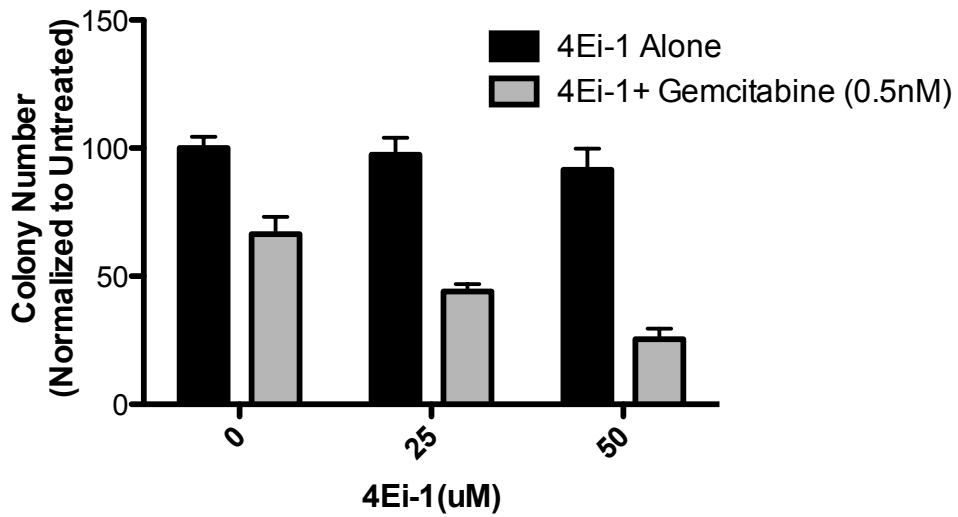
d)

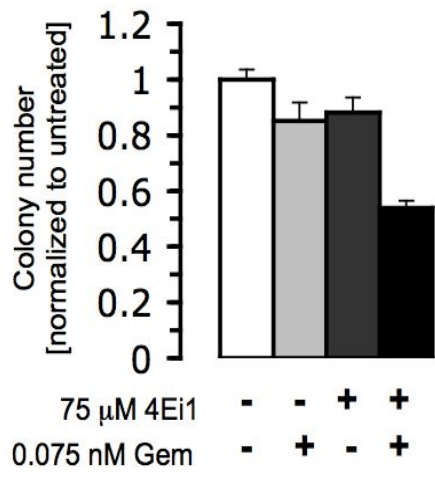
A549 Colony Forming Assay



e)

838 Colony Forming Assay





3.4 Discussion

In this paper we demonstrated that a pronucleotide of eIF4E antagonist, 4Ei-1, synergistically chemosensitized the prevalent lung and breast cancer cells to the traditional chemotherapy gemcitabine in hope to improve current chemotherapy. Compound 4Ei-1 is a prodrug of Bn⁷GMP, a known antagonist of the validated anti-cancer target eIF4E. As the rate-limiting factor of cap-dependent translation initiation, eIF4E is tightly regulated in normal cells and cap-dependent translation is constitutively upregulated in most human malignancies [3, 42-47]. Enhanced expression of eIF4E promotes malignancy and reduced eIF4E expression suppresses malignancy in experimental cancer models [48, 49]. Though the mechanism by which dysregulated eIF4E promotes oncogenesis is still not completely understood, the efficiency of cap-dependent translation varies greatly among various mRNA transcripts. Activated eIF4E disproportionately and dramatically stimulates the translation of a defined set of mRNAs, weak mRNAs, which encode malignancy-related proteins that control cell survival and proliferation [50, 51]. Compound 4Ei-1 is a synthesized phosphoramidate, which is converted to Bn⁷GMP by HinT1.

As a standard for cap analog development, Bn⁷GMP (IC₅₀ = 15.5 μM) became the foundation for a recent SAR study of N-7 substituted GMP analogs. By substituting the N-7 benzyl with a *para*-chloro or *para*-bromo phenoxyethyl group, the IC₅₀ values were decreased to 0.059±0.02 μM and 0.041±0.02 μM, respectively. The ~300-fold improvements in efficacy placed the two compounds in the lead for eIF4E antagonists. Phosphoramidates of the phenoxyethyl analogs have not been developed, but we plan to do so in the future [52].

Previous studies have suggested that 4Ei-1 was able to inhibit cap-dependent translation in a dose-dependent manner in rabbit reticulocyte lysates [13]. In this paper, we quantified the amount of both 4Ei-1 and Bn⁷GMP inside MDA-MB-231, H460, H838, and H2009 cells directly incubated with 100 μM 4Ei-1 using a capillary HPLC-ESI-MS/MS method. Our data suggested that the uptake and conversion of 4Ei-1 occurred as early as after 5-minute incubation. Four-hour incubation yielded the maximum uptake and an extended 24-hour incubation did not give more Bn⁷GMP accumulation. Our studies suggest that phosphoramidates indeed represent a group of cell permeable prodrugs for delivering nucleotides into mammalian cancer cells. Our results are consistent with earlier studies of the metabolism of AZT phosphoramidates which acted as prodrugs through converting to the corresponding AZT monophosphate [15, 53].

Gemcitabine is the first line of treatment for many cancers, but high levels of resistance in tumors limit the efficiency of gemcitabine in clinical use. Our colony forming assays demonstrated that 4Ei-1 significantly enhanced (> 30%) cell killing resulting from gemcitabine treatment in MDA-MB-231, H460, H549, and H838 cells, while 4Ei-1 had little or no toxicity when used alone. Western blot analysis suggested that 4Ei-1 suppressed the intracellular level of eIF4E, which might contribute to the improved gemcitabine efficacy. Heat shock protein 27 (Hsp27) is phosphorylated in response to cellular stress to form oligomers that prevent aggregation and/or regulate activity and degradation of certain client proteins [34, 54, 55]. Phosphorylated Hsp27 increases Hsp27-eIF4E interaction and is necessary to confer gemcitabine resistance in prostate cancer [34]; similar to eIF4E expression, Hsp27 was also overexpressed in tumor tissues [56-62]. Intracellular level of Hsp27 affects that of eIF4E directly, and vice versa

[34]. In their recent work, Mori-Iwamoto *et al.* demonstrated Hsp27 as a biomarker for prostate cancer cell's resistance to gemcitabine [63, 64]. Indeed, overexpression of Hsp27 inhibits gemcitabine-induced apoptosis [34]. These findings suggest that gemcitabine resistance might involve eIF4E, and by inhibiting eIF4E expression 4Ei-1 sensitized H460 cells to low dosage of gemcitabine. Silverstrol and cisplatin were both able to enhance chemosensitivity through inhibiting initiation-mediated pathways [2, 65].

Compound 4Ei-1 was subjected to a stability test in cell media and most 4Ei-1 degraded into the monophosphate after 12 hours of incubation at 37 degrees (data not shown). At first glance this contradicted with our colony forming assays, which were 9-day experiments. However, the fixed agar plates were much more stable environments than media; the chance of 4Ei-1 hydrolysis, its most common degradation pathway, is significantly lower in solid agar than aqueous solution. Overall our studies suggested that the regulation of intracellular eIF4E might be involved in the synergistic effect of 4Ei-1 and gemcitabine treatment on H460 cells. Still, the detailed mechanism remains to be elucidated.

Our western blot analysis suggested that not only does 4Ei-1 inhibit eIF4E activity *in vivo* [13], but also reduces the intracellular eIF4E protein levels. When we first decided to examine the endogenous eIF4E levels in H2009 cells over the period of 24 hours, cell cultures were treated with 100 μ M 4Ei-1 in media at 37°C and harvested at 30 minutes, 2 hours, 4 hours, and 24 hours. Phosphoramidate 4Ei-1 slowly degrades into monophosphate in cell media at 37°C (data not shown) after 12 hours. Although cell permeable at first, as the actual concentration of 4Ei-1 decreases due to hydrolysis the effectiveness of the pro-drug also declines after 12 hours. Western blots showed

continuous decrease of endogenous eIF4E after 30 minutes and restoration at 24 hours (**Figure 5b**). This is consistent with the phosphoramidate stability test and hinted 4Ei-1-dose-dependent eIF4E degradation. This result led us to a more substantial inspection consisting three different time points (24, 48, and 72 hours) and four concentrations (500, 200, 100, and 50 μ M). The amount of endogenous eIF4E in H2009 cells did correlate to the concentration and treatment length of 4Ei-1. Knockdown of eIF4E in H2009 cells was observed at 500 μ M 4Ei-1, and the addition of proteasome inhibitor MG132 completely reversed that effect (**Figure 5e**). Normally eIF4E is produced through cap-dependent protein synthesis, and knockdown of eIF4E would terminate the synthesis of the initiation factor itself. It then follows that cap-dependent protein synthesis, as well as cell division, would also come to a halt. Interestingly, MTS cytotoxicity assay (data not shown) with identical time points and concentrations of 4Ei-1 yielded similar cell-dividing rates for both untreated and treated H2009 cells. Given the fact that 4Ei-1 induces eIF4E degradation through the proteasome pathway, as suggested by the restoration of eIF4E in the presence of MG132, cap-independent eIF4E synthesis must be involved to achieve cell survival [66, 67].

The eukaryotic initiation factor 4E antagonist 4Ei-1 synergistically chemosensitized non-small lung cancer cells to the gemcitabine treatment with 4Ei-1 being not toxic by itself. As suggested by our western blot, the mechanism of this synergistic effect may involve the inhibition and subsequent degradation of eIF4E by 4Ei-1 (data not shown). More studies need to be carried out to confirm our hypothesis and pinpoint the mechanism of degradation. Overall, our studies demonstrated the first example of successful chemosensitization by regulating cap-dependent translation using a

pronucleotide of eIF4E antagonist 4Ei-1. Whether this strategy can be applied to chemosensitization of other chemotherapeutics or to overcome their resistance remains to be studied.

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