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Chemical Genetics Identify eIF2α Kinase Heme Regulated Inhibitor as Anti-Cancer Target

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Abstract

Translation initiation plays a critical role in cellular homeostasis, proliferation, differentiation and malignant transformation. Consistently, increasing the abundance of the eIF2·GTP·Met-tRNA$_i$ translation initiation complex transforms normal cells and contributes to cancer initiation and the severity of some anemia. The chemical modifiers of the eIF2·GTP·Met-tRNA$_i$ ternary complex are therefore invaluable tools for studying its role in the pathobiology of human disorders and for determining if this complex can be pharmacologically targeted for therapeutic purposes. Using a cell based assay, we identified *N*,*N*'-diarylureas as novel inhibitors of the ternary complex abundance. Direct functional-genetics and biochemical evidence demonstrated that the *N*,*N*'-diarylureas activate heme regulated inhibitor kinase, thereby phosphorylate eIF2α and reduce abundance of the ternary complex. Using tumor cell proliferation in vitro and tumor growth in vivo as paradigms, we demonstrate that *N*,*N*'-diarylureas are potent and specific tools for studying the role eIF2·GTP·Met-tRNA$_i$ ternary complex in the pathobiology of human disorders.

Differential translation of eukaryotic mRNAs, regulated at the level of initiation, critically affects gene expression and plays an important role in cellular homeostasis, proliferation, differentiation, malignant transformation, and the maintenance of malignant phenotype[1-6]. A

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AUTHOR CONTRIBUTIONS

T.C., Characterized hits compounds, carried out experiments to determine the mechanism of action of hit compounds including DART assay, analyzed the data, and contributed to writing the paper and assembling the Figures and tables; D.O., generated the screening assay, helped initial compound characterization; Y.Q., generated and characterized PC-3 cell lines where endogenous eIF2α is replaced by recombinant eIF2α; F.H., Carried out screening campaign; L.C., carried out cell growth experiments; S.D., carried out small scale re-synthesis of compounds used in this study; Z.H., N.Z., and J.G.S., developed and validated an LC-MS method and analyzed mouse plasma samples for drug concentrations; M.C., supervised compound synthesis, discussed data, contributed to writing paper; J.A.H., discussed the data, contributed to writing the manuscript; B.H.A., conceived, designed and supervised all aspects of research, analyzed the data, and wrote the manuscript.

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key regulatory step in the translation initiation cascade is the assembly of a ternary complex formed by the eukaryotic translation initiation factor 2 (eIF2), GTP and the initiator methionine tRNA (Met-tRNA$_i$). The eIF2·GTP·Met-tRNA$_i$ ternary complex interacts with the 40S ribosomal subunit and other translation initiation factors to form the 43S pre-initiation complex, which recruits the mRNA and scans through 5' untranslated region (5'UTR). Hydrolysis of GTP and release of Pi phosphate are critical for translation initiation and subsequent start site selection [7]. The GDP of eIF2·GDP complex released from the pre-initiation complex must be exchanged for GTP for a new round of translation initiation. The GDP-GTP exchange reaction is catalyzed by eIF2B, the eIF2 guanidine nucleotide exchange factor. Phosphorylation of the alpha subunit of eIF2 (eIF2α) on S51 reduces the abundance of the ternary complex by inhibiting the guanine nucleotide exchange activity of eIF2B[8-10].

The ternary complex plays critical roles in normal physiology and participates in the pathogenesis of several human disorders. For example, forced expression of eIF2α-S51A, a non-phosphorylatable eIF2α mutant[11] or of Met-tRNA$_i$ transforms normal cells[1]. Consistently, overexpression of eIF2 and inactivating mutations eIF2α kinases that cause unrestricted translation has been reported in various cancers[12-15]. The ternary complex also plays an important role in the development and/or progression of other human disorders[16]. For example, heme regulated inhibitor kinase (HRI), an eIF2α-kinase, couples hemoglobin synthesis to heme availability and influences the severity of hemolytic anemia such as β-thalassemia by regulating the abundance of the ternary complex[17-19]. The eIF2α-kinases Protein Kinase R (PKR), General Control Nonderepressible (GCN) 2, and PKR-like Kinase (PERK) are activated to shut down protein synthesis in response to viral infections, amino acid starvation or ER-stress, respectively[18,20-22]. Inactivating mutations of PERK allow uncontrolled insulin synthesis, induces ER-stress and apoptosis of pancreatic β-cells, causing permanent neonatal diabetes in the human Wolcott-Rallison syndrome[23].

To generate molecular probes for studying the normal and patho-biology of the eIF2·GTP·Met-tRNA$_i$ ternary complex and to determine if it can be pharmaceutically targeted for treatment of human disorders we developed a cell-based dual luciferase high-throughput assay. For this, we took advantage of the paradoxical increase in the translation of the activating transcription factor-4 (ATF-4) mRNA when the abundance of the ternary complex is reduced, a property that can be imparted on heterologous ORFs fused to the 5'UTR of the ATF-4 mRNA[24-26]. We report here that our screening of ~102,000 small molecule compounds in the ternary complex assay resulted in the identification of the *N,N'*-diarylureas as a privileged scaffold that reduces the abundance of the ternary complex. Specifically, *N,N'*-diarylureas activate HRI thereby causing the phosphorylation of eIF2α. Consistently, these agents activate downstream effectors of eIF2α phosphorylation, reduce the expression of oncogenic proteins and potently inhibit proliferation of human cancer cell lines and growth of xenografted human breast tumors in mice; all with no apparent toxicity. We provide direct biochemical evidence that *N,N'*-diarylureas interact with HRI and direct genetic evidence that all downstream effects of the *N,N'*-diarylureas are dependent on the activation of HRI and consequent phosphorylation of eIF2α.

RESULTS

Design, development and adaptation of screening assay

To develop the ternary complex assay, we constructed a bi-directional plasmid in which a common tetracycline-regulated transactivator (tTA)[27] dependent promoter/enhancer complex drives the transcription of the firefly luciferase (F-luc) ORF fused to the 5'UTR of ATF-4 on one side and of a renilla luciferase (R-luc) ORF fused to a 90-nucleotide 5'UTR on the other side (pBISA-DL$^{(ATF-4)}$, Fig. 1a). We generated stable KLN cells expressing tTA (KLN-tTA), which were then transfected with pBISA-DL$^{(ATF-4)}$ to establish stable KLN-tTA/pBISA-DL$^{(ATF-4)}$ cell lines. For assay validation, we used thapsigargin (TG) or tunicamycin (TU), two ER-stress inducing agents that cause phosphorylation of eIF2α[8,24] Treatment with either TG or TU increased the ratio of F-luc to R-luc activity that resulted from the increased expression of F-luc and the reduced expression of R-luc (Supplementary Results, Supplementary Fig. 1a). The activity of TG or TU in the ternary complex assay is due to the presence and organization of multiple uORFs in the 5'UTR of ATF-4 because elimination of uORF-2 by insertion of a single nucleotide that puts it in-frame with the bona-fide ORF completely reversed the increase in the normalized F-luc/R-luc ratio induced by TG or TU (Supplementary Fig. 1b). Furthermore, this activity is not secondary to inhibition of cell growth because other anti-proliferative agents such as etoposide had no activity in the ternary complex assay (Supplementary Table 1). Assay validation studies with TG and DMSO in a 384-well format demonstrated that the assay has a high signal to background ratio (~100 for both luciferases), and an excellent Z factor of 0.58 as calculated from the scattered plots (Supplementary Fig. 1c).

Screening

The results of the screening campaign, defined as activity score, are reported as the F-luc/R-luc ratio of the wells treated with test compounds relative to the F-luc/R-luc ratio of wells treated with DMSO (control) in the same plate. Based on preliminary screening of a diversity library, we established an activity score of 3 or higher for qualifying compounds as a hit. The primary screening of the ~102,000 compounds conducted at a single concentration of 10 μM identified ~1200 hits. Six hundred and six initial hits were confirmed in a dose-response ternary complex assay (Supplementary Dataset 1, Supplementary Table 2).

Identification and validation of *N,N*'-diarylurea hits

Analysis of the screening data revealed a high prevalence of *N,N*'-diarylureas, a known privileged scaffold, among the hits. To further assess this scaffold, we assembled a diversity library of 180 *N,N*'-diarylureas and tested them in the ternary complex assay. Based on their activity as well as on structural features we selected one inactive, 1-(2-chloro-5-nitrophenyl)-3-(3,4-dichlorophenyl)urea (**1**, NCPdCPU), and three active, 1-(benzo[d][1,2,3]thiadiazol-6-yl)-3-(3,4-dichlorophenyl)urea (**2**, BTdCPU), 1-(benzo[d][1,2,3]thiadiazol-6-yl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (**3**, BTCtFPU), 1-(benzo[c][1,2,5]oxadiazol-5-yl)-3-(4-chlorophenyl)urea (**4**, BOCPU), *N,N*'-diarylureas for further evaluation (Fig. 1b). Dose-dependent activities of these *N,N*'-diarylureas in the ternary complex assay are shown in Figure 1c.

To validate these compounds as bona fide inhibitors of the ternary complex formation, we took advantage of the fact that reducing the abundance of the ternary complex up-regulates CHOP mRNA and protein expression, a direct transcriptional target of ATF-4[24]. We measured the effect of the *N,N*'-diarylureas on the expression of CHOP mRNA by real time PCR and CHOP protein by Western blot of KLN-tTA/pBISA-DL$^{(ATF\text{-}4)}$ cells. Results of these secondary assays showed that *N,N*'-diarylureas active in the ternary complex assay also induce expression of both CHOP protein, and mRNA (Fig. 1d, Fig. 1e, Supplementary Fig. 2) without any effect on the expression of the housekeeping protein β-actin. These *N,N*'-diarylureas displayed similar activities in the ternary complex and secondary assays in CRL-2351 breast, PC-3 prostate, and CRL-2813 melanoma human cancer cell lines that were co-transfected with the tTA and the pBISA-DL$^{(ATF\text{-}4)}$ dual luciferase expression vector (Supplementary Fig. 3a-d).

N,N'-diarylureas induce eIF2α phosphorylation—The availability of the ternary complex can be reduced by phosphorylation of eIF2α, by reduced expression of Met-tRNA$_i$ or by eIF2α phosphorylation-independent reduction in the activity of eIF2B, the eIF2 guanine nucleotide exchange factor. To explore these possibilities, we determined the effect of *N,N*'-diarylureas on phosphorylation of eIF2α by Western blot analysis of KLN-tTA/pBISA-DL$^{(ATF\text{-}4)}$ and PC-3 human prostate cancer cells. The three active *N,N*'-diarylureas caused phosphorylation of eIF2α whereas, the inactive *N,N*'-diarylurea, NCPdCPU, had a negligible effect (Fig. 2a, Supplementary Fig. 4). To determine if phosphorylation of eIF2α is necessary for the activity of *N,N*'-diarylureas in the ternary complex assay, we took advantage of the transgenic PC-3 human prostate cancer cell lines in which endogenous eIF2α is replaced by either a non-phosphorylatable eIF2α mutant (eIF2α-S51A) or a recombinant wild type eIF2α (eIF2α-WT). These cells were genetically engineered by transducing PC-3 cells with lentiviral expression vectors that co-express an shRNA that specifically targets only the endogenous eIF2α and HA-tagged recombinant eIF2α-S51A or eIF2α–WT. These transgenic cells were co-transfected with tTA and pBISA-DL$^{(ATF\text{-}4)}$ and treated with four *N,N*'-diarylureas or vehicle. Replacement of endogenous eIF2α by the non-phosphorylatable eIF2α-S51A mutant, but not with eIF2α-WT, significantly reduced the activity of *N,N*'-diarylureas in the ternary complex assay (Fig. 2b). Similarly, expression the eIF2α-S51A mutant but not eIF2α-WT compromised the induction of CHOP mRNA expression by these agents (Fig. 2c). These findings demonstrate conclusively that phosphorylation of eIF2α mediates the activity of the *N,N*'-diarylureas in the ternary complex assay. Phosphorylation of eIF2α and inhibition of translation initiation selectively reduces the expression of many oncogenic proteins such as cyclin D1 with less prominent effect on that of housekeeping proteins[10,28]. Consistently, active *N,N*'-diarylureas reduced the expression of cyclin D1 with minimal effect on expression of proteins such p27^{Kip1} or β-actin (Supplementary Fig. 5). These findings indicate that *N,N*'-diarylureas preferentially reduce the expression of oncogenic proteins.

HRI mediates phosphorylation of eIF2α by N,N'-diarylureas—To elucidate the mechanism by which *N,N*'-diarylureas induce eIF2α phosphorylation, we first knocked down the expression of each one of the four eIF2α kinases individually or in all combinations by transfecting mouse KLN-tTA/pBISA-DL$^{(ATF\text{-}4)}$ and human CRL-2813

melanoma cells with siRNAs targeting PKR, GCN2, PERK, HRI or combinations thereof. The knocked down efficiency was 70-80% for all four kinases (Supplementary Table 3). We treated co-transfected cells with vehicle or BTdCPU, an active *N,N'*-diarylurea, and determined the normalized F-luc/R-luc ratio. Reducing the expression of HRI significantly interfered with the activity of BTdCPU in the ternary complex assay. In contrast, knocking down PKR, PERK, or GCN2 expression either individually or in double or triple combination had no effect on the activity of BTdCPU (Fig. 3a). Consistently, silencing HRI but not the other eIF2α kinases significantly reduced the increased expression of CHOP mRNA in cells treated with BTdCPU (Fig. 3b). We then compared the effect of knocking down PERK or HRI expression on the induction of eIF2α phosphorylation by tunicamycin or BTdCPU. Knocking down HRI expression significantly reduced BTdCPU induced eIF2α phosphorylation without any apparent effect on the tunicamycin induced eIF2α phosphorylation. In contrast, knocking down PERK expression significantly reduced tunicamycin induced eIF2α phosphorylation without any apparent effect on BTdCPU induced eIF2α phosphorylation (Fig. 3c and Supplementary Fig. 6, Supplementary Fig. 7). Furthermore, studies in KLN-tTA/pBISA-DL$^{(ATF\text{-}4)}$ and CRL-2813 cell lines with all four *N,N'*-diarylureas showed that knocking-down expression of HRI, but not other eIF2α kinases significantly reduced the effect of all three active *N,N'*-diarylureas on the ternary complex abundance (Fig. 3d and Fig. 3e, respectively). Similarly we demonstrate that knocking down HRI expression in MCF-7 human breast cancer cells fully abrogated the effect of all three active *N,N'*-diarylureas on the ternary complex abundance and reduced the induction of CHOP mRNA (Supplementary Fig. 8a and 8b), consistent with the very high HRI knockdown efficiency in MCF-7 cells. Taken together, these data demonstrate that activation of HRI specifically mediates *N,N'*-diarylurea-induced phosphorylation of eIF2α, reduces the abundance of the ternary complex and its downstream effects.

N,N'-diarylureas interact directly with HRI—To determine directly if active *N,N'*-diarylureas directly interact with HRI, we expressed recombinant HRI and investigated interactions of BTdCPU with HRI by proton NMR and by drug affinity responsive target stability (DARTS) assay. The proton NMR relies on the fact that BTdCPU has a unique NMR signature that would be lost upon binding to HRI because the ligand/receptor interaction causes broadening of compound specific proton signals. However, addition of aqueous buffers reduced BTdCPU specific signals below the detection limit of NMR, likely due to gradual compound aggregation on NMR time-scale. As an alternative approach we carried out the recently described DARTS assay in which binding of a small molecule to a protein target imparts on to the protein resistance to certain bacterial proteases such as thermolysin and subtilisin[29]. We digested HRI with subtilisin in the presence of increasing concentrations of BTdCPU or vehicle. To demonstrate the specificity of the DARTS assay we incubated recombinant eIF4E with 4EGI-1, a small molecule that interacts with eIF4E[30] or BTdCPU. BTdCPU renders recombinant HRI but not eIF4E resistant to proteolysis (Supplementary Figure 9a and 9b). In contrast 4EGI-1 protects recombinant eIF4E from subtilisin digestion confirming the specificity of DARTS assay. These data indicate that BTdCPU directly interacts with HRI.

N,N'-diarylureas do not cause oxidative stress—HRI can be activated in intact cells but not in cell lysates by cytoplasmic stress-inducing agents such as arsenate or H_2O_2 [18,31]. To determine directly if *N,N'*-diarylureas activate HRI by causing oxidative stress, we incubated CRL-2813 cells with various doses of BTdCPU using sodium arsenite and H_2O_2 as positive controls. As shown in Supplementary Figure 9c, BTdCPU does not cause oxidative stress, ruling out the possibility that oxidative stress mediates activation of HRI by active *N,N'*-diarylureas.

N,N'-diarylureas induce eIF2α phosphorylation in cell-free lysates—Cytoplasmic stress-inducing agents activate HRI thereby cause eIF2α phosphorylation in intact cells but not in cell-free extracts[31] indicating that activation of HRI is secondary to the perturbations in cellular homeostasis. To determine if the *N,N'*-diarylureas activate HRI directly or as a consequence of their effects on cellular stress, we treated lysates of CRL-2813 human melanoma cancer cells or rabbit reticulocytes with BTdCPU and determined phosphorylation of eIF2α by Western blot analysis. BTdCPU caused phosphorylation of eIF2α in cell-free extracts in a dose dependent manner (Supplementary Figure 9d and Supplementary Figure 9e), ruling out the possibility that *N,N'*-diarylureas activate HRI by causing cellular stress. Taken together with our demonstration that BTdCPU interacts directly with HRI but does not cause oxidative stress, these data demonstrate that direct interaction of *N,N'*-diarylureas with HRI (or HRI containing molecular complexes) is responsible for their activity.

Specificity of N,N'-diarylureas—The *N,N'*-diarylureas can be utilized for studying the normal- and pathobiology of the HRI and/or the ternary complex or developed for treatment of human disorders only if they demonstrate reasonable specificity. We expected the *N,N'*-diarylureas to inhibit cell proliferation because they reduced the abundance of the ternary complex which is shown to results in inhibition of cell proliferation[9,10]. However, cell proliferation could also be inhibited by other (off-target) effects of *N,N'*-diarylureas independently of the HRI dependent phosphorylation of eIF2α. We therefore, choose cell proliferation as a biological response parameter to demonstrate target specificity of the *N,N'*-diarylureas. We tested the effects of the *N,N'*-diarylureas on the proliferation of KLN mouse squamous cell carcinoma, CRL-2351 human breast, CRL-2813 melanoma, A549 lung and PC-3 prostate cancer cell lines. Remarkably, the *N,N'*-diarylureas active in the ternary complex assay were also potent inhibitors of cancer cells proliferation (Table 1). To determine if the *N,N'*-diarylureas inhibit cell proliferation by reducing the abundance of the ternary complex, we compared their effect on the proliferation of the transgenic PC-3 human prostate cancer cell lines expressing either the non-phosphorylatable eIF2α-S51A mutant or the eIF2α–WT. The results of these studies (Fig. 4a and Supplementary Fig. 10a) demonstrate clearly that PC-3 cancer cells expressing the non-phosphorylatable eIF2α-S51A mutant were resistant, while those expressing eIF2α–WT were sensitive to the inhibition of cell proliferation by the *N,N'*-diarylureas that induce eIF2α phosphorylation. Reducing the expression of HRI, the eIF2α kinase that mediates the phosphorylatation of eIF2α by the *N,N'*-diarylureas, also significantly reduced the inhibition of cell proliferation by these agents in CRL-2813 human melanoma (Fig. 4b and Supplementary Fig. 10b) and MCF-7 human breast cancer cells (Supplementary Fig. 10c). These data indicate that the *N,N'*-

diarylureas posses sufficient specificity that makes them invaluable probes for studying the biology of the HRI and/or the ternary complex.

Activity N,N'-diarylureas correlates with HRI expression—Based on our demonstration that anti-proliferative effects of the *N,N'*-diarylureas are mediated by HRI, we postulated that cancer cells expressing high levels of HRI are, in general, more susceptible to inhibition of cell proliferation than those expressing low levels of HRI. To test this hypothesis we determined the level of HRI expression in a panel of breast, melanoma and prostate cancer cell lines by Western blot analysis using β-actin levels as internal standard. We also determined the potency of the *N,N'*-diarylureas in abrogating proliferation of these cells by SRB assay. Our results showed that the sensitivity of the various cancer cell lines to the anti-proliferative effects of the *N,N'*-diarylureas correlates well with the expression of HRI. KLN cells, which express undetectable levels of HRI are least sensitive to inhibition of cell proliferation by the *N,N'*-diarylureas whereas CRL-2813 or CRL-2351 cells that express high level of HRI are most sensitive (Fig. 5a). Taken together, these data demonstrate that the *N,N'*-diarylureas posses the required potency and specificity to interrogate the role of the eIF2·GTP·Met-tRNA$_i$ ternary complex and/or HRI in normal- and patho-biology of human disorders.

N,N'-diarylureas inhibit tumor growth without toxicity—To further demonstrate that the *N,N'*-diarylureas can be utilized for studying the biology of the HRI and/or the ternary complex *in-vivo* we used inhibition of tumor growth as an *in vivo* paradigm. For this purpose we first investigated *in vivo* safety of *N,N'*-diarylureas. Briefly, we treated mice with various doses of BTdCPU or vehicle for seven consecutive days and measured the weight of animals and observed mice for frank signs of toxicity. Treatment with 100, 200 or 350 mg/kg/d of BTdCPU had no discernable adverse effect on weight gain and mice did not display any outward signs of toxicity even at the highest dose (Fig. 5b). To determine the plasma drug levels we undertook a limited study by treating mice with a single 175mg/kg dose of BTdCPU and measured the plasma drug concentrations by liquid chromatography mass-spectroscopy (LC-MS). Based on the one hour plasma concentration of 1.4 μM, four hour plasma concentration of 0.4 μM and twenty four hour plasma concentration of 0.3 μM of BTdCPU, we expect the mice to attain a steady state plasma concentration of ~0.4-2 μM. We then tested the anti-cancer efficacy of BTdCPU against xenografted breast tumors. Briefly, we treated mice carrying human breast tumors xenografts (~150 mm^3) with 175 mg/kg/d BTdCPU in 15 μl DMSO or 15μl DMSO alone; both by i.p. injection. Mice were observed daily, and weighed twice weekly, and tumor dimensions were measured weekly. Administration of 175 mg/kg/d of BTdCPU caused a total tumor stasis starting one week after the first injection (Fig. 5c). This complete tumor stasis persisted for the remainder of the 3-week study. Importantly, Western blot analysis of tumors treated for three weeks demonstrated that treatment with compound BTdCPU significantly elevated phosphorylation of eIF2α (Fig. 5d, Supplementary Fig. 11), suggesting that *in vivo* and *in vitro* anti-tumor effects of the *N,N'*-diarylureas are mediated by the same mechanism.

To determine if long term (21 days) administration of BTdCPU causes any macro- or micro-toxicity we collected blood from tumor-bearing mice on day 21st of treatment, sacrificed the

animals and submitted the whole mice for necropsy. Blood was processed in the Hematology core facility and full necropsy and histopathology was carried out at the core Rodent Pathology Laboratory. This analysis demonstrated that BTdCPU had no effect on macroscopic and microscopic appearance of any organs (Supplementary Fig. 12). The results showed further that the administration of BTdCPU did not have any negative effect on red and white blood cells, platelet and reticulocyte counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin or any other blood parameters measured (Supplementary Fig. 13). These data indicate that the *N,N'*-diarylureas may be safely administered to mice for studying the role of HRI or the eIF2·GTP·Met-tRNA$_i$ ternary complex *in vivo* or evaluating their efficacy in mouse model of human diseases such as cancer.

DISCUSSION

Tight regulation of the eIF2.GTP.Met-tRNA$_i$ ternary complex enables cells to rapidly regulate translation initiation in response to changes in the cellular environment[18,20,24,32,33]. Removing the physiological restraints on the abundance of the ternary complex, on the other hand, causes malignant transformation,[2,4,11,34]. These findings indicate that the ternary complex plays a crucial role in normal- and patho-physiology of human disorders. The studies reported here were undertaken to obtain potent and selective chemical modulators of the ternary complex abundance to study its normal- and patho-biology *in vitro* and *in vivo* and to test the hypothesis that eIF2.GTP.Met-tRNA$_i$ ternary complex can be pharmaceutically targeted for treatment of human disorders.

The cell-based dual luciferase high throughput screening assay described here enables us to quantify the relative abundance of the ternary complex. This assay is particularly robust because the bidirectional nature of our expression system allows the same enhancer/promoter complex to control transcription of both luciferases, thereby eliminating artifacts that may result from the modulation by test compounds of transcription, translation elongation or of mRNA or protein stability. The *N,N'*-diarylurea privileged scaffold identified in the screening campaign is shared by several biologically active agents that modulate the activities of different targets, depending on the specific substitution pattern of the aryl rings. Our assembly and testing of an diversity *N,N'*-diarylurea-library lead to the selection of one inactive (NCPdCPU) and three active (BTCtFPU, BTdCPU, and BOCPU) compounds for in depth characterization in target identification.

Our studies demonstrated that the active *N,N'*-diarylureas induce eIF2α phosphorylation by specifically activating only one of four eIF2α kinase, HRI. Active *N,N'*-diarylureas interact directly with HRI as demonstrated by: i) BTdCPU protects HRI but not eIF4E, an unrelated protein, from proteolysis in DARTS assay (Supplementary Fig. 9a and 9b), ii) BTdCPU does not cause oxidative stress (Supplementary Fig. 9c), and iii) while agents causing cellular stress activate HRI in intact cells but not cell-free lysates[31], BTdCPU activates HRI in cell-free lysates (Supplementary Figs. 9d and 9e). The correlation of anti-proliferative activity of the *N,N'*-diarylureas with the level of HRI in cancer cells (Fig. 5a) and much lower sensitivity of isogenic cells in which either endogenous eIF2α is replaced by eIF2α-S51A or HRI expression knocked down compared to eIF2α-WT cells or cells without HRI

knockdown (Fig. 4a-b, and Supplementary Fig. 10c) indicates that the *N,N'*-diarylureas have limited off-target effects and posses the necessary specificity for studying the normal- and pathobiology of the ternary complex.

At least one of the *N,N'*-diarylureas, BTdCPU, potently inhibits growth of xenografted human breast carcinoma without any macro-, micro-, or bone marrow toxicity (Fig. 5b and Supplementary Fig. 12 and 13). While these findings indicate that the *N,N'*-diarylureas are excellent candidates for the development of potent mechanism specific anti-cancer agents, whether the tumor cells will develop resistance to the *N,N'*-diarylureas, for example by mutating S51 of eIF2α to a non-phoshorylatable residue is not known. This will depend, at least in part, on whether or not this residue is susceptible to mutation (hot-spot) and whether the mutation itself will have any adverse consequences for the tumor under certain circumstances (for example under chemotherapy or in the highly hypoxic tumor regions).

The lack of apparent toxicity as well as specificity of the *N,N'*-diarylureas for a single eIF2α kinase distinguishes them from other agents that cause eIF2α phosphorylation and suggest that they may be utilized to study the role of the ternary complex in the development of hemolytic anemias such as β-thalassemia[17-19], diabetes and anti-viral defense [18,20-23]. Other agents that cause eIF2α phosphorylation either have pleiotropic effects and/or lack specificity (i.e. TG) or impinge on other aspects of cellular metabolism (i.e. salubrinol)[35,36]. This precludes development of those agents (i.e. TG or salubrinol) for treatment of human disorders or their use for *in vivo* studies aimed at understanding the biology of eIF2·GTP·Met-tRNA$_i$ ternary complex.

In conclusion, we propose that the *N,N'*-diarylureas described here represent invaluable tools for investigating the role of the HRI and the ternary complex in various human disorders [37,38,17-19,39-40] and may form the basis of a drug development program that will bring novel treatments for human disorders such as cancer, and certain anemias.

METHODS

Cell growth assay

Cell growth was measured by the SRB assay as described elsewhere [41].

Plasmids

The pBISA plasmid contains tetracycline regulated transactivator response element (TRE), flanked on both sides by minimal human cytomegalovirus (CMV) minimal promoters, allowing bi-directional transcription and two multiple cloning sites (MCS) [27]. Firefly and renilla luciferases were subcloned into MCS-I and MCS-II, respectively (Fig. 1). Generation of this expression plasmid, called pBISA-DL$^{(ATF-4)}$, is described in detail under Supplementary Methods.

Stable and transient transfection

Cells were seeded at a density of 2×10^5 in 60-mm (stable transfection) or 10^4 cells per well in 96-well plates (transient transfection) and transfected using the Qiagen transfectamine

transfection kit. For selection of stable cell lines, transfected cells were transferred to 100-mm plates and selected with appropriate antibiotics.

Western blotting

Cell extracts were separated by SDS-PAGE and probed with anti-phosphoserine-51-eIF2α (pS51-eIF2α Epitomics Inc, CA), anti-total eIF2α-specific antibodies (eIF2α Biosource International, Hopkinton, MA), anti-CHOP, or anti-β-actin (Santa Cruz Biotechnology, CA) as described elsewhere [42].

Real time PCR

Total RNA was extracted with TaqMan Gene Expression Cells-to-Ct™ Kit (Applied Biosystems, Branchburg, NJ) and DNAse I treated according to manufacturer's recommendations. 1-Step Real-time PCR was performed on a Bio-Rad iCycler IQ5 system by using B-R 1-Step SYBR Green qRT-PCR Kit (Quanta BioSciences, Gaithersburg, MD) according to manufacturer's specifications. The thermal cycler conditions and the primers utilized are detailed under the Supplementary Methods. All PCRs were performed in triplicate in at least two independent PCR runs. Mean values of these repeated measurements were used for calculation. To calibrate the results, all the transcript quantities were normalized to 18S rRNA (18S ribosomal RNA-like mRNA in mouse).

RNAi transfection

The siRNA pools against Human PKR, PERK, GCN2 and HRI and Mouse PKR, PERK, GCN2 and HRI were obtained from Dharmacon. Cells were plated in 96-well plates (1×10^4 cells/well) together with 25nM of siRNA Smartpool and 0.2 μl/well Lipofectamine RNAiMax (Invitrogen) incubated for 24 hours, then treated with compounds, and harvested at 6, 16, and 72 h after treatment for Real-time PCR, luciferase, and viability assays. The siRNA pools and transfections reagents are further described under Supplementary Methods.

High throughput screening and dual luciferase assay

Liquid handling was conducted on a Biomek FX (Beckman Coulter). Luminescence measurements were conducted on a Microbeta Trilux (Perkin Elmer). Screening was conducted in 384-well white opaque plates (Nalge Nunc), 100 μl RPMI + 10% fetal bovine serum. The details of screening procedure and dual luciferase assay are described under Supplementary Methods. The F-luc/R-luc (F/R) ratio in each well of a plate was normalized to the F/R ratio of vehicle treated wells of that plate.

DARTS assay

Twelve μg recombinant HRI or 5 μg recombinant eIF4e was incubated with DMSO, BTdCPU (5, 50, and 500 μM) or 4EGI1 (500 μM) for 2 h at 4 °C, followed by digestion with subtilisin at room temperature. 1:800 (wt:wt) subtilisin:HRI or 1:500 (wt:wt) subtilisin:eIF4E for 1 h. The reactions were stopped by adding 12 μl SDS loading buffer and boiling for 5 min. Samples were loaded onto a 12% acrylamide SDS-PAGE gel, followed by staining with Coomassie brilliant blue to visualize the banding pattern.

In vivo toxicity and efficacy testing

Five female nude mice each were treated with 200 mg/kg, 100 mg/kg BTdCPU in 15 μl DMSO or 15 μl DMSO daily for seven days. Mice were observed daily for signs of toxicity and weighed every other day for total of 15 days and then necropsy was performed. The average body of each group is plotted against the time.

Female nude mice were implanted with a slow release estradiol 17-β pellet in the sub-scapular region. The MCF-7 human breast cancer cells were transplanted to the mammary fat pad of the 4th inguinal gland of these mice. Tumor bearing mice were randomly distributed to the vehicle or treatment group, mice in the treatment group received 175 mg/kg compound BTdCPU in 15 μl DMSO and those in the vehicle group received the same amount of DMSO alone. Mice were observed daily, and weighed twice weekly, and tumor dimensions were measured weekly.

Pharmacokinetics studies

Plasma concentration-time profiles were determined by treating mice with a 175 mg/kg of compound BTdCPU by IP injection in 15 μL of DMSO. Blood samples were obtained from sacrificed mice at 1, 4, and 24 hours postinjection. Plasma was prepared by spinning the fresh blood containing 1000 unit/ml heparin. Analytical methods based upon high performance liquid chromatography coupled with electrospray ionization mass spectrometry were developed and validated for the determination of compounds BTdCPU in mouse plasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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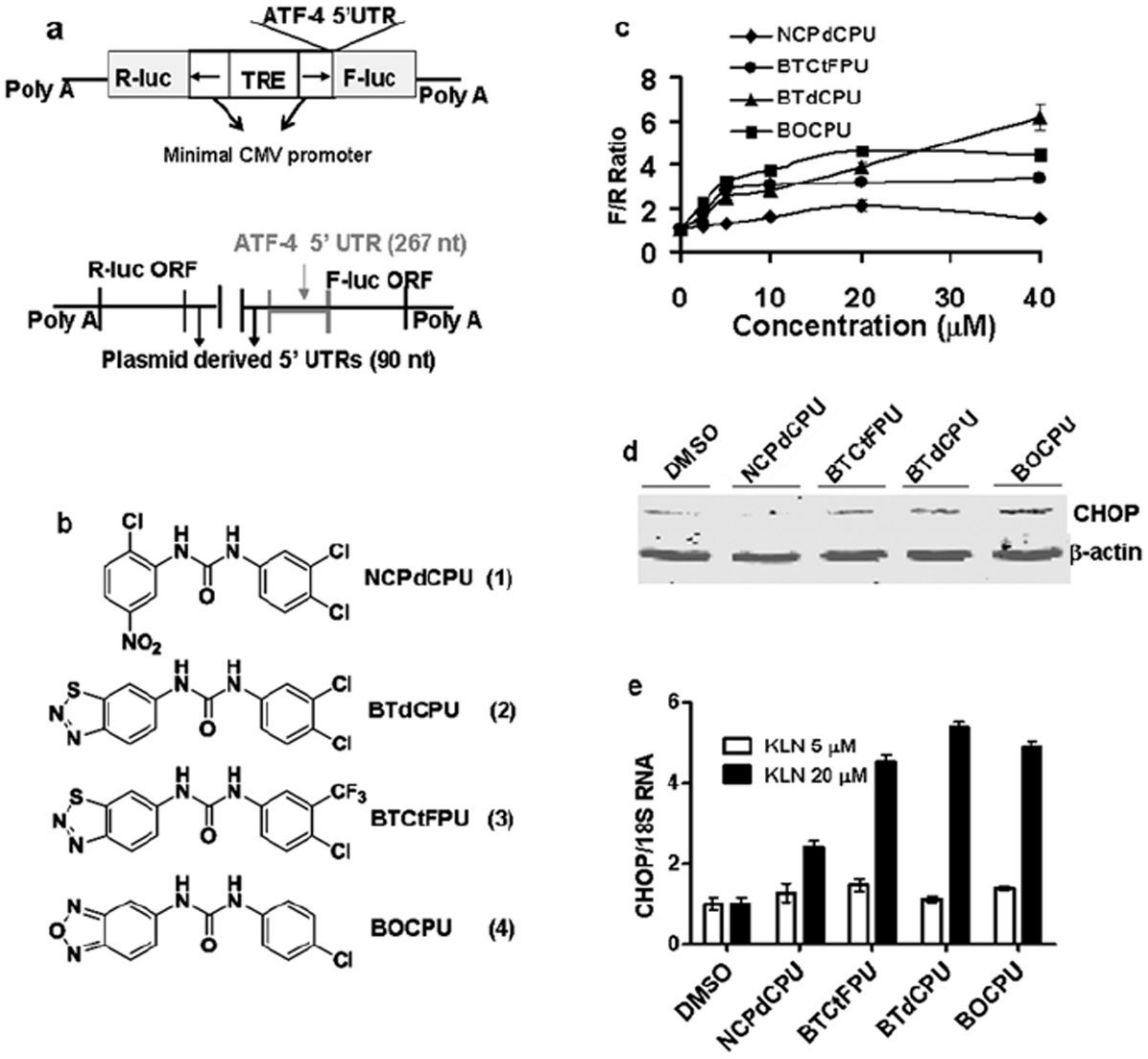

Figure 1. Identification and validation of the *N,N'*-diarylureas as modifiers of the ternary complex abundance

a) F-luc and R-luc ORFs were cloned into pBISA plasmid to transcribe two reporter mRNAs. The 5'UTR of the mouse ATF-4 mRNA including first two codons of bona-fide ORF was cloned in frame with respect to the start codon of F-luc ORF (pBISA-DL$^{(ATF-4)}$). The mRNA products of pBISA-DL$^{(ATF-4)}$ plasmid are shown. **b)** The structure of three active (a) and one inactive (i) *N,N'*-diarylureas. **c)** KLN-tTA/pBISA-DL$^{(ATF-4)}$ cells were incubated with the indicated concentrations of each *N,N'*-diarylurea and the normalized F/R ratio was determined by DLR assay. **d)** KLN-tTA/pBISA-DL$^{(ATF-4)}$ cells were incubated with the indicated concentrations of each *N,N'*-diarylurea and expression of endogenous CHOP protein was determined by Western blot analysis. **e)** KLN-tTA/pBISA-DL$^{(ATF-4)}$ cells were incubated with 5 or 20 μM of each *N,N'*-diarylurea and expression of endogenous CHOP mRNA was determined by real-time PCR. 3 replicates in each experimental and control group and each experiment was independently performed 3 times.

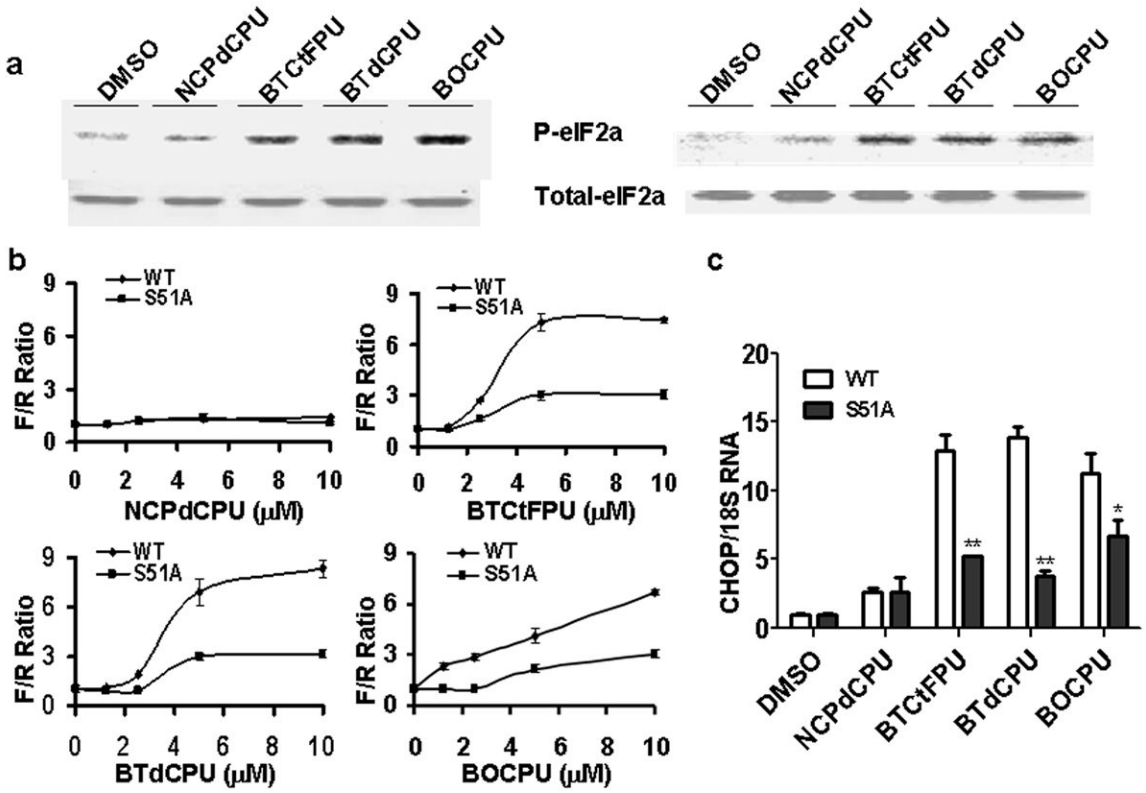

Figure 2. *N,N'*-diarylureas reduce the abundance of the ternary complex by causing phosphorylation of eIF2α

a) KLN-tTA/pBISA-DL$^{(ATF\text{-}4)}$ (left) or PC-3 cell (right) lines were incubated with selected *N,N'*-diarylureas, levels of phosphorylated (p-eIF2α) and total eIF2α (eIF2α) were determined by Western blot analysis with pS51-eIF2α specific rabbit monoclonal antibodies or with total eIF2α specific mouse monoclonal antibodies; respectively. **b**) The PC-3 cells in which endogenous eIF2α is replaced by recombinant WT or non-phosphorylatable eIF2α-S51A mutant were co-transfected with tTA and pBISA-DL$^{(ATF\text{-}4)}$ dual luciferase expression vector and treated with the indicated concentrations of *N,N'*-diarylureas. The normalized F/R ratio was determined by DLR assay and standard error of mean are shown. **c**) Genetically engineered PC-3 cells in (b) were treated with *N,N'*-diarylureas (10 μM) and the expression of CHOP mRNA was determined by real-time PCR. The experiment was conducted in triplicates and each experiment was independently performed three times.

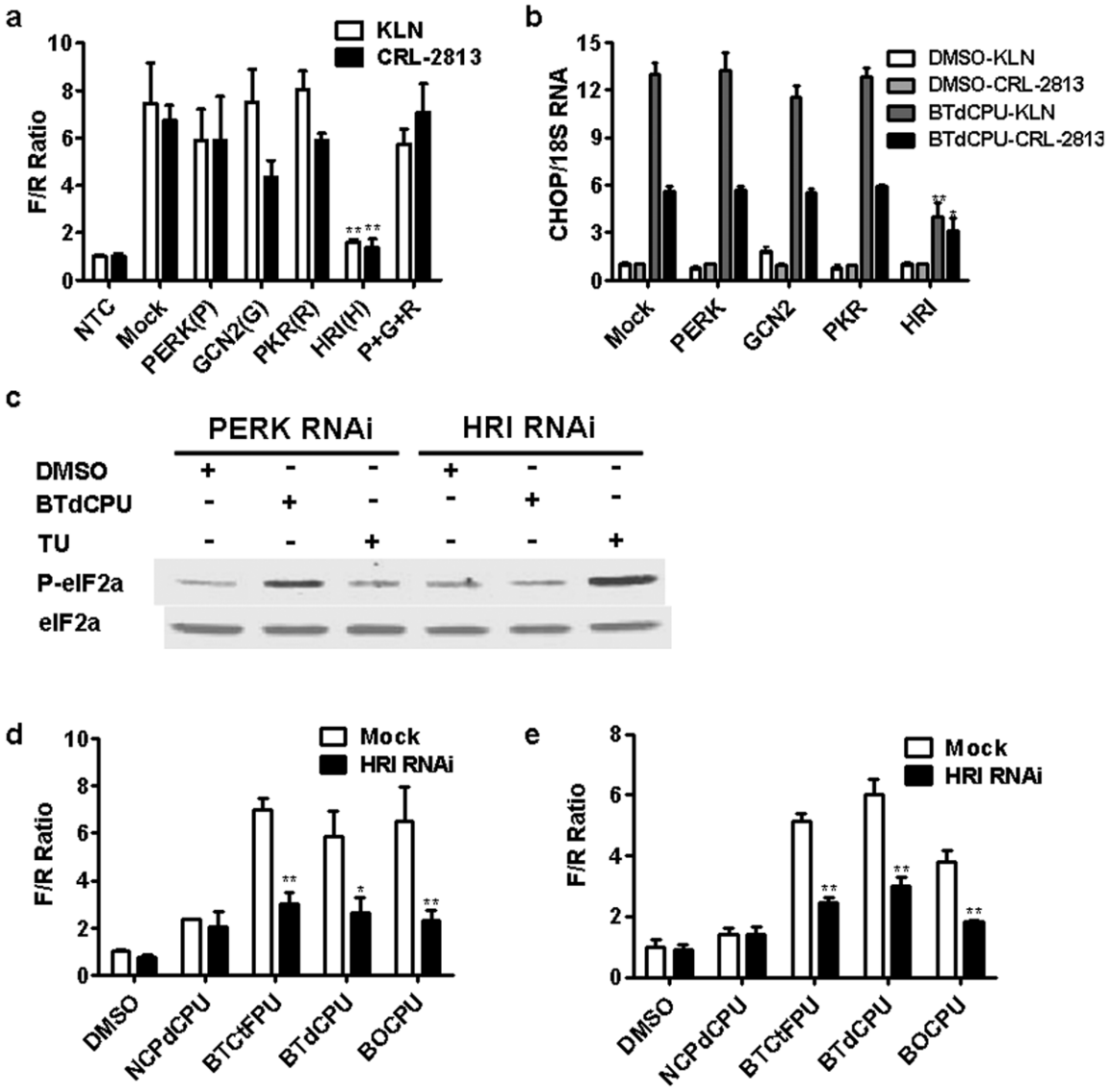

Figure 3. The *N,N'*-diarylureas specifically activate HRI kinase

a) KLN-tTA/pBISA-DL$^{(ATF-4)}$ cells were transfected with mock siRNA or siRNA targeting PKR, PERK, GCN2, or HRI individually or simultaneously in all combinations (only PKR, PERK, and GCN2 combination is shown). CRL-2813 cells were transfected in the same manner except that the transfection mixture also contained the pBISA-DL$^{(ATF-4)}$ and tTA plasmids. Cells were treated with compound BTdCPU or with DMSO and the normalized F/R ratio was determined by DLR. **b)** KLN-tTA/pBISA-DL$^{(ATF-4)}$ or CRL-2813 cells were transfected with siRNAs targeting each of the eIF2α kinases and treated with compound BTdCPU or with DMSO. Expression of CHOP mRNA was determined by real-time PCR. **c)** CRL-2813 cells were transfected with mock, PERK or HRI siRNA, treated with tunicamycin, compound BTdCPU or vehicle, and the levels of phosphorylated (p-eIF2α) and total eIF2α (eIF2α) were determined by Western blot. **d**) KLN-tTA/pBISA-DL$^{(ATF-4)}$ cells were transfected with mock or HRI-targeting siRNA, treated with four *N,N'*-diarylurea

compounds or vehicle and the normalized F/R ratio was determined by DLR. **e**) CRL-2813 cells were transfected with mock or HRI targeting siRNA, treated with four *N,N'*-diarylurea compounds or vehicle and the normalized F/R ratio was determined by DLR.

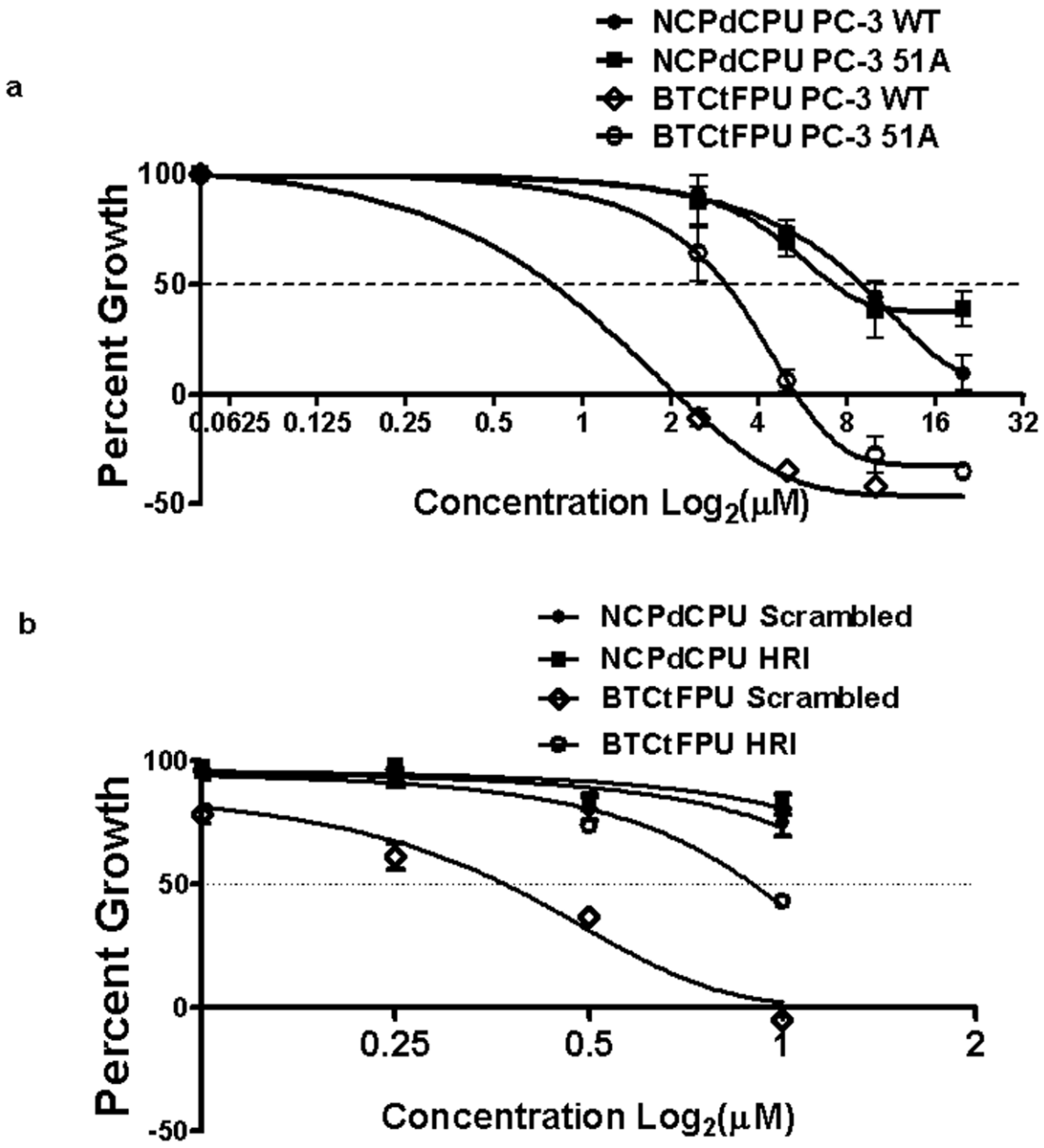

Figure 4. Phosphorylation of eIF2α by HRI mediates inhibition of cancer cell proliferation by *N,N'*-diarylureas

a) The PC-3 human prostate cancer cells in which endogenous eIF2α is replaced by recombinant WT or the non-phosphorylatable eIF2α-S51A mutant were treated with the indicated concentrations of *N,N'*-diarylureas and cell proliferation was measured by SRB assay. Panel **a** shows the growth inhibition curve for one active (BTCtFPU) and one inactive (NCPdCPU) *N,N'*-diarylurea. Calculated IC_{50} for all four compounds in these genetically engineered cell lines are shown in Supplementary Fig. 10a. b) CRL-2813 human melanoma cancer cells were transfected with HRI or mock siRNA, treated with the indicated concentrations of *N,N'*-diarylureas and cell proliferation was measured by SRB assay. The panel **b** shows the growth inhibition curve for one active (BTCtFPU) and one inactive

(NCPdCPU) *N,N'*-diarylurea, calculated IC_{50} for all four compounds in cells transfected with HRI or mock siRNA is shown in Supplementary Fig. 10b. The experiment was conducted in triplicates and each experiment was independently performed three times.

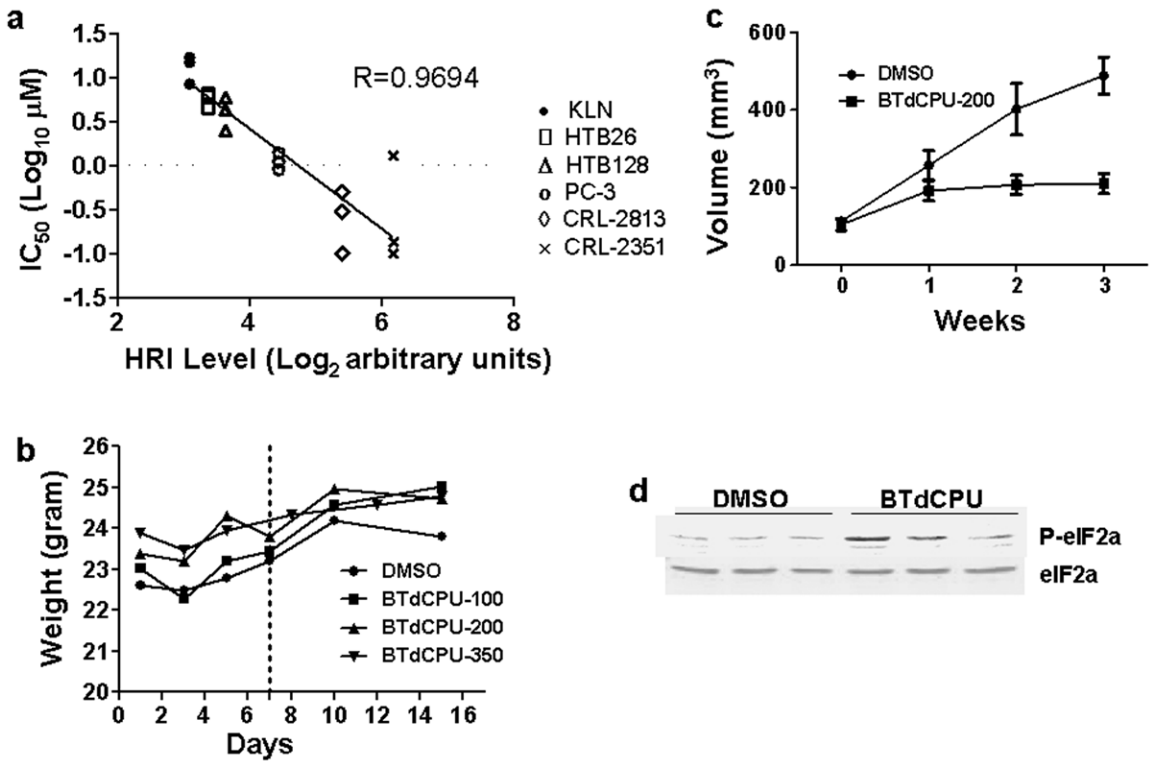

Figure 5. The in vitro and in vivo anti-cancer activity of *N,N'*-diarylureas

a) Lysates were prepared from KLN mouse squamous cell carcinoma, HTB-26, HTB-128, and CRL-2351 human breast, PC-3 human prostate, and CRL-2813 human melanoma cancer cell lines were separated by SDS-PAGE and probed with antibodies specific to HRI or β-actin and quantified. The concentration of the three active *N,N'*-diarylureas that inhibit proliferation of these cells by 50% (IC_{50}) were plotted against the levels of HRI (normalized for β-actin levels) in the cancer cell lines. Each experiment was independently performed three times. **b)** Five female nude mice each were treated with 200 mg/kg/d, 100 mg/kg/d and 350 mg/kg/d BTdCPU in 15 μl DMSO or 15 μl DMSO daily for seven days, weighed every other day for total of 15 days and then necropsy was performed. The average body weight of each group is plotted against the time. **c)** Female nude mice xenografted with MCF-7 human breast cancer cells were randomly distributes to two groups and treated with 175 mg/kg/d BTdCPU in 15 μl DMSO or DMSO alone. Mice were observed daily, and tumor dimensions were measured weekly. **d)** Lysates prepared from three excised tumors in the treatment and control groups, separated by the SDS-PAGE and blotted with antibodies specific to phosphorylated (P-eIF2α) or total eIF2α (eIF2α) and ratio of phosphorylated eIF2α to total eIF2α was quantified (see Supplementary Fig. 11).

Table 1

Effect of *N.N'*-diarylureas on proliferation of human cancer cells.

	IC_{50} *(μM)			
	NCPdCPU	**BTCtFPU**	**BTdCPU**	**BOCPU**
PC-3	8.6	0.9	1.1	1.4
KLN	>20	14.8	17.1	8.5
2813	20	0.1	0.5	0.3
2351	9.5	1.3	3.0	0.1
A549	>20	0.8	1.2	1.3

* Concentration of compound that inhibit cell proliferation by 50%.