Reversing chemoresistance by small molecule inhibition of the translation initiation complex eIF4F

Regina Cencic1, David R. Hall2, Francis Robert1, Yuhong Du1, Jaeki Min1, Lian Li1, Min Qui1, Iestyn Lewis1, Serdar Kurtkaya2, Ray Dingledine3, Haian Fu3, Dima Kozakov3, Sandor Vajda4, and Jerry Pelletier1,a,1

1Department of Biochemistry; 2Department of Oncology; and 3The Rosalind and Morris Goodman Cancer Research Center, 355 Promenade Sir William Osler, McIntyre Medical Sciences Building, McGill University, Montreal, QC, Canada H3G 1Y6; 4Department of Biomedical Engineering, Boston University, Boston, MA 02215; and 5Emory Chemical Biology Discovery Center, Emory University, Atlanta, GA 30322

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Deregulation of cap-dependent translation is associated with cancer initiation and progression. The rate-limiting step of protein synthesis is the loading of ribosomes onto mRNA templates stimulated by the heterotrimeric complex, eukaryotic initiation factor (eIF)4F. This step represents an attractive target for anticancer drug discovery because it resides at the nexus of the TOR signaling pathway. We have undertaken an ultra-high-throughput screen to identify inhibitors that prevent assembly of the eIF4F complex. One of the identified compounds blocks interaction between two subunits of eIF4F. As a consequence, cap-dependent translation is inhibited. This compound can reverse tumor chemoresistance in a genetically engineered lymphoma mouse model by sensitizing cells to the proapoptotic action of DNA damage. Molecular modeling experiments provide insight into the mechanism of action of this small molecule inhibitor. Our experiments validate targeting the eIF4F complex as a strategy for cancer therapy to modulate chemoresistance.

Eukaryotic translation initiation is tightly regulated at the step of ribosome recruitment. This process involves binding of eukaryotic initiation factor (eIF)4F complex to mRNA cap structures (m7GpppN; where N is any nucleotide). eIF4F is composed of: eIF4E, the cap-binding protein; eIF4A, a DEAD-box RNA helicase; and eIF4G, a scaffolding protein that bridges the interaction between eIF4E and eIF4A (1). Binding of eIF4F to the cap structure (via eIF4E) delivers eIF4A to the 5′ end of the mRNA template, an event required to disrupt RNA structure or RNP complexes to prepare a ribosome landing pad. eIF4E is the least abundant initiation factor, is rate-limiting for eIF4F complex assembly, and its availability for translation is regulated by the PI3K/Akt/mTOR pathway (1). The binding of one of three eIF4E negative regulators (eIF4E-Binding Proteins [4E-BPs]) to eIF4E is controlled by mTOR—with mTOR-dependent phosphorylation of 4E-BPs leading to their dissociation from the binary 4E-BP:eIF4E complex. Because 4E-BPs and eIF4G (there are two isoforms, called eIF4GI and eIF4GII, that share all structural features and show 46% sequence identity) compete for binding to a common site on eIF4E, 4E-BP binding to eIF4E decreases its availability for incorporation into the eIF4F complex and reduces translation initiation rates—with modest consequences on global translation but more pronounced effects on translation of specific mRNAs (1). This discriminatory effect on translation initiation is dependent on the amount of secondary structure present in the 5′ UTRs of mRNAs—with mRNAs harboring more secondary structure being more dependent on eIF4F for ribosome loading (2, 3). Gene expression profiling has identified mRNA transcripts whose translation is preferentially stimulated by altered eIF4E levels, indicating that eIF4E can affect the expression of a large gene set that impinge on several signaling nodes.

Several lines of evidence indicate that translational regulation is usurped in human tumors. Many cancers harbor lesions in the PI3K/Akt/mTOR pathway, which predictably affect eIF4F activity (4). eIF4E is overexpressed in many human cancers and hyper-phosphorylated 4E-BP is associated with tumor progression and adverse prognosis (5). Ectopic overexpression of eIF4E in vitro (6) and in vivo (7) is oncogenic and associated with chemoresistance. Hence, targeting eIF4F activity has been pursued to determine the consequences on tumor cell growth and chemoresistance.

Conceptually, the mRNA-ribosome loading step of eukaryotic translation initiation can be blocked at several points, and include inhibiting mRNA cap-eIF4E interaction with cap analogues, interfering with eIF4F subunit interaction (i.e., eIF4E:eIF4G or eIF4E:eIF4A), blocking eIF4A RNA helicase activity, and preventing eIF4F subunit synthesis. Although some of these approaches are beginning to be explored, it is not clear that they will exert similar effects on cell proliferation in vitro or that they will allow for a therapeutic response in vivo. In cell culture, sequestration of eIF4E by ectopic overexpression of 4E-BP1 in eIF4E transformed cells can partially reverse tumorigenicity (8). As well, antisense RNA oligonucleotides directed to eIF4E (9), peptides directed to the eIF4E:eIF4G interaction site (10), or small molecule inhibition of the eIF4E:eIF4G interaction (11) suppress transformation and induce apoptosis in vitro, although the potential of these approaches has not been tested in vivo. Importantly, and more clinically relevant, antisense targeting of eIF4E (12) or small molecule inhibition of eIF4A helicase activity (13, 14) show promising efficacy in blocking tumor growth in vivo in several preclinical cancer models. Herein, we explore the consequences of blocking eIF4E:eIF4G interaction in vivo on chemoresistance in a genetically defined preclinical lymphoma model.

Results

Ultra-High-Throughput Screening Identifies eIF4E:eIF4GI Inhibitors. A previous high-throughput screen (HTS) for eIF4E:eIF4G inhibitors probed a small collection of ~16,000 compounds and identified a molecule that binds to eIF4E, called 4EGI-1 (11). This compound inhibits eIF4E:eIF4G interaction, yet stimulates eIF4E:eIF4G:eIF4A interaction (11). To explore the therapeutic potential of uncoupling eIF4E from both eIF4G and 4E-BP, we sought to identify new compounds that could block interaction of eIF4E with eIF4G and 4E-BP. To this end, a library of 217,341 compounds was screened using a time resolved (TR)-FRET based assay consisting of His-tagged eIF4E and a glutathione S-transferase (GST)-tagged fragment of 4EGI (GST-4EGI157-606) (Fig. 1 A). Following dose-response analysis of 798 primary hits, 120 compounds showed an IC50 < 20 μM (Fig. 1 A). These com-


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1To whom correspondence should be addressed. E-mail: jerry.pelletier@mcgill.ca.

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pounds were further evaluated in an in vitro translation assay to score for their ability to inhibit cap-dependent but not Hepatitis C Virus internal ribosome entry site (HCV IRES) mediated translation (Fig. 1B). A counterscreen to identify false positives that nonspecifically quenched the luciferase enzymatic reaction rather than inhibiting cap-dependent translation was also performed (Fig. 1B). After testing all other compounds (harboring or lacking quenching activity) in an in vitro translation assay using [35S]-methionine to monitor protein synthesis, we identified three that inhibited cap-dependent translation, one of which we describe in detail herein (called 4E1RCat; PubChem ID 16195554). 4E1RCat displayed an IC₅₀ < 10 μM in the TR-FRET assay (Fig. 1C) and its resynthesis confirmed its activity (Fig. 1C; IC₅₀ ~ 4 μM). We performed structure-activity relationship (SAR) analysis on 4E1RCat using the TR-FRET assay and tested 16 analogs containing modifications at two positions of the molecule (Fig. S1A). Structure confirmation, resynthesis, and SAR analysis led to the identification of several oxo-pyrrolyl benzoic acid analogs that showed low micromolar potency inhibition for the eIF4E:eIF4G interaction based on the initial hit structure. However, none of the modifications to the “A” or “B” moieties improved the IC₅₀ compared to 4E1RCat in the TR-FRET assay (Fig. S1B).

Modeling Binding of 4E1RCat to eIF4E Predicts Interference with eIF4G and 4E-BP Binding. In silico probing of the three-dimensional surface of eIF4E for potential drug binding sites by computational solvent mapping (15) revealed the presence of five shallow pockets that form an elongated binding site and could support small molecule interactions (Fig. 2A). 4E1RCat shows potential for binding to four of these pockets (Fig. 2B). Both 4E-BP1 and eIF4G bind to a common site on the dorsal side of eIF4E (modeled in Fig. 2C) and it is clear that 4E1RCat is predicted to clash with this interaction (Fig. 2C). This modeling data offers a potential mechanism by which 4E1RCat inhibits eIF4E:eIF4G and eIF4E:4E-BP interaction.

Inhibition of Cap-Dependent Translation Initiation by 4E1RCat. Much of our current understanding of factor assembly on mRNA templates during translation initiation is based on reconstitution and
biochemical approaches that have dissected various steps of this process. One very useful assay for monitoring ribosome binding to mRNA is the visualization of 80S complexes on radiolabeled mRNA templates following sedimentation velocity centrifugation. In this assay, 4E1RCat reduced 80S ribosome complex formation (Fig. 3A), albeit not to the same extent as observed for m7GTP (Fig. S2). The inhibition of ribosome recruitment to mRNA by 4E1RCat was cap-specific, because this compound showed no inhibitory effect on 80S complex formation on the GpppG-HCV IRES (Fig. 3A).

4E1RCat blocked eIF4E:eIF4G and eIF4E:eIF4GI interaction (Fig. 3B). This compound also inhibited the ability of full-length eIF4GI to bind to GST-eIF4E (Fig. S3). To determine if 4E1RCat could disrupt preformed eIF4F complexes, we used m7GTP-agarose to purify the eIF4F complex from Ribosome Salt Wash (RSW) (Fig. 3C) and from extracts prepared from 4E1Cat treated MDA-MB-231 cells (Fig. 3D). Western blot analysis probed for the presence of eIF4E and the copurifying subunits, eIF4A and eIF4GI, in the m7GTP eluents. The amount of eIF4GI present in the eIF4F complex was reduced in vitro (Fig. 3C) and in vivo (Fig. 3D).

In vitro translations were performed to assess the effects of 4E1RCat on bicistronic mRNAs harboring IRESes that do not require eIF4F for ribosome recruitment (HCV and CrPV) (16) (Fig. 4A and Fig. S4) and on a bicistronic mRNA harboring an IRES that requires eIF4G but not eIF4E for ribosome recruitment (17) (EMCV, Fig. S4). As expected, m7GDP inhibited cap-dependent firefly (FF) expression, whereas the general translation inhibitor, anisomycin, inhibited production of both FF and renilla (Ren) from FF/HCV/Ren mRNA (Fig. 4A, lanes 2 and 4, respectively). 4E1RCat inhibited cap-dependent translation from FF/EMCV/Ren but did not affect translation initiated by the EMCV IRES (Fig. S4B). 4E1RCat inhibited protein synthesis in vivo in MDA-MB-231 and HeLa cells, but did not significantly affect RNA or DNA synthesis (Fig. 4B). Inhibition of protein synthesis by 4E1RCat in vivo was readily reversible (Fig. S5). 4E1RCat decreased polysomes, increased the fraction of 80S ribosomal subunits (Fig. 4C), and decreased levels of Mcl-1 and c-Myc proteins, two eIF4E-dependent mRNAs (19, 20) (Fig. 4D).

4E1RCat Reverses Chemoresistance. The Eph-Myc lymphoma model is a powerful, genetically defined system for studying drug action in vivo. Activation of mTOR signaling in this preclinical model through constitutive activation of the serine/threonine-specific kinase AKT or loss of Pten accelerates tumorogenesis and promotes chemoresistance—effects that have been attributed to increased eIF4F activity (7, 21). In this model Pten+/− Eph-Myc and Tsc2+/− Eph-Myc tumors are resistant to doxorubicin and we have previously shown that inhibition of mTOR signaling (7, 22) or eIF4A activity (13) can dramatically impact on this and significantly extend tumor-free survival. We therefore used this model to assess the consequences of 4E1RCat on chemoresistance (Fig. 5A).

Treatment of mice bearing Pten+/− Eph-Myc or Tsc2+/− Eph-Myc lymphomas with 4E1RCat alone was not effective in inducing any noticeable remission, whereas doxorubicin (Dxr) or rapamycin (Rap) induced a short-lived remission (Fig. 5B and Fig. S6). Dxr and 4E1RCat synergized in mice and extended tumor-free remissions for up to 14 d (Fig. 5B, p < 0.001; Fig. S6B, p < 0.001), similar to what was observed with Dxr and Rap. This effect was unlikely due to 4E1RCat nonspecifically increasing Dxr efficacy because we did not observe synergy between 4E1RCat and Dxr in mice bearing Eph-Myc lymphomas (Fig. S6B). Nor did we observe synergy in vitro on Eph-Myc lymphomas, in contrast to the effects of 4E1RCat and Dxr on Tsc2+/− Eph-Myc lymphomas (Fig. S6C). Analysis of Pten+/− Eph-Myc tumor samples 6 h after treatment revealed an increase in the number of apoptotic cells for 4E1RCat + Dxr treated samples, compared to Dxr or 4E1RCat only samples (Fig. 5C). As expected, levels of Mcl-1 were decreased in tumors following treatment of mice with 4E1RCat (Fig. 5D). These results indicate that 4E1RCat sensitizes Pten+/− Eph-Myc and Tsc2+/− Eph-Myc lymphomas to the cytotoxic effects of Dxr by inhibition of a prosurvival pathway. 4E1RCat was targeting translation in vivo as determined by poly-some profiles analysis (Fig. 5E).

Discussion

We have identified and characterized a small molecule inhibitor that blocks interaction of eIF4E with two of its binding partners, eIF4G and 4E-BP1. Molecular modeling of 4E1RCat indicates that it binds to eIF4E to the region that is also utilized by eIF4G and 4E-BP1 for binding (Fig. 2). The binding site is also located at residues that show preferential broadening of heteronuclear single quantum coherence peaks by the small molecule inhibitor 4EGI-1 (11). However, the inhibitory properties of 4E1RCat are different from 4EGI-1, because the latter blocks eIF4E:eIF4G but paradoxically increases eIF4E:4E-BP1 interaction (11). These results indicate that 4E1RCat and 4EGI-1 share overlapping but nonidentical sites.
We note that 4E1RCat appeared to be a weaker inhibitor than m\(^7\)GTP in preventing 80S complex formation (Fig. S4 and Fig. S2). The lower efficiency in inhibition may be due to differences in binding affinities, the inability of 4E1RCat to efficiently disrupt all preformed eIF4F complex, or the ability of the newly released eIF4E/eIF4A dimers to partially compensate for loss of eIF4F activity. Along these lines, we note that eIF4G can function in stimulating mRNA translation independent of eIF4E. Truncated mutants of eIF4G that lack the eIF4E-binding site have been shown to stimulate translation of uncapped mRNAs in rabbit reticulocyte lysate (RRL) (23), restored translation of capped mRNAs in eIF4F-depleted RRL (24), and in vivo can stimulate initiation of translation (25). In reconstituted systems, eIF4G (lacking the eIF4E-binding site) and eIF4A can efficiently load 48S complexes on capped and uncapped \(\beta\)-globin mRNA (26). These observations are consistent with reports demonstrating that translation initiation is reduced, but not abolished, by removal of the cap structure (27). Hence, we do not expect complete inhibition of translation by 4E1RCat upon exposure to cells, which is what was observed (Fig. 4B).

We find that 4E1RCat can reverse chemoresistance in a Myc-driven lymphoma model, consistent with the idea that deregulated translation plays a role in this phenomenon (7). Because 4E1RCat prevents eIF4E from interacting with two known protein partners, our data does not allow us to discriminate between which interaction is responsible for the biological effects observed. However, we favor the interpretation that 4E1RCat acts through disruption of eIF4E:eIF4G interaction because this would be consistent with results showing that blocking the eIF4A subunit of eIF4F from loading onto mRNA templates (13, 14) shows similar chemosensitizing properties.
Fig. 4. Effects of 4E1RCat on translation. A. Effect of 4E1RCat on in vitro translations performed in Krebs extracts programmed with FF/HCV/Ren. A schematic representation of FF/HCV/Ren mRNA is provided (top). In vitro translations were performed in the presence of 35S-Met and a representative autoradiograph of the products after fractionation by 10% SDS-PAGE is provided (bottom). Reactions contained vehicle (1% DMSO) (lane 1), 500 μM mGDP (lane 2), 500 μM GDP (lane 3), and 50 μM anisomycin (lane 4), the indicated concentrations of 4E1RCat (lanes 5–10), or lacked input mRNA (lane 11). Center: FF and Ren RLU values (relative to DMSO controls) from two independent experiments with the SEM indicated. B. 4E1RCat inhibits protein synthesis in vivo. The rate of incorporation of each radiolabeled tracer into TCA-insoluble material was monitored and is expressed relative to vehicle (DMSO) treated cells, which is set at 1. Results are the average of three experiments with the error of the mean shown. C. Polysome profiling analysis of Jurkat cells treated with 50 μM 4E1RCat. D. 4E1RCat inhibits c-Myc and Mcl-1 production. Jurkat cells were treated with 4E1RCat for 1 h, cell extracts prepared, and analyzed by Western blotting for c-Myc (Santa Cruz SC-40), Mcl-1 (Rockland), and actin (Sigma A5441) expression.

Fig. 5. 4E1RCat alters chemosensitivity of Pten+/−/Etp-Myc tumors in vivo. A. Representation of Etp-Myc model and treatment response. B. Kaplan-Meier plot showing tumor-free survival of mice bearing Pten+/−/Etp-Myc tumors following treatment with doxorubicin (Dxr, solid black line; n = 10), rapamycin (Rap, solid green line; n = 9), Rap and Dxr (dashed black line; n = 10), 4E1RCat (4E1RCat, solid red line; n = 10), or 4E1RCat and Dxr (dashed red line; n = 10). C. Combination treatment of 4E1RCat and doxorubicin increases the percentage of apoptotic cells. Representative TUNEL staining on sections of Pten+/−/Etp-Myc tumors following treatments (original magnification × 20-fold). D. The percentage of cells that stained positive represents the average of four different fields, where 500 cells were counted per field. D. 4E1RCat inhibits Mcl-1 production. E. 4E1RCat inhibits protein synthesis in vivo.
elF4F Pull-Down Experiments. Pulldown experiments of elF4F were performed as previously described (14). In the case of RSW, this was treated for 1 h with either 1% DMSO or 50 μM 4E1RCat, whereas for cell extracts, MDA-MB-231 extracts prepared from cells treated with either 1% DMSO or 50 μM 4E1RCat for 4 h. Primary antibodies used were anti-elF4E (Santa Cruz), anti-elF4GI (Bethyl), and anti-elF4A (29). Secondary antibodies were from Jackson Immuno-Research.

Treatment Studies. One million secondary Pten+/−/Ez-Myc Tsc2−/−/Ez-Myc, or Ez-Myc lymphoma cells were injected into the tail vein of 6–8 w old female C57BL/6 mice. When tumors were palpable, mice were treated with rapamycin (4 mg/kg daily for 5 d), 4E1RCat (15 mg/kg daily for 5 d), or doxorubicin (once at 10 mg/kg). Compounds were administered via intraperitoneal (i.p.) injection in 5.2% PEG 400/5.2% Tween 80. For combination studies, rapamycin or 4E1RCat were injected i.p. daily for five consecutive days, with doxorubicin being administered once on day two. Animals were palpated daily to monitor for the onset of tumors. Tumor-free survival was defined as the time between disappearance and reappearance of tumors. Data was analyzed using the log-rank (Mantel-Cox) test for statistical significance (SigmaStat software) presented in Kaplan-Meier format.

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Corrections

IMMUNOLOGY

The authors note that Heiko Hermeking should be added to the author line immediately following Kiichi Murakami, and Bert Vogelstein should be added to the author line immediately preceding Pamela Ohashi. Heiko Hermeking and Bert Vogelstein should be credited with contributing new reagents/analytic tools. The online version has been corrected. The corrected author and affiliation lines and author contributions appear below.

Yu-Wen Suab, bZhenyue Hao, Atsushi Hiraoab, cKazuo Yamamoto, bWen-Jye Lin, Ashley Young, bGordon S. Duncan, Hiroki Yoshidaab, dAndrew Wakeham, Philipp A. Lang, eKiichi Murakami, fHeiko Hermeking, gBert Vogelsteina, hPamela Ohashia and iTak W. Mak.

aCampbell Family Cancer Research Institute, Ontario Cancer Institute, University Health Network, Toronto, ON, Canada MSG ZC1; bDepartment of Immunology Research Center, National Health Research Institute, Zhunan 350503, Taiwan; cDivision of Molecular Genetics, Center for Cancer and Stem Cell Research, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan; dDepartment of Biomolecular Sciences, Faculty of Medicine, Saga University, Nabeshima, Saga 849-8501, Japan; eExperimental and Molecular Pathology, Institute of Pathology, Ludwig-Maximilians-University, D-80337 Munich, Germany; and fSidney Kimmel Comprehensive Cancer Center, Baltimore, MD 21231


NEUROSCIENCE

The authors note that, due to a printer’s error, on page 3787, right column, first paragraph, lines 8–20, “Although significant evidence supports a primary role for the 5-HT transporter in the reinforcing properties of psychostimulants (35–37), SERT blockade also appears to contribute to reinforcement (38–40). Indeed, SERT appears to be primarily responsible for the sustained reinforcing properties of cocaine in the 5-HT transporter–KO mouse (22, 23, 39–41). The significant compensatory alterations evident in 5-HT transporter–KO mice encouraged Chen and colleagues (42, 43) to develop a mouse bearing knock-in mutations in 5-HT transporter that, in vitro, reduced cocaine potency. Studies with these mice have yielded convincing evidence that 5-HT transporter is a key determinant of many synaptic and behavioral actions of cocaine” should instead appear as “Although significant evidence supports a primary role for the DA transporter in the reinforcing properties of psychostimulants (35–37), SERT blockade also appears to contribute to reinforcement (38–40). Indeed, SERT appears to be primarily responsible for the sustained reinforcing properties of cocaine in the DA transporter–KO mouse (22, 23, 39–41). The significant compensatory alterations evident in DA transporter–KO mice encouraged Chen and colleagues (42, 43) to develop a mouse bearing knock-in mutations in the DA transporter that, in vitro, reduced cocaine potency. Studies with these mice have yielded convincing evidence that the DA transporter is a key determinant of many synaptic and behavioral actions of cocaine.”

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The authors note that, due to a printer’s error, a footnote for Supporting Information appeared at the end of the first page. There is no Supporting Information accompanying the article, and the online version has been updated by removing the footnote.

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Supporting Information

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SI Materials and Methods

Ultra-high-throughput screening in 1,536-well format. A time resolved (TR)-fluorescence resonance energy transfer (FRET) based high-throughput assay that monitors the interaction between eIF4E and eIF4G has been previously described (1). The assay is in a homogenous format and has been further miniaturized and optimized for ultra-high-throughput screening (uHTS) in 1,536-well format. To this end, His-tagged eIF4E (4.8 nM) and glutathione S-transferase (GST)-tagged eIF4G157-606 (100 nM) were incubated with 1 nM Eu-W1024 labeled anti-6xHis antibody and 50 nM anti-GST IgG antibody conjugated to SureLight-Allophycocyanin (APC) (Perkin Elmer) in TR-FRET buffer (20 mM Tris, 50 mM NaCl, and 0.01% NP40). Reactions were dispensed into black 1,536-well plates (Corning Costar 3724#) using a Multidrop Combi (Thermo-Fisher Scientific). Compounds were added using a Beckman NX liquid handling station (Beckman Coulter). Reactions were incubated at room temperature (RT) for 3 h and TR-FRET signal was measured using an Emulsion multilabel plate reader (Perkin Elmer Life Sciences) with laser excitation at 337 nm and emission filters at 615/8.5 nm and 665/7.5 nm, as well as a LANCE/DELFIA dual mirror (D400/D630). A delay time of 50 μs was used. Due to the time delay, only the longer-lived FRET signal is detected, eliminating short-lived background fluorescence.

FRET signals were expressed as FRET ratios: FRET = F665 nm (fluorescence counts at 665 nm emission)/F615 nm (fluorescence counts at 615 nm emission) * 10,000 cps. Each plate contained DMSO positive control reactions, as well as negative control reactions with His-eIF4E, His-Eu, and GST-APC but no GST-eIF4G157-606. Data were analyzed using BioAssay software (CambridgeSoft). Percent inhibition was calculated for each plate using the equation: %Inhibition = 100 – (FRET compound/ FRET negative control) * FRET positive control/ FRET negative control. FRET compound was the FRET ratio from wells with a test compound, FRET positive control was an average FRET ratio from wells containing DMSO, defining the maximum FRET within each plate, and FRET negative control was the average FRET ratio from wells containing reactions without GST-eIF4G157-606, defining the minimum FRET within each plate. Compounds that caused >30% inhibition were defined as active.

Using this miniaturized TR-FRET assay, a library of 217,341 compounds was screened with the Molecule Library Screening Center Network. The plate Z’ values were all above 0.6 and S/B ratios above 10. The primary hit rate was 0.37%. From the primary screen, 798 compounds were identified as inhibiting the TR-FRET signal by at least 30% (Fig. 1A). The primary data are available at: http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=782&loc=ea_ras.

Synthesis of cpd 4E1RCat and analogs. 4E1RCat, 4-[3E]-3-[[5-(4-nitrophenyl)furran-2-yl]methylidene]-2-oxo-5-phenylpyrrol-1-yl] benzoic acid, is commercially available through various vendors.

Modeling of 4E1RCat and eIF4E. The site that binds 4E1RCat was identified using computational solvent mapping of the three-dimensional structures of eIF4E (4). The method places molecular probes—small organic molecules containing various functional groups—on a dense grid defined around the protein, finds favorable positions using empirical free energy functions, refines the selected poses by free energy minimization, clusters the low energy conformations, and ranks the clusters on the basis of the average free energy (4, 5). We used 16 small molecules as probes (ethanol, isopropanol, tert-butanol, acetone, acetaldehyde, dimethyl ether, cyclohexane, ethane, acetonitrile, urea, methylamine, phenol, benzaldehyde, benzene, acetamide, and N,N dimethylformamide). To determine the binding site, we first find consensus sites, i.e., regions on the protein where clusters of different probes overlap, and rank these sites in terms of the number of overlapping probe clusters. The highly ranked consensus sites define energetically important binding regions or hot spots (6). The main hot spot with the largest number of probe clusters and other hot spots within a 7 Å radius predict the site that can potentially bind drug-size ligands (4).

The available X-ray structures of human eIF4E were downloaded from the Protein Data Bank and were mapped after removing all other molecules. The only a priori constraint was blocking the m7GTP binding site, but 4E1RCat does not compete with the mRNA cap. Based on the mapping, a box for docking was created around this putative binding site with 4 Å padding on each side. The initial atomic coordinates of 4E1RCat were based on the conformer provided by PubChem, but the molecule was considered flexible in docking. The ligand and receptor were prepared for docking using version 1.5.4 of MGL Tools, the graphical front-end for setting up and running the AutoDock docking software. The docking was carried out using the standard settings of AutoDock Vina 1.1.0 (7), and the nine low energy binding modes were retained. The most likely binding pose was selected as the lowest energy pose, which aligned well with the predicted positions of the hot spots.

Ribosome binding assays. Rabbit reticulocyte lysate was preincubated with 0.6 mM cycloheximide (CHX) ±50 μM 4E1RCat for 5 min at 30°C. [32P]-radiolabeled mRNA was then added...
to the reaction and the incubation continued for an additional 10 min at 30 °C. The mRNA templates used in the binding reactions were mGpppG-terminated firefly luciferase A+ and GpppG-terminated Hepatitis C Virus internal ribosome entry site, respectively. Translation complexes were resolved by centrifugation through 10%–30% glycerol gradients in an SW40 rotor at 187,000 × g for 3 h. Fractions from each gradient were collected using a Brandel Tube Piercer connected to an ISCO fraction collector. Fractions of 500 µL were collected, and radioactivity determined by scintillation counting.

**GST-pull downs.** For pull-down experiments, 2.5 µg GST-fusion proteins were incubated in the presence of 100 µM 4E1RCat and 0.25 µg elF4E. Following incubation for 1 h at RT in Binding Buffer [20 mM Tris–HCl, 100 mM KCl, 10% glycerol, and 0.1% nonidet P-40 (NP-40)], the reaction was incubated with glutathione beads for another hour. Beads were washed three times with 10-column volumes of Binding Buffer and eluted for 1 h using 10 mM reduced glutathione. The eluents were separated on a 10% SDS-polyacrylamide gel followed by transfer to a PVDF membrane (Millipore) and Western blot analysis. Anti-GST and anti-elF4E antibodies were from Santa Cruz. Secondary antibodies were from Jackson Immuno Research.

For GST-pulldown experiments using radiolabeled elF4GI, full-length elF4GI was generated by in vitro transcription from a full-length cDNA clone containing a T7 RNA polymerase promoter. The resulting mRNA was translated in rabbit reticulocyte lysate (Promega) in the presence of [35S]-methionine. For pull-down experiments, 2.5 µg GST-elF4E or GST prebound to glutathione beads were incubated with radiolabeled elF4GI in the absence or presence of 100 µM 4E1RCat in Binding Buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA) for 2 h at 4 °C. Following three washes with 10-column volumes Binding Buffer, bound proteins were eluted for 1 h using 10 mM reduced glutathione. The eluents were analyzed on a 10% SDS-polyacrylamide gel followed by transfer to a PVDF membrane (Millipore) and Western blot analysis. Anti-GST and anti-elF4E antibodies were from Santa Cruz. Secondary antibodies were from Jackson Immuno Research.

In vivo metabolic labeling. For [35S]-methionine labeling in vivo, 60,000 cells/well were seeded in 24-well plates 24 h prior to treatment. Cells were treated for 4 h in the presence of increasing concentrations of compound. For reversibility assays, cells were incubated for 4 h with 50 µM 4E1RCat, washed with prewarmed PBS, followed by addition of DMEM without 4E1RCat. Cells were allowed to recover for the indicated time points. For the last hour, medium was replaced by methionine-free DMEM supplemented with 10% dialyzed serum and for the last 15 min, cells were labeled with [35S]-methionine (150–225 µCi/ml). Medium was removed, cells washed with PBS and lysed in RIPA buffer (20 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM PMSF, 4 µg/mL aprotinin, 2 µg/mL leupeptin, and 2 µg/mL pepstatin).

For RNA and DNA labeling, cells were treated for 4 h with 50 µM 4E1RCat in the presence of [3H]-uridine (24 µCi/mL) or [3H]-thymidine (48 µCi/mL). Cells were washed in PBS and lysed in RIPA buffer as described above. Samples were trichloroacetic acid (TCA) precipitated and radioactivity determined by scintillation counting. Protein content in each sample was measured using the BioRad DC Protein Assay (BioRad Laboratories) and used to standardize the counts obtained after TCA precipitation.

**Polysome profile analysis.** Twelve million Jurkat cells were seeded in 15 cm2 dishes in the presence of 50 µM 4E1RCat or vehicle (DMSO) for 1 h. Cells were harvested and processed as described previously (8). For polysome profiling of liver extracts, extracts were prepared as described previously (9).

**TUNEL assays.** For TUNEL assays, 6–8 wk old female C57Bl/6 mice bearing well palpable Pten+/−/Eµ-Myc lymphomas, were treated three times 1 h apart with 4E1RCat (15 mg/kg) or twice with rapamycin (4 mg/kg). Doxorubicin (10 mg/kg) was included with the last treatment. Six hours after the last injection, tumors were removed and fixed in 10% Neutral Buffered Formalin overnight and embedded in paraffin. Tumor sections (4 µm) were used in TUNEL assays performed according to the manufacturer’s instructions (Roche Applied Science), followed by staining of the sections with Hematoxylin.

**Cell proliferation and median effect analysis.** TSC2+/−/Eµ-Myc and Eµ-Myc lymphomas were seeded in 96-well plates at 104 cells/mL in the presence of increasing concentrations of Dxr (ranging from 3.9 nM to 250 nM) and 4E1RCat (ranging from 78.13 nM to 10 000 nM) at a constant ratio of either 20:1 or 40:1. Twenty four hours later, a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed. To this end, CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was added to the plates and the plates further incubated for up to 3 h, followed by measuring the OD50+ on a SpectramaxPlus384 (Molecular Devices) using Softmax Pro 4.8.2 software. Values obtained were standardized against DMSO controls. Median Effect Analysis was determined using the method of Chou and Talalay (10) using CompuSyn 1.0 (ComboSyn Inc, NJ).
A

![Chemical structure of 4E1RCat](image)

**Fig. S1.** SAR analysis of 4E1RCat. A. Schematic diagram of 4E1RCat denoting moieties “A” and “B” that were targeted for SAR analysis. B. Structures of 4E1RCat analogs and corresponding IC₅₀’s from the TR-FRET assay.

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Fig. S2. Inhibition of cap-dependent ribosome binding by m⁷GTP. ³²P-labeled m⁷GpppG-FF Luc A⁺ RNA was incubated with CHX and vehicle (1% DMSO) or 1.5 mM m⁷GTP in rabbit reticulocyte lysate. Following separation on sucrose gradients, fractions were collected and radioactivity determined. Total counts recovered from each gradient and the percent mRNA bound in 80S complexes were: m⁷GpppG-FF/mRNA + 1% DMSO [63,096 cpm, 28% binding] and m⁷GpppG-FF/mRNA + m⁷GTP [55,045 cpm, 16.4% binding].
**Fig. S3.** Inhibition of GST-elf4E and elf4G1 interaction by 4E1RCat. GST-pull downs were performed with radiolabeled elf4G1 produced from in vitro translation reactions in rabbit reticulocyte lysate (RRL). Lane 1, 1/5 of input material used for pull-down experiments; Lanes 2-5 are eluents from GST-pull-down experiments utilizing GST (lanes 2, 3) or GST-elf4E (lanes 4, 5) as bait. The presence or absence of 4E1RCat is indicated. Top: Autoradiography of gel showing radiolabeled elf4G1. Bottom: Coomassie brilliant blue staining of input RRL (lane 1) and GST-tagged proteins (lanes 2-5).

**Fig. S4.** In vitro translations performed in Krebs extracts programmed with Ren/CrPV/FF or FF/EMCV/Ren. A schematic representation of Ren/CrPV/FF (A) and FF/EMCV/Ren (B) mRNAs is provided (top). In vitro translations were performed as described for Fig. 4A. Bottom: FF and Ren, renilla (Ren) luciferase (luc) activity (RLU), values (relative to DMSO controls) from two independent experiments with the SEM are provided.
Fig. S5. Inhibition of translation by 4E1RCat is reversible. The rate of $^{35}$S-Met incorporation into TCA-insoluble material in HeLa cells was measured and is expressed relative to vehicle (DMSO) treated cells. Results are the average of three experiments with the SEM shown.

Fig. S6. 4E1RCat synergizes with Dxr in vitro and in vivo in Tsc2$^{+/−}$-Eμ-Myc lymphomas. (A) Kaplan-Meier plot (left) showing tumor free survival of mice bearing Tsc2$^{+/−}$-Eμ-Myc tumors following treatment with Dxr (solid black line; n = 4), Rap (solid green line; n = 5), or Rap + Dxr (dashed black line; n = 5; p = 0.003). Right: Tsc2$^{+/−}$-Eμ-Myc or Eμ-Myc cells were treated for 2 h with 20 nM rapamycin followed by Western blot analysis probing for phospho-S6 (p-S6). (B) Kaplan-Meier plot showing tumor free survival of mice bearing Tsc2$^{+/−}$-Eμ-Myc or Eμ-Myc tumors following treatment with 4E1RCat, Dxr or 4E1RCat + Dxr (For Tsc2$^{+/−}$-Eμ-Myc lymphomas; p < 0.001). n = 5 in all cohorts (C) Lymphoma cells of the indicated genotypes were treated with a constant ratio of Dxr and 4E1RCat for 24 h, at which point cell viability was determined using a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and Median Effect Analysis calculated as described in SI Materials and Methods. Combination Index (CI) values below 1 indicate synergism, whereas CI values above 1 indicate antagonism. Average of duplicates are presented.
Fig. S7. Representative synthetic scheme for 4E1RCat and its analogs. Reagents and conditions: (i) TEA/DCM, 5 h, 25 °C; (ii) substituted anilines, MW, 150W, 2 h, 180 °C.

Probe: $R_1 = \text{p-NO}_2$
$R_2 = \text{p-CO}_2\text{H}$