



Emerging Therapeutics Targeting mRNA Translation

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A defining feature of many cancers is deregulated translational control. Typically, this occurs at the level of recruitment of the 40S ribosomes to the 5'-cap of cellular messenger RNAs (mRNAs), the rate-limiting step of protein synthesis, which is controlled by the heterotrimeric eukaryotic initiation complex eIF4F. Thus, eIF4F in particular, and translation initiation in general, represent an exploitable vulnerability and unique opportunity for therapeutic intervention in many transformed cells. In this article, we discuss the development, mode of action and biological activity of a number of small-molecule inhibitors that interrupt PI3K/mTOR signaling control of eIF4F assembly, as well as compounds that more directly block eIF4F activity.

It would seem fitting to finish this collection with an article on the topic of “emerging therapeutics in mRNA translation” because much of our current thinking into the molecular biology of this rich field owes a great debt to inhibitors of protein synthesis. The experimental use of antibiotics that block the ribosome at key enzymatic steps has been instrumental in defining general features of translation and identifying paradigms of translational control. From a therapeutic perspective, however, it has only been those antibiotics that inhibit prokaryotic protein synthesis that have had any medical success, contributing significantly to the treatment of bacterial infectious disease (Chambers 2001a,b). It thus had seemed that the use and

potential of eukaryotic protein synthesis inhibitors were limited to tools in basic research. This view has changed during the last 20 years of research. We now have a more profound understanding of the complex regulatory apparatus that the eukaryotic cell uses to control messenger RNA (mRNA) translation and, with the emergence of several novel compounds that target steps other than elongation, a greater appreciation of the rewiring of translation factors that occurs in human disease, and most notably, cancer (Silvera et al. 2010). This latter and exciting development forms the basis of this article. For a more complete description of other known protein synthesis inhibitors we refer the reader to Pelletier and Peltz (2007).

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TARGETING TRANSLATION INITIATION AS AN ANTINEOPLASTIC APPROACH

The earliest, most varied and most widely studied inhibitors of eukaryotic protein synthesis are those that target the elongation step of mRNA translation (Pestka 1977; Vazquez 1979). Most of them act either by impairing peptidyl transferase function, impeding translocation, or interfering with aminoacyl-tRNA (transfer RNA) binding or accommodation. Despite their structural diversity and nuanced specificities, most eukaryotic elongation inhibitors have shown limited therapeutic value especially when compared to their prokaryotic counterparts, most likely owing to nonspecific toxicity derived from blocking global protein synthesis in nontransformed cells at the doses tested. Even with these apparent limitations, there has been renewed interest in using translation elongation inhibitors as anti-neoplastics. For example, homoharringtonine (omacetaxine mepesuccinate or HHT), a tree-derived alkaloid from the conifer *Cephalotaxus harringtonia*, has shown promise in several clinical trials for myelodysplastic syndrome and in phase II clinical trials in patients with gleevec-resistant chronic myelogenous leukemia and in acute myeloid leukemia (AML) (Kantarjian et al. 2001; Kim et al. 2011). HHT is thought to inhibit peptide chain elongation by binding the A site of the ribosome, blocking aminoacyl-tRNA binding, and halting peptide chain elongation (Gurel et al. 2009). Recent experiments have provided some understanding of the possible mechanism of HHT's mode of action. On treatment of leukemic cells with HHT, the short-lived antiapoptotic protein Mcl-1 was observed to be rapidly degraded, an effect that was solely attributed to the acute loss in overall protein synthesis and that was reversed upon proteasome inhibition (Tang et al. 2006; Robert et al. 2009; Chen et al. 2011a). This, and perhaps the degradation of other short-lived prosurvival factors, explains some of the synergistic effects observed with different inhibitors of translation elongation in the Burkitt's-like E μ -Myc lymphoma mouse model and would warrant greater study and further

evaluation of these drugs in combination therapies (Robert et al. 2009).

Other elongation inhibitors have had less clinical success when applied toward the treatment of cancer owing to unacceptable toxicities or poor pharmacological properties limiting their therapeutic window (Dumez et al. 2009). Not surprisingly, the current trend in the development of therapeutic agents that disrupt translation has thus drifted away from inhibitors of elongation to those that target initiation, potentially shifting the pharmacological response from global protein synthesis to a more selective (and possibly more cancer cell-dependent) translationally regulated effect.

eIF4F and Tumorigenesis

The eIF4F translation complex is currently at the forefront of the development of pharmacological agents that block initiation. Briefly, eIF4F is a heterotrimeric complex composed of eIF4E, the m⁷GpppN cap-binding protein that anchors the complex to the 5'-end of the mRNA; eIF4A, an RNA DEAD box helicase that is thought to unwind RNA secondary structure surrounding the cap and increase the efficiency of ribosome binding; and eIF4G, a large scaffolding protein that bridges eIF4F to the 43S ribosomal complex (see Lorsch et al. 2012). Given eIF4E's limited abundance, the recruitment of the 43S ribosomal complex to the mRNA by eIF4F is thought to be rate limiting for translation initiation (Duncan et al. 1987). The interaction of eIF4F with the cap structure is affected by mRNA proximal secondary structure with increased structure diminishing the efficiency of the eIF4E-cap interaction and/or imposing a structural barrier to the weak eIF4A helicase activity (Pelletier and Sonenberg 1985b; Lawson et al. 1986). Consequently, altering the levels of eIF4F can influence which mRNAs are more readily translated (Lawson et al. 1986, 1988), correlating with the degree of secondary structure in the 5'-untranslated regions (5'-UTRs) of the mRNA—the greater the thermal stability, the more poorly the transcript is translated, presumably by obstructing efficient ribosome loading and 48S complex



formation (Pelletier and Sonenberg 1985a,b; Lawson et al. 1986; Babendure et al. 2006). Increasing the amounts of cellular eIF4E can thus disproportionately stimulate the expression of messages that were once outcompeted by unstructured mRNAs, encoding proteins that are often progrowth and prosurvival in nature. In fact, this is thought to be the underlying mechanism behind eIF4E's tumorigenic properties, the selective increase in translation of a limited set of oncogenic and metastatic transcripts (Lazaris-Karatzas et al. 1990; Ruggiero et al. 2004; Wendel et al. 2004). The role that eIF4E plays in cancer has received much broader scientific and clinical interest of late since the discovery that its function is regulated by mTOR (mammalian target of rapamycin), a master regulator of cellular homeostasis and key signaling node often up-regulated in cancers, and that the chemotherapeutic rapamycin, its highly specific inhibitor, can block its activity (Kunz et al. 1993; Sabatini et al. 1994; see Dobson et al. 2012 for details).

Therapeutic Strategies Targeting Regulation of eIF4F Assembly

Rapalogs (Rapamycin Analogs)

mTOR is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family and is the catalytic subunit of two functionally distinct complexes: mTORC1 and mTORC2—defined by the nature of interacting accessory proteins. mTORC2 integrates cell survival, proliferation, lipogenesis (as well as other catabolic processes), and cytoskeletal organization to growth factor signaling, primarily through phosphorylation of members of the AGC class of kinases upstream of mTORC1. mTORC1 serves as a regulator of eIF4F assembly and translation initiation, sensing the energy and nutrient status of a cell and transmitting either a growth or starvation response to the translation apparatus (Sengupta et al. 2010). Briefly, mTOR stimulates eIF4F formation through two mechanisms: (1) It phosphorylates eIF4E-binding proteins (4E-BPs; of which there are three, the most prominent being 4E-BP1

and 4E-BP2), which prevents them from disrupting the eIF4E/eIF4G interaction, and (2) mTOR signals the degradation of PDCD4, which interferes with the eIF4A/eIF4G interaction (Gingras et al. 1999b; Dorrello et al. 2006). Thus, active mTOR promotes eIF4F formation and translation initiation.

The identification of germline mutations in genes that encode negative regulators of mTOR signaling (e.g., *Pten* and *Tsc1/2*), the fact that rapamycin possesses antiproliferative activity against a number of cancer cell lines (Hidalgo and Rowinsky 2000), and the observation that a majority of human cancers arise owing to activated mTORC1 signaling (Yuan and Cantley 2008), emphasized the need to uncover the role of mTOR in human cancer. Importantly, it is well established that mTOR control of eIF4F assembly acts as a critical node for cancer cell survival and proliferation (Wendel et al. 2004; Mills et al. 2008).

The rapalogs temsirolimus and everolimus have been clinically approved for the treatment of metastatic renal cell carcinoma, whereas temsirolimus has also been approved for the treatment of mantle cell lymphoma (Table 1). Currently, rapalogs are under investigation as cancer therapies in numerous clinical trials (www.clinicaltrials.gov). There are, however, limitations to rapamycin-based therapies. Among these is the presence of a p70^{S6K}-IRS-1 (insulin receptor substrate-1) negative-feedback loop (Fig. 1A) (Harrington et al. 2005). The mTOR substrate p70^{S6K} normally suppresses PI3K signaling by inactivating IRS-1 as well as platelet-derived growth factor receptor (PDGFR) and uncoupling PI3K from upstream growth factor signals (Harrington et al. 2005). Thus, exposure of tumor cells to rapamycin leads to inhibition of p70^{S6K} activity and subsequent stimulation of upstream PI3K signaling—an unfavorable situation for cancer therapy because this activates several mTORC1-independent prosurvival and proliferative signals and is associated with treatment failure (O'Reilly et al. 2006). Additionally, the mitogen-activated protein kinase (MAPK) pathway can be activated upon mTORC1 inhibition via the p70^{S6K}/PI3K/Ras pathway and indeed MAPK activation has been noted in

Table 1. Compounds that affect eIF4F assembly or activity

Targets	Compound	Sponsor	Stage of development	
mTORC1	Rapamycin (Sirolimus)	Pfizer	FDA approved	
	RAD001 (Everolimus)	Novartis	FDA approved	
	CCI-779 (Temsirolimus)	Pfizer	FDA approved	
	AP23573 (Ridaforolimus)	Merck/ARIAD	Accepted for FDA approval	
TOR-KI	Torin1	N/A	Ex vivo cell culture	
	INK128	Intellikine	Phase I	
	AZD8055	AstraZeneca	Phase I/II	
	AZD2014		Phase I	
	OSI027	OSI Pharmaceuticals	Phase I	
PI3K/TOR-KI	PI-103	N/A	Ex vivo cell culture	
	NVPBEZ235	Novartis	Phase I/II	
	SF1126	Semafore	Phase I	
	GSK2126458	Glaxo Smith Kline	Phase I	
	XL765	Exelixis	Phase I/II	
	BGT226	Novartis	Phase I/II	
	GDC0980	Genentech	Phase I	
	PF04691502	Pfizer	Phase I	
	PKI587	Pfizer	Phase I	
	eIF4F	Cap analogs	N/A	In vitro studies
4Ei-1		N/A	In vitro studies	
4EGI-1		N/A	Ex vivo cell culture	
4E1RCat		N/A	Active in preclinical models	
4E2RCat		N/A	Active in preclinical models	
ISIS-eIF4ERx		Eli Lilly/ISIS	Phase II	
Pateamine A		N/A	Ex vivo cell culture	
DMDA-Pat A		N/A	Active in preclinical models	
Hippuristanol		N/A	Active in preclinical models	
Rocaglamides (Silvestrol)		N/A	Active in preclinical models	
Mnk		Cercosporamide	Eli Lilly	Active in preclinical models
		CGP57380	Novartis	Ex vivo cell culture

human tumors following RAD001 treatment (Fig. 1A) (Carracedo et al. 2008).

Rapamycin resistance can also be imparted by overexpression of eIF4E, which has been identified as a genetic modifier of the rapamycin response (Fig. 1B) (Wendel et al. 2004, 2006; Mills et al. 2008). Given that neither 4E-BP phosphorylation status nor p70^{S6K} activity serve as reliable predictive markers of rapamycin sensitivity in human cancers (Noh et al. 2004; Satheesha et al. 2011), eIF4E expression levels should be assessed as a potential marker to inform on rapamycin resistance (Satheesha et al. 2011). These results suggest that direct inhibitors of eIF4E (and eIF4F) may synergize with rapamycin to diminish resistance.

TOR-Kinase Inhibitors (TOR-KI)

Rapamycin acts through an unusual allosteric mechanism wherein it binds FKBP12 and it is this dimer that then interacts with mTOR (Chen et al. 1995). Binding of FKBP12-rapamycin to mTOR induces a conformational change that specifically weakens the mTOR-Raptor interaction (Kim et al. 2002). In vitro kinase assays with mTORC1 have shown that prolonged incubation with rapamycin is necessary to inhibit 4E-BP1 phosphorylation (Burnett et al. 1998), which differs kinetically from what is observed with p70^{S6K}, where rapamycin rapidly blocks mTORC1 phosphorylation of p70^{S6K}. This is recapitulated in vivo, where rapamycin fully

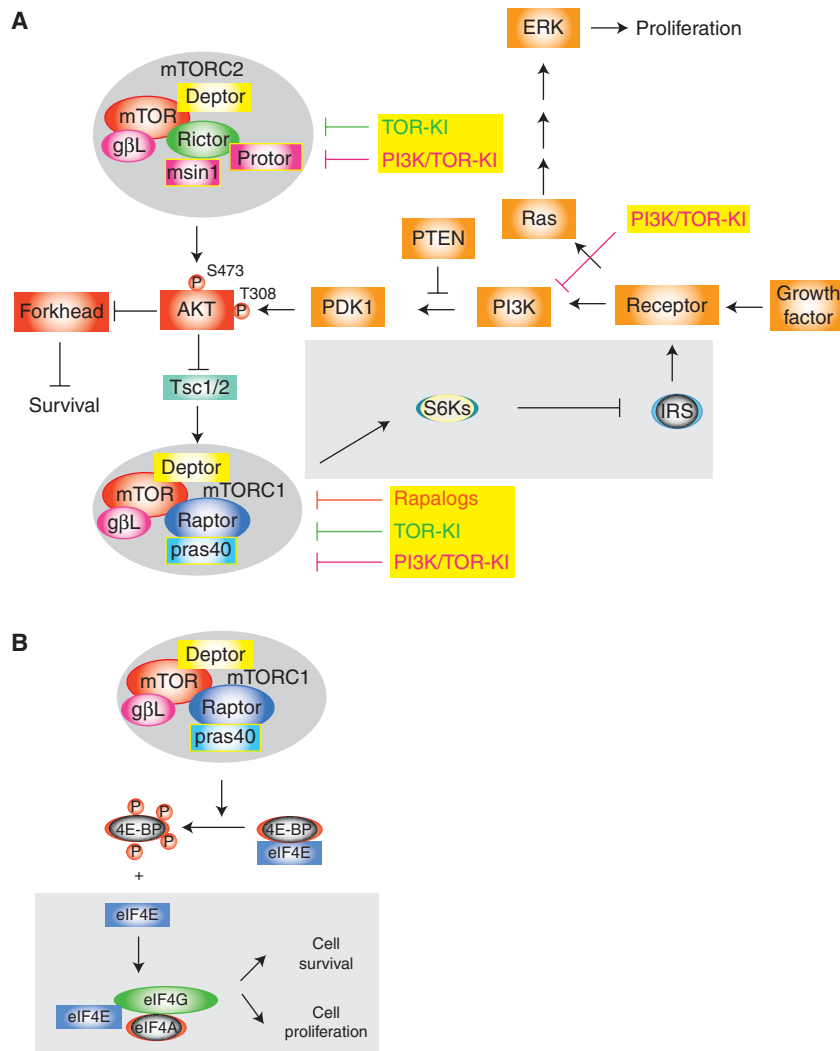


Figure 1. Diagram illustrating rapamycin-resistance pathways. (A) Inhibition of mTORC1 by rapalogs leads to dampening of the S6K/IRS feedback loop (in gray box) and increased signaling flux to PI3K and Ras. (B) Increased levels of eIF4E can circumvent effects on cell proliferation and cell survival (in gray box) mediated by inhibition of mTORC1 by rapamycin. See text for details.

suppresses p70^{S6K} phosphorylation but fails to completely dephosphorylate 4E-BP1 (Choo et al. 2008). The molecular basis responsible for these different phosphorylation kinetics is not well understood but may relate to rapamycin-induced structural changes in mTOR that exert more profound effects on mTOR-p70^{S6K} association than mTOR-4E-BP association, allowing uninterrupted signaling flux in the pres-

ence of rapamycin. Consistent with such a model is the finding that raptor binds 4E-BP1 more efficiently than p70^{S6K} (Hara et al. 2002). Furthermore, the FKBP12-rapamycin complex fails to effectively suppress mTORC2 kinase activity, limiting its use as a fully active inhibitor of mTOR (Sarbassov et al. 2004).

These issues prompted development of mTOR kinase inhibitors to block both mTOR

complexes. Indeed several novel mTOR inhibitors were identified that specifically compete with ATP for access to the kinase active site and some of these compounds are currently under intensive clinical development (Table 1) (Wander et al. 2011). The TOR-KIs are superior to rapalogs in their ability to suppress growth, proliferation, and protein synthesis (Feldman et al. 2009; Thoreen et al. 2009) and they target mTOR irrespective of its protein-binding partners. One important and unanticipated result from these studies is that at least two TOR-KIs, Torin1 and PP242, more potently inhibit 4E-BP1 phosphorylation than do the rapalogs (Feldman et al. 2009; Thoreen et al. 2009). These results prompted a reexamination of how 4E-BP phosphorylation is regulated by mTOR. It is known that mTORC1 phosphorylates 4E-BP1 in a hierarchical manner, although there is some disagreement in the literature about how this occurs (Gingras et al. 1999a, 2001; Choo et al. 2008). In vitro, mTORC1 can phosphorylate 4E-BP1 on Thr37/46 (Burnett et al. 1998). Yet in some settings, rapamycin fails to block the phosphorylation of these sites in vivo (Choo et al. 2008). In contrast, rapamycin is very effective at allowing dephosphorylation of 4E-BP1 on Ser 65 and Thr 70 in vivo (Gingras et al. 1999a; Choo et al. 2008). The ability of rapamycin to suppress translation is variable between cell lines and correlates with differences in the extent of 4E-BP1 phosphorylation (Beretta et al. 1996; Choo et al. 2008).

In contrast, TOR-KIs rapidly and fully inhibit mTOR-mediated phosphorylation of both 4E-BP1 and p70^{S6K} and notably offer an improvement in their ability to suppress translation initiation (Feldman et al. 2009; Garcia-Martinez et al. 2009; Thoreen et al. 2009; Yu et al. 2010). Fully dephosphorylated 4E-BP1 is much better at inhibiting eIF4E function and translation initiation than its partially dephosphorylated variants (Mothe-Satney et al. 2000). Inhibition of mTORC1 with TOR-KIs suppresses cell proliferation in an mTORC1/4E-BP1/2-dependent manner, whereas cell growth inhibition is mediated by mTORC1/p70^{S6K} (Dowling et al. 2010). Oddly, however, in the absence of 4E-BP1/2, rapamycin can still medi-

ate a partial block of cell proliferation suggesting that rapalogs influence mTOR activity through a more complex mechanism than is currently appreciated (Dowling et al. 2010).

Dual-Specificity PI3K/TOR-KI

A more recent class of mTOR inhibitors that also target PI3K has been characterized and entered into clinical development (Table 1) (Wander et al. 2011). The added advantage of these compounds over rapalogs is that they have the potential to block reactivated PI3K following p70^{S6K} inhibition. One limitation of these inhibitors is specificity. It turns out that some of these drugs have off-target inhibitory effects for related PIKK family members owing to shared structural overlap (Toledo et al. 2011). Although the effectiveness of this class of compounds at inhibiting eIF4F formation remains to be carefully assessed, an interesting link between the PI3K pathway and MYC has recently emerged. Elevated eIF4E and c-MYC levels can lead to PI3K/TOR-KI resistance (Ilic et al. 2011). In a breast cancer setting, MYC amplification leads to PI3K-independent tumor cell survival and resistance to PI3K inhibitors (Liu et al. 2011). A chemical genetics screen further validated c-MYC activation as a means to overcome the proliferative block induced by PI3K/TOR-KIs (Muellner et al. 2011). Elevated MYC and eIF4E levels are expected to increase eIF4F formation independent of mTOR (Jones et al. 1996; Rosenwald 1996; Lin et al. 2008) suggesting that inhibition of translation initiation may be a prominent mechanism by which PI3K/TOR-KIs exert their effects. Taken together, these results suggest that targeting components of the translation apparatus directly under mTOR control would be one avenue to overcome this resistance.

Therapeutic Strategies Directly Targeting eIF4F

There are several strategies under way to develop drugs that impede eIF4F activity, with efforts targeting different functions: (1) competing for eIF4E binding to the m⁷GpppN cap structure,

(2) uncoupling the eIF4E-eIF4G interaction, (3) targeting eIF4E production, (4) blocking eIF4A activity, and (5) inhibiting eIF4E phosphorylation. All of these have diverse effects on cellular and therapeutic outcomes and all have shown some promising results.

Blocking eIF4E-Cap Interaction

The archetypal inhibitors of eIF4E activity in the field of mRNA translation are undoubtedly the cap analogs (Shatkin et al. 1982; Grudzien-Nogalska et al. 2007b). At the level of translation, these act by outcompeting nascent capped mRNA transcripts for eIF4E binding and preventing the preinitiation ribosomal complex from binding and commencing translation. The original competitors, first described over 30 years ago, were simple m^7GDP or m^7GTP derivatives. Now more than 75 analogs have been synthesized and extensively tested in vitro, with diverse chemical modifications from simple substitution of the methylated residue to bulkier aromatic groups and remodeling of the nucleotide backbone (Hickey et al. 1977; Adams et al. 1978; Darzynkiewicz et al. 1981, 1987, 1989; Cai et al. 1999; Grudzien-Nogalska et al. 2007a,b; Kowalska et al. 2008; Jemielity et al. 2010; Su et al. 2011). The best compounds that bind to eIF4E are, not surprisingly, those that model the natural mRNA cap residue—minimally, a m^7GpppN -like molecule—preserving the contacts between the π - π electron stacking of the eIF4E tryptophan residues (W56 and W102) and the m^7G ring, as well the electrostatic attractions between the basic residues of eIF4E (R122, R157, and K162) and the 5'-5' triphosphate bridge (Marcotrigiano et al. 1997; Niedzwiecka et al. 2002).

These compounds have been invaluable for the biophysical and biochemical study of eIF4E in vitro but their application in vivo has been limited owing to poor cross-membrane transport and low cellular stability arising from both intracellular and extracellular hydrolysis (Wagner et al. 2000; Jemielity et al. 2010). Two recent synthetic routes have been developed to circumvent such limitations, both involving modification of the phosphate back-

bone with nonnatural functional groups. One study focused primarily on altering the α , β , and γ phosphate groups to prevent hydrolysis by the decapping enzymes DcpS and/or Dcp1/2 (Grudzien-Nogalska et al. 2007a; Kowalska et al. 2008; Su et al. 2011). When attached at the 5' end of a luciferase-driven construct, a phosphoborate moiety introduced at the β position supported translation efficiencies similar to that of the parent $m_2^{7,2'}GpppG$ compound but imparted greater mRNA stability in vivo (Su et al. 2011)—the latter attributed to a combined effect of protection from decapping by association with eIF4E (Schwartz and Parker 2000) and increased resistance to endogenous Dcp1/2 (and probably DcpS as well) degradation.

Similarly, but with an emphasis toward more therapeutically viable eIF4E cap analog-based inhibitors, Ghosh et al. (2009) have published the synthesis of phosphoramidate derivatives of m^7GTP . These “pronucleotides” harbor protecting groups that, on removal by endogenous phosphoramidasases (histidine triad nucleotide-binding proteins), are converted to active derivatives. These cap analogs were able to inhibit cap-dependent translation in vitro in reticulocyte lysates, as well as in vivo when co-injected with reporter constructs into fertilized zebrafish eggs (Ghosh et al. 2009). Intriguingly, one of these (4Ei-1) was also able to block eIF4E-dependent epithelial-to-mesenchymal (EMT) transition of these eggs. Although promising, their therapeutic relevance remains unclear because both classes of cap derivatives appear unable to cross phospholipid bilayers (requiring either lipofection or ectopic injection), which precludes their use in more clinically relevant assays. Moreover, the use of cap analogs could influence cellular physiology more broadly than just via eIF4E-dependant protein synthesis: For example, processes that rely on the nuclear cap-binding proteins CBP20 and CBP80, such as NMD, nuclear export, splicing, mRNA 3'-end formation, and miRNA processing, may also be affected (Kim et al. 2008; Maquat et al. 2010). Alternate pharmacological routes may therefore be required to more directly inhibit eIF4E function.

Uncoupling eIF4E-eIF4G Interaction

As mentioned previously, a majority of the driver mutations in cancers up-regulate mTOR signaling, which ultimately promotes the eIF4E-eIF4G interaction. Reverting this dysregulation using nonphosphorylatable 4E-BP constructs often halts tumor cell proliferation and limits the extent of disease progression in mouse models, suggesting that inhibitors designed to prevent the binding of eIF4E to eIF4G would be therapeutically beneficial. Several modified peptides encompassing the eIF4G/4E-BP hydrophobic eIF4E-binding motif coupled to cell-penetrating moieties have been developed and most have proven successful in various model systems with few nonspecific effects (Herbert et al. 2000; Ko et al. 2009; Brown et al. 2011). Most of these peptides bind fairly efficiently to tagged recombinant eIF4E (in the low- to mid-nanomolar range), can inhibit cap-dependent translation in vivo and induce cancer cell lines to undergo apoptosis. In one impressive example, a 4E-BP-like peptide coupled to a gonadotropin-receptor agonist dramatically decreased tumor burden following intraperitoneal (i.p.) injection in an ovarian cell xenograft mouse model with seemingly no detectable toxicity (Ko et al. 2009).

In a search for small molecules that can disrupt the eIF4E-eIF4G interaction, the Wagner group has designed a high-throughput screen. To identify such compounds, chemical libraries were assayed that decreased the fluorescence polarization of a labeled eIF4G-like peptide when bound by recombinant eIF4E (Moerke et al. 2007). The most potent hit, called 4EGI-1, was evaluated further and found to inhibit cap-dependent translation in extracts, down-regulate known eIF4E-dependent transcripts in vivo, and trigger apoptosis in several tumor lines (Moerke et al. 2007). Based on nuclear magnetic resonance (NMR) spectra, 4EGI-1 was shown to directly bind eIF4E. Counterintuitively, 4EGI-1 did not appear to prevent 4E-BP1 from binding to eIF4E but rather promoted the interaction. Whether or not this “gain-of-function” activity contributes to some of the in vitro and in vivo effects observed remains to be tested. 4EGI-1

has biological properties associated with it that appear to be independent of its effects on cap-dependent translation (Fan et al. 2010; Pruvot et al. 2011) and medicinal chemistry efforts are now required to remove these features from 4EGI-1.

We used a similar strategy in which the eIF4E-eIF4G interaction was monitored using time-resolved fluorescence resonance energy transfer and identified several eIF4E-eIF4G inhibitors, among which the most potent were 4E1RCat and 4E2RCat (Cencic et al. 2011a,b). 4E1RCat blocked cap-dependent but not hepatitis C virus (HCV) internal ribosome entry site (IRES)-dependent translation of a bicistronic dual-luciferase reporter mRNA with an IC_{50} of $\sim 25 \mu\text{M}$. Like 4EGI-1, 4E1RCat can prevent the association of eIF4G with $m^7\text{GTP}$ -Sepharose-bound eIF4E but, unlike 4EGI-1, it also blocked 4E-BP1 binding with nearly the same efficacy (Cencic et al. 2011b). 4E1RCat was also pharmacologically active in cells and in mice, where it decreased the rate of overall protein synthesis by $\sim 30\%$. Most strikingly, 4E1RCat was able to improve the response to chemotherapy in the $E\mu$ -myc mouse lymphoma model and thus prolong tumor-free survival. This synergy was probably owing to 4E1RCat’s ability to lower Mcl-1 levels, an effect that it shares in common with other inhibitors of translation in vivo.

Targeting eIF4E Levels with Antisense Oligonucleotides (ASOs)

Early proof of principle using antisense-based approaches showed the feasibility of knocking down eIF4E levels to limit the tumorigenic potential of transformed cells, although these experiments often suffered from lack of specificity controls or used short-lived biomolecules (De Benedetti et al. 1991; Rinker-Schaeffer et al. 1993; DeFatta et al. 2000). Second generation ASOs against eIF4E have largely overcome such pitfalls by incorporating extensive phosphate backbone modifications imparting improved nuclease resistance and tissue stability to allow for effective systemic therapeutic delivery. Second generation ASOs targeting eIF4E have been made and results are encouraging (Graff et al.



2007). Despite decreasing overall protein synthesis only minimally (<20%), inhibition of known eIF4E-specific pro-growth and pro-survival genes decreased in a dose-dependent manner, halting proliferation and eliciting apoptosis. Impressively, administration of the most potent eIF4E ASO (4E-ASO4, >80% reduction in eIF4E expression) showed pronounced *in vivo* activity in breast and prostate xenograft models, blunting tumor onset and growth and restricting endothelial cell tube formation, suggesting that eIF4E may also be a possible anti-angiogenic target. Thus, targeting eIF4E expression directly might prove to be more sensitive and selective toward tumors through specific down-regulation of oncogenes under eIF4F translational control and afford a much wider therapeutic index and control.

Inhibiting eIF4A Helicase Activity

Another way to inhibit eIF4F-dependent translation initiation is to target eIF4A, the key enzymatic component of the complex. Although both eIF4A's helicase and ATPase functions are required for efficient ribosome loading and scanning, the consequences of eIF4A enzymatic activity in the mechanism of translation initiation is yet to be precisely defined. It has been suggested that eIF4A is required for unwinding 5'-proximal secondary structure to facilitate 40S ribosome recruitment; that eIF4A actively participates in the preinitiation complex scanning of the 5'UTR; and/or that hydrolysis of ATP may simply be necessary to rearrange protein-protein or protein-mRNA interactions (Rogers et al. 2002; Kapp and Lorsch 2004).

Three small-molecule inhibitors, pateamine A (Pat A), hippuristanol, and silvestrol (and related rocaglamide family members) have been characterized extensively as inhibitors of eIF4A and are currently being explored as potential chemotherapies. All of these compounds were originally discovered and purified from natural sources and all were initially characterized as having strong tumor cell-line-specific cytotoxic properties even though their shared molecular target was then unknown (Higa et al. 1981; Gonzalez et al. 2001; Hood et al. 2001; Hwang

et al. 2004). It was fortuitous that all three were identified in a screen designed to identify compounds that could differentially distinguish cap-driven versus IRES-driven translation in Krebs II ascites cell extracts (Novac et al. 2004; Bordeleau et al. 2005, 2006a, 2008). More specifically, they were all able to inhibit the expression of the first cap-dependent luciferase cistron (as part of a dual-luciferase bicistronic capped mRNA transcript) but not the eIF4A-independent HCV-IRES-driven downstream cistron, which provided the first clue that all three were specifically blocking an eIF4A-dependent process. NMR (Lindqvist et al. 2008), affinity selection using immobilized compounds (Bordeleau et al. 2005), and *in vitro* assays using purified eIF4A (Bordeleau et al. 2008) showed that each was able to bind and affect eIF4A activity directly. Even though all three compounds share a common target, they each have distinct mechanisms of action.

Hippuristanol is an allosteric inhibitor of eIF4A: it prevents both free eIF4 (eIF4A_f) and eIF4F complex bound eIF4A (eIF4A_c) from binding RNA, which in turn inhibits eIF4A helicase and ATPase activities (Bordeleau et al. 2006a). Consequently, ribosome loading on exogenous transcripts is prevented as eIF4F activity is greatly reduced. Pat A and silvestrol, on the other hand, promote the binding of recombinant eIF4A to RNA and stimulate by several fold the ATP hydrolysis activity of eIF4A (Bordeleau et al. 2005, 2006b, 2008; Low et al. 2005, 2007). Although perhaps perplexing at first glance, because both are as efficient in blocking ribosome loading as hippuristanol, it seems that either Pat A or silvestrol can only exert this effect on eIF4A_c, effectively limiting the amount of eIF4A_c by sequestering eIF4A_f nonspecifically on cellular RNAs (Bordeleau et al. 2005). Intriguingly, Pat A and hippuristanol bind to different regions on eIF4A (the binding of silvestrol has not yet been determined). Hippuristanol appears to make specific contacts in the carboxy-terminal domain (CTD) of eIF4A (Lindqvist et al. 2008), and whereas the exact binding site of Pat A is not well defined, Pat A does not appear to interact with the CTD of eIF4A (M Oberer, J Pelletier, and G Wager, unpubl.) and mutational analysis of



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eIF4A indicates that it requires the amino-terminal ATP-binding domain of eIF4A for its activity (Low et al. 2005). Similarly, a recently characterized bacterial toxin, BPSL1549, from *Burkholderia pseudomallei* was shown to deamidate Gln339 of eIF4A, a conserved residue in motifs V and VI within a loop flanking eIF4A's ATP- and RNA-binding sites (Cruz-Migoni et al. 2011). In fact, this maps precisely to the same region whose residues experienced the strongest chemical shifts in NMR on addition of hippuristanol. The Gln339 modification appeared to transform endogenous eIF4A into a dominant-negative mutant, robustly blocking cellular protein synthesis while inhibiting eIF4A helicase activity and preventing eIF4A recycling (Cruz-Migoni et al. 2011).

Hippuristanol suffers from poor solubility and low potency and thus in vivo studies are scarce, although novel synthetic routes will hopefully resolve these issues (Li et al. 2009; Ravindar et al. 2010, 2011). In one study, hippuristanol was able to selectively inhibit the proliferation of HTLV-1-infected T-cell leukemia cells, both in culture as well as when injected into immunodeficient mice (Tsumuraya et al. 2011). Pat A suffers from a different problem in that it seems to inhibit protein synthesis in an irreversible manner, both in vitro and in vivo, which might make Pat A more difficult to develop for further clinical use (Bordeleau et al. 2005). Nevertheless, in one report, a desmethyl desamino analog of Pat A (DMDA-Pat A) displayed single-agent activity against several human cancer cell xenografts in nude mice, although it appears to inhibit DNA synthesis as well, substantially differing in its mechanism of action from its precursor (Kuznetsov et al. 2009).

In terms of preclinical mouse models, silvestrol is at present the best-studied eIF4A inhibitor, mostly owing to good pharmacological tolerance and little apparent nonspecific toxicity in animals (Cencic et al. 2009a; Saradhi et al. 2011). Silvestrol has been tested in multiple settings, from cancer cell line xenografts to oncogene-derived genetic mouse models with encouraging results. It has been tested in xenografts of acute lymphoblastic leukemia (697 ALL), prostate (PC-3), and breast (MDA-MBA-231) cancer lines as

well as the E μ -Tcl-1 (CLL) and E μ -Myc lymphoma mouse models (Bordeleau et al. 2008; Cencic et al. 2009a; Lucas et al. 2009). In almost all of these cases, administration of silvestrol as single-agent chemotherapy elicited apoptosis and caused tumor regression. In one study, it was observed that silvestrol appeared to be far more toxic toward B cells than T cells, an effect that was magnified in blood samples of CLL patients relative to normal healthy individuals, which suggests preferential killing of proliferating leukemic cells by the drug (Lucas et al. 2009). Likewise, silvestrol treatment alone showed nanomolar in vitro potency against Tsc2^{-/-} E μ -Myc lymphoma cultures, even in cells rendered rapamycin-resistant by overexpression of the PIM2 kinase (Schatz 2011). In contrast to these studies, silvestrol treatment of Pten^{+/-} E μ -Myc-derived tumors had minimal effect when used alone, perhaps as a result of the lower doses used (Bordeleau et al. 2008). When combined with doxorubicin, however, silvestrol greatly prolonged the tumor-free survival of mice, matching the synergy observed for combination rapamycin treatment (Bordeleau et al. 2008). Moreover, this combination therapy was even effective against eIF4E-driven E μ -Myc lymphomas, which do not respond to monotherapy or combination chemotherapy treatment (Bordeleau et al. 2008). This is also what is seen for human AML cell lines, where silvestrol synergizes with the standard-of-care drugs daunorubicin and cytarabine (Cencic et al. 2009b). Finally, as with other inhibitors of translation, silvestrol's ability to chemosensitize tumors correlates with its ability to inhibit synthesis of Mcl-1 protein. Further improvement of silvestrol's pharmacology is becoming more feasible as new synthetic routes are helping uncover which structural features determine the activity of rocaglamides (El Sous et al. 2007; Gerard et al. 2007; Roche et al. 2010).

Preventing eIF4E Phosphorylation through Mnk Inhibition

Unlike the 4E-BP/mTOR axis of eIF4E regulation, the molecular and biological consequences of direct phosphorylation of eIF4E have

only recently begun to be understood. eIF4E is phosphorylated on a single residue, Ser 209, by two kinases: the MAP kinaselike interacting kinases (Mnk) 1/2 (Pyronnet et al. 1999), which are both activated by the MAPK growth-induced and p38 stress-induced signaling pathways (Buxade et al. 2008). Exactly how the added phosphate residue alters eIF4E activity is not well understood. Theories ranging from increased “clamping” of eIF4E to the cap to greater stability in the eIF4F complex have thus far yielded few conclusive results. In lower organisms, eIF4E phosphorylation seems vital for organismal development, yet in mouse models loss of eIF4E phosphorylation has no noticeable phenotype (Ueda et al. 2004). What is emerging, however, is that eIF4E phosphorylation appears to be important in cancer—overexpression or knock-in of an S209A eIF4E mutant profoundly blunts tumor formation (Topisirovic et al. 2004; Wendel et al. 2007; Furic et al. 2010), which, in some circumstances, appears to rely on phospho-eIF4E’s ability to regulate Mcl-1 expression levels (Wendel et al. 2007). This suggests that small-molecule inhibitors of the Mnk kinases might yield novel anti-cancer agents. Recently, a novel Mnk inhibitor, cercosporamide, was identified from a screen of 300,000 compounds using an *in vitro* Mnk1 kinase reaction (Konicek et al. 2011). Cercosporamide is more selective for Mnk2 than Mnk1 ($IC_{50} = 0.011 \mu\text{M}$ versus $IC_{50} = 0.116 \mu\text{M}$), also potently inhibits Jak3 ($IC_{50} = 0.031 \mu\text{M}$), and is orally bioavailable, acting on tissues (e.g., liver) within 30 min after administration (Konicek et al. 2011). The compound was found to inhibit eIF4E phosphorylation in a dose-dependent manner, block cellular proliferation, and engage apoptosis (Konicek et al. 2010). In a xenograft colon (HCT116) and B16 lung metastasis model, treatment reduced tumor progression and incidence only modestly, probably reflecting the rebound in eIF4E phosphorylation seen after >4 h treatment in mouse tissues. Defining which cancers depend on eIF4E phosphorylation for tumor maintenance will be key in determining whether Mnk1/2 inhibition will become a therapeutically viable option.

Strategies Targeting Ternary Complex Formation

A second key point of regulation of translation initiation is through phosphorylation of eIF2 α , an event that limits the availability of the ternary complex, (eIF2•GTP•tRNA^{Met}), integral for proper start codon selection and the formation of a competent 43S ribosomal scanning complex. eIF2 α can be phosphorylated by four different intracellular kinases (PERK, PKR, GCN2, and HRI), which respond to a variety of cellular stress conditions (e.g., amino acid starvation, viral infection, oxidative stress, excess unfolded proteins, heme deficiency) to limit the protein synthetic load in a cell. Given the fact that cancer cells continuously grow, divide, and catabolize, they are generally under tremendous stress to maintain appropriate protein homeostatic load, a complex balancing act between protein biosynthesis, folding, translocation, assembly/disassembly, and clearance. Thus, relative to normal cells, tumors have constitutively higher levels of eIF2 α phosphorylation (Rosenwald et al. 2003, 2008). Compounds designed to exploit this discrepancy between malignant and normal cells by tipping the scales of eIF2 α phosphorylation could yield therapeutic benefit (and, arguably, may already form part of the mechanism of action of the approved drug bortezomib). Classic reagents used to induce the phosphorylation of eIF2 α in cell culture are often too toxic for animal use (e.g., tunicamycin or thapsigargin) and thus finding novel compounds that can hinder ternary complex formation through alternative means is necessary. In a recent screen aimed to ferret out such chemicals, a group of novel activators of HRI was discovered (Chen et al. 2011b). These similarly structured *N,N'*-diarylurea compounds, the most potent being BTdCPU, were able to specifically activate eIF2 α phosphorylation and downstream stress-associated pathways in a variety of conditions. Importantly, all appeared to cause eIF2 α phosphorylation through direct activation of HRI and not through nonspecific oxidative damage, the usual route to HRI activation. Moreover, BTdCPU inhibited the growth of tumor cell lines, both in culture and in xenografts

in nude mice, an effect that correlated with the induction of eIF2 α phosphorylation. BTdCPU appeared to be well tolerated in vivo with little apparent weight loss and surprisingly minimal impact on the hematopoietic system. Whether BTdCPU will develop into an effective anti-cancer agent will depend crucially on the levels of expression of HRI in a given tumor cell. Still, its relatively potent in vivo activity means that it will prove a valuable tool in elucidating the impact of eIF2 α phosphorylation and ternary complex formation on both normal cellular physiology and in various disease settings. It will also be interesting to see whether more direct and specific inhibitors of ternary complex formation (like NSC119889 [Robert et al. 2006]) will be able to target a more diverse set of tumor cell types.

FUTURE PERSPECTIVES

As the development of compounds targeting protein synthesis begins to transition away from preclinical models, the need for assays and biomarkers to reliably gauge drug efficacy and patient stratification becomes most pressing. Unlike the PI3K/TOR-KI inhibitors, where recent work on new pathway-based phosphoproteomics methodologies has proven quite successful in translation into the clinic (Andersen et al. 2010), biomarker readouts for eIF4F activity are less obvious. Polymerase chain reaction (PCR)-based methodologies coupled to next-generation sequencing are sensitive enough to measure polysome-bound mRNAs and may enable one to correlate drug treatment to tumor response on small biopsy specimens while informing on the relative tumorigenic contribution of cap-dependent versus cap-independent translation. In terms of patient stratification, clinical testing will be required to determine whether aberrant PI3K/mTOR activation in tumors can guide treatment modality involving translational inhibitors, particularly in cases of relapse, where increased eIF4F activity has been associated with chemoresistance.

We also anticipate continued screening for novel drug leads that can influence other translation initiation processes and factors, such as

(1) AUG selection, which potentially can alter the spectrum of therapeutically targeted mRNAs (Takacs et al. 2011); (2) translation reinitiation, which appears to contribute to the translation of a significant number of mRNA transcripts containing upstream open reading frames (ORFs), as recent deep sequencing data of ribosome-protected mRNA fragments suggests (Ingolia et al. 2011); (3) eIF3, a multisubunit complex that associates with the mTOR/raptor, eIF4G, and eIF4A (Korneeva et al. 2000; Holz et al. 2005) and has been implicated in the transformation process (Marchetti et al. 1995; Zhang et al. 2007, 2008); and (4) eIF4B, an essential factor whose regulation is under p70^{S6K} regulation (Raught et al. 2004; Shahbazian et al. 2006). Mammalian genetic screens using short hairpin RNA (shRNA) libraries will further tease out the unique oncogene and non-oncogene exploitable dependencies of mRNA translation factors and regulators (Solimini et al. 2007). Ultimately, these screens will need to be accomplished in more clinically relevant in vivo model systems that better replicate the natural tumor microenvironment and non-cell-autonomous interactions. Looking back at the evolution of this area of translation over the last 10 years and the remarkable progress that has been made, we are excited about the years to come and the potential that these inhibitors hold for both clinical use and in gaining further insight into the fundamental mechanisms of translational control.

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