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Selective Pharmacological Targeting of a DEAD Box RNA Helicase

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Abstract

RNA helicases represent a large family of proteins implicated in many biological processes including ribosome biogenesis, splicing, translation, and mRNA degradation. However, these proteins have little substrate specificity, making inhibition of selected helicases a challenging problem. The prototypical DEAD box RNA helicase, eIF4A, works in conjunction with other translation factors to prepare mRNA templates for ribosome recruitment during translation initiation. Herein, we provide insight into the selectivity of a small molecule inhibitor of eIF4A, hippuristanol. This coral-derived natural product binds to amino acids adjacent to, and overlapping with, two conserved motifs present in the carboxy-terminal domain of eIF4A. Mutagenesis of amino acids within this region allowed us to alter the hippuristanol-sensitivity of eIF4A and undertake structure/function studies. Our results provide an understanding into how selective targeting of RNA helicases for pharmacological intervention can be achieved.

Introduction

Helicases and translocases are classified into 6 superfamilies (SF1–SF6) based on the arrangement of conserved sequence motifs, with many providing essential functions in nucleic acid metabolic processes [1]. Members of the SF2 family consist of RNA helicases implicated in transcription, RNA export, splicing, translation, ribosome biogenesis, miRNA processing, and RNA decay [2–4]. Eukaryotic initiation factor (eIF) 4A is one of the archetypical founding members of the DEAD box helicase family, the largest subclass of the SF2 family. eIF4A is an abundant translation factor that exists in free form (referred to herein as eIF4A0) or as a subunit of the heterotrimeric cap binding complex, eIF4F (referred to herein as eIF4A1) [5,6]. It participates in the ribosome recruitment phase of translation and is delivered to the cap structure (m7GpppN, where N is any nucleotide) of mRNA templates as a subunit of eIF4F. It is thought to prepare the mRNA template for 43S pre-initiation complex (40S ribosome and associated factors) binding by unwinding local secondary structure. The helicase activity of eIF4A is ~20-fold more efficient than eIF4A0 [7,8] and during initiation eIF4A is thought to cycle through the eIF4F complex [9–12]. There are two highly related isoforms, eIF4AI and eIF4AII (85–90% sequence identity) which are thought to be functionally interchangeable for translation initiation [12,13]. A third protein, called eIF4AIH (DDX48), has ~65% sequence identity to eIF4AI and is part of the exon junction complex that participates in nonsense mediated decay [14,15]. The helicase activity of eIF4A is inhibited when associated with the tumor suppressor gene product, Pdcd4, an event that is regulated by the mammalian target of rapamycin (mTOR) [16,17]. This underscores an important link between cellular homeostasis and translational control at the level of eIF4A availability.

In a screen aimed at identifying novel inhibitors of translation initiation, we identified and characterized two marine-derived natural products, pateamine and hippuristanol, that modulate eIF4A activity [18–20]. The binding site of pateamine on eIF4A is not defined, although its activity is dependent on the nature of the linker region joining the amino-terminal (NTD) and carboxy-terminal domains (CTD), a region with significant sequence variation among DEAD-box family members [21]. On the other hand, hippuristanol interacts with eIF4AI-CTD (residues 237–406) and blocks the RNA-dependent ATPase, RNA binding, and helicase activities of eIF4AI [20]. Herein, we define the hippuristanol-binding site on eIF4A. The site displays extensive sequence variation among DEAD box RNA helicases and provides a framework for understanding the selectivity of hippuristanol. We utilize this information to generate eIF4A alleles with reduced sensitivity to this small molecule and capable of rescuing hippuristanol-induced inhibition of translation. This allowed us to probe structure-function relationships of eIF4A in translation.
Results
Defining the eIF4A hippuristanol binding site

To identify the amino acids involved in hippuristanol binding, a series of NMR experiments were undertaken in which 1H-15N-HSQC spectra of uniformly labelled eIF4AI-CTD were obtained in the absence or presence of compound (Fig. 1A). Residues that experienced significant chemical shift changes (≥mean plus standard deviation) are indicated in grey whereas those displaying direct NOE contacts (<5A) are highlighted in yellow (Fig. 1B). Hippuristanol binds directly (exhibits NOEs) to the N-terminal elements (Figs. 1A–C). Furthermore, adjacent regions undergo significant chemical shift changes (Fig. 1C; highlighted in blue).

We analyzed the position of the hippuristanol binding site in the context of a model based on the domain orientation of eIF4AIII in the exon junction complex (EJC) (PDB code 2HYI) [22,23] and the exon junction complex (EJC) (PDB code 2G9N) [20] was used and the holomodell for eIF4AI [24]. The positions of the RNA and ADPNP are taken from the EJC [22]. Accordingly, the hippuristanol-binding site on the eIF4AI-CTD is directly adjacent to the ATP-binding site in the NTD. Since hippuristanol does not inhibit ATP crosslinking to eIF4A [20], it may perturb the interface between the NTD and CTD domains. The hippuristanol-binding site is far from the RNA-binding face and allows us to conclude that hippuristanol inhibits eIF4A RNA binding in an allosteric manner.

Selectivity of hippuristanol for eIF4A

With the exception of R247eIF4AI and T329eIF4AI/H, all the hippuristanol binding residues are present in murine eIF4AI, eIF4AII, and the yeast eIF4A homolog Tif1p/2p (Fig. S1). This hippuristanol binding site however is not conserved in eIF4AIII, and we note 7 amino acid differences (Fig. S1: R247eIF4AI is changed to T247eIF4AI/H and T329eIF4AI to T329eIF4AI/H). The 338VQ339 eIF4A amino acid pair immediately downstream of motif V in eIF4A was altered to 338IP339 (present in Ded1p) or 338IG339 (for future NMR studies; since proline residues do not have amide protons and are not visible in 1H-15N HSQC spectra). These mutants also harboured a G363T alteration in motif VI (eIF4AIG/T), so we generated mutants harbouring only a G363T or a 338VQ339 to 338IG339 alteration (Fig. S1; eIF4AIG and eIF4AIIG/T). IP/T variants of eIF4AII and eIF4AIII were also generated (Fig. S1). We also addressed whether we could increase the sensitivity of eIF4AII to hippuristanol by rebuilding a complete hippuristanol site (Fig. S1; eIF4AIIITL1-LQQ; eIF4AIIG/T and eIF4AIIIP/T are more active than eIF4AI in an RNA-dependent ATPase assay (Fig. 3A and data not shown). The increased ATPase activity was principally a consequence of the 338VQ339 to 338IG339 alteration (Fig. S3). Hippuristanol inhibited eIF4A ATPase activity but had little effect on the ATPase activity of eIF4AIG/T and eIF4AIIIP/T (Fig. 3A and B). Similarly, eIF4AII was sensitive to inhibition by hippuristanol whereas eIF4AIIP/T was not (Fig. 3B). Titration of hippuristanol revealed that the ATPase activity of eIF4AIIIP/T was resistant to hippuristanol at concentrations up to 75 μM with a slight inhibition of activity at 100 μM (Fig. 3C). In contrast, eIF4AIIITL1-LQQ was more sensitive to hippuristanol than eIF4AII (Fig. 3C), showing a dose-response profile that resembled that of eIF4AI and eIF4AII (Fig. 2A). We further characterized the eIF4AI and eIF4AII hipuristanol-resistant alleles in RNA binding and helicase assays. As expected, hippuristanol reduced the ability of eIF4AI and eIF4AII to interact with RNA (Fig. S4A, compare lanes 2 and 8 to 1 and 7, respectively) but did not affect the RNA binding activities of eIF4AIG/T, eIF4AIIP/T, and eIF4AIIIP/T (Fig. S4A, compare lanes 4, 6, and 10 to 3, 5, and 9, respectively). The helicase activity of eIF4AI and eIF4AII is blocked by hippuristanol, whereas both eIF4AIG/T and eIF4AIIIP/T are resistant to inhibition (Fig. S4B). Taken together, these results demonstrate the feasibility of generating mutant alleles of DEAD-box helicase members with increased or reduced sensitivity to hippuristanol. This provides a powerful means by which to investigate the function of individual members of this family of proteins.

Structure/Function Studies of eIF4A

We used the ability to generate hippuristanol-resistant alleles of eIF4A to probe structure-function relationships in vitro. Specifically, we asked: (i) if the helicase activity of eIF4A is required for translation (or is its ATPase activity sufficient); (ii) if eIF4A:eIF4G interaction is essential for translation, and (iii) whether eIF4AI and eIF4AII are functionally interchangeable. The design of a helicase deficient mutant of eIF4A, eIF4AIVh/eIF4/G/T, was guided by a previously described Vasa mutation in which this alteration abolished helicase activity but only reduced ATPase activity by 50% (Fig. S1) [25]. eIF4AIVh/eIF4/G/T contains 4 missense

S2B). Hippuristanol did not inhibit the RNA binding properties of hDDX19 (Fig. 2B) nor the ATPase activity of hDDX52 or hDDX19 (Fig. 2C). These results provide insight into why hippuristanol is selective for eIF4A since the amino acids that define the hippuristanol binding site are not well conserved among other DDX family members.

Modulating eIF4A Hippuristanol Sensitivity

Using the mapping information from these NMR studies, we addressed the feasibility of modulating hippuristanol sensitivity among eIF4A family members (Fig. S1). Given that Ded1p is resistant to inhibition by hippuristanol (HippR), we used information obtained from the sequence comparison of the hippuristanol binding site to guide us in our mutagenesis approach [20] (Fig. S1). The 338VQ339 eIF4AI amino acid pair immediately downstream of motif V in eIF4A was altered to 338IP339 (present in Ded1p) or 338IG339 (for future NMR studies; since proline residues do not have amide protons and are not visible in 1H-15N HSQC spectra). These mutants also harboured a G363T alteration in motif VI (eIF4AIG/T), so we generated mutants harbouring only a G363T or a 338VQ339 to 338IG339 alteration (Fig. S1; eIF4AIG and eIF4AIIG/T). IP/T variants of eIF4AII and eIF4AIII were also generated (Fig. S1). We also addressed whether we could increase the sensitivity of eIF4AII to hippuristanol by rebuilding a complete hippuristanol site (Fig. S1; eIF4AIIITL1-LQQ).

eIF4AIG/T and eIF4AIIP/T are more active than eIF4AI in an RNA-dependent ATPase assay (Fig. 3A and data not shown). The increased ATPase activity was principally a consequence of the 338VQ339 to 338IG339 alteration (Fig. S3). Hippuristanol inhibited eIF4A ATPase activity but had little effect on the ATPase activity of eIF4AIG/T and eIF4AIIP/T (Fig. 3A and B). Similarly, eIF4AII was sensitive to inhibition by hippuristanol whereas eIF4AIIP/T was not (Fig. 3B). Titration of hippuristanol revealed that the ATPase activity of eIF4AIIIP/T was resistant to hippuristanol at concentrations up to 75 μM with a slight inhibition of activity at 100 μM (Fig. 3C). In contrast, eIF4AIIITL1-LQQ was more sensitive to hippuristanol than eIF4AII (Fig. 3C), showing a dose-response profile that resembled that of eIF4AI and eIF4AII (Fig. 2A). We further characterized the eIF4AI and eIF4AII hippuristanol-resistant alleles in RNA binding and helicase assays. As expected, hippuristanol reduced the ability of eIF4AI and eIF4AII to interact with RNA (Fig. S4A, compare lanes 2 and 8 to 1 and 7, respectively) but did not affect the RNA binding activities of eIF4AIG/T, eIF4AIIP/T, and eIF4AIIIP/T (Fig. S4A, compare lanes 4, 6, and 10 to 3, 5, and 9, respectively). The helicase activity of eIF4AI and eIF4AII is blocked by hippuristanol, whereas both eIF4AIG/T and eIF4AIIIP/T are resistant to inhibition (Fig. S4B). Taken together, these results demonstrate the feasibility of generating mutant alleles of DEAD-box helicase members with increased or reduced sensitivity to hippuristanol. This provides a powerful means by which to investigate the function of individual members of this family of proteins.

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Figure 1. Hippuristanol binds to elf4AI-CTD. (A) Chemical shift changes of $^{1\text{H}}-^{15\text{N}}$-HSQC peaks, ($\Delta\delta^{(\text{H})}+0.2\Delta\delta^{(\text{N})}$), of elf4AI-CTD (52 µM) upon addition of hippuristanol (100 µM). Free and bound forms are in slow exchange and the resonances of elf4AI-CTD had to be assigned in both states. The locations of secondary structures were identified by NMR and are indicated with magenta arrows (β-strands) and yellow rectangles (helices). (B) Primary amino acid sequence of elf4AI indicating residues involved in hippuristanol binding. NOEs are highlighted in yellow, whereas those within 5Å are in grey and correspond to regions a, b, and c in A. Residues in bold denote conserved amino acids that define motifs V (ARGID) and VI (HRIGRGGRFG) of DEAD box family members [40]. Arrows denote residues identified in Vasa that interact with ATP (red), RNA (blue), or are involved in interdomain interaction (green)[25]. (C) Surface and ribbon representations of the model for elf4AI-CTD. The CTD is viewed from the position of the NTD. Residues of elf4AI-CTD that show NOEs to hippuristanol are coloured yellow, those exhibiting major chemical shift changes but no NOEs are coloured blue. Residues contacting elf4G are in red [24]. The β-sheets (E1–E6) and α-helices (H1–H6) are labelled and refer to the locations marked in A. RNA and ADPNP are shown as sticks models. (D) Location of the hippuristanol-binding site in a model for elf4AI complexed with RNA and ADPNP. The model is composed of the crystal structure of human elf4AI-NTD (PDB #2G9N) and the homology model of the elf4AI-CTD [24]. The two domains are aligned to the structure of elf4AIII from the EJC from which the RNA and ADPNP binding sites are adapted (PDB/#2HYI) [22]. Color scheme of amino acid residues is as in C.

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Figure 2. Selectivity of hippuristanol for eIF4A. (A) Inhibition of eIF4A RNA-dependent ATPase activity by hippuristanol. ATPase assays were performed with 0.1 μg of His6-eIF4A1 or His6-eIF4AII at 25 °C or with 0.1 μg of His6-eIF4AIII at 37 °C for 2 h with 0.1 μCi [γ-32P]ATP (10 Ci/mmole). Following analysis by TLC and quantitation using a Fuji BAS 2000 phosphorimager, the percent hydrolysis was determined and set relative to the DMSO vehicle control reactions. Each value represents the average of three measurements with the error shown as the standard deviation. (B) Crosslinking of recombinant proteins to RNA in the presence of hippuristanol. 32P-labelled CAT RNA was cross-linked to 0.5–1 μg of the indicated recombinant protein in the presence or absence of hippuristanol, separated by SDS-PAGE, and visualized by autoradiography. [Note that in our hands, recombinant hDDX52 did not crosslink to RNA.] (C) Relative ATPase activity of eIF4A1, hDDX19, and hDDX52 in the presence of 50 μM hippuristanol, eIF4A1 and hDDX19, and hDDX52 where performed at 25 °C for 5 minutes while hDDX52 was incubated for 60 minutes to allow for analysis to be in the linear range of ATP hydrolysis. The percent ATP hydrolysis was determined in the presence of hippuristanol and set relative to the DMSO vehicle control reactions. The results represent the average of 3 experiments with error bars signifying the standard deviation.
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mutations previously shown to inhibit interaction with eIF4G [24]. eIF4A1Hel/IG/T showed a reduction in the rate of RNA-dependent ATP hydrolysis compared to eIF4AIIG/T - bringing it to levels similar to wild-type eIF4A1 (Fig. S5A). As expected, eIF4A1Hel/IG/T does not display helicase activity (Fig. S5B, compare lanes 5 to 3), and its RNA binding activity is resistant to hippuristanol (Fig. S5C). eIF4A1Quad/IG/T has a similar rate of RNA-dependent ATP hydrolysis as eIF4AIIG/T (Fig. S5A), possesses helicase activity (Fig. S5D), and its RNA binding activity is resistant to hippuristanol (Fig. S5C). As predicted, it is impaired in its ability to interact with eIF4G1 (Fig. S6).

We tested whether the Hipp eIF4A alleles could rescue translation when this process is inhibited with hippuristanol (Fig. 4). In vitro translations were performed in rabbit reticulocyte lysate (RRL) programmed with the bicistronic reporter mRNA FF/HCV/Ren (Fig. 4A) [20]. Here, Renilla (Ren) luciferase expression is HCV-driven and not eIF4A-dependent [20], thus serving as an internal control. Firefly (FF) luciferase expression is inhibited by >90% in the presence of 5 μM hippuristanol, whereas that of Renilla is slightly reduced (Fig. 4B, compare lane 2 to 1). Renilla luciferase RLU readings from the translation products of this experiment are consistent with a 2-fold reduction in activity (LL, data not shown). Addition of wild-type eIF4AI does not rescue the inhibition by hippuristanol, whereas eIF4AIQuad/IG/T restored translation to ~60% of normal levels (Fig. 4B, compare lanes 6 and 4 to 5 and 3, respectively). These results are consistent with the idea that inhibition of translation by hippuristanol in vitro is a direct consequence of impaired eIF4A activity. Neither eIF4A1Quad/IG/T or eIF4A1Hel/IG/T are able to rescue translation inhibition by hippuristanol [Fig. 4B] indicating that eIF4A's helicase activity and its ability to interact with eIF4G are necessary for its role in translation.

Next, we tested if eIF4AI and eIF4AII are functionally redundant for translation. To this end, we assessed the ability of eIF4AIHel/IG/T and eIF4AIIG/T to rescue hippuristanol-induced translation inhibition (Fig 4C). Like eIF4AIHel/IG/T (Fig. 4B), both eIF4AIIG/T and eIF4AIIIG/T rescued to the same extent (Fig. 4C, compare lanes 10 and 6 to 8 and 4, respectively). The rescue by the HippR mutants in these experiments was specific for hippuristanol, since it was not observed when translation was inhibited by pateamine - another eIF4A small molecule activity modulator (Fig. 4D, compare lane 9 to 8).

Hippuristanol targets eIF4A in vivo

We used a genetic approach to demonstrate that hippuristanol targets eIF4A in vivo. S. cerevisiae contains two eIF4A orthologues of identical amino acid sequence, called Tif1p and Tif2p [26]. We first assessed whether hippuristanol can block translation in an in vitro S. cerevisiae system programmed with Renilla mRNA (Fig. 5A). Concentrations of 1 μM hippuristanol were sufficient to inhibit protein synthesis (Fig. 5A). If Tif1/2p is the relevant biological target of hippuristanol in vivo, then Saccharomyces cerevisiae strains showing reduced activity of Tif1/2p should be more sensitive to growth inhibition by this compound than the wild-type (wt) strain [26]. The growth of 4A-ts, which contains the temperature sensitive V69S allele of Tif1p, was more sensitive than the wild-type parental strain or strains containing a deletion of Tif1p (Δtif1) or Tif2p (Δtif2) (Figs. 5B–C). These results imply that hippuristanol targets the yeast homolog of eIF4A (Tif1/2p) in vivo to affect growth.

These findings prompted us to use the Xenopus laevis translation system to assess if hippuristanol-induced inhibition of translation could be relieved by eIF4AHel/IG/T in vivo (Fig. 6). The hippuristanol binding site is 100% conserved between the murine and X. laevis eIF4AI proteins (data not shown). Addition of 5 μM hippuristanol inhibited cap-dependent translation of injected FF/HCV/Ren mRNA by 95% (Fig. 6A). Co-injection of recombinant eIF4AI slightly relieved the inhibition by hippuristanol to 30% of vehicle treated cells, whereas introduction of eIF4AHel/IG/T completely rescued the effect (Fig. 6A). Equivalent amounts of recombinant eIF4AI and eIF4AIHel/IG/T were delivered to the cells upon micro-injection, as assessed by western blot analysis of extracts prepared from the injected eggs (Fig. 6B).

Discussion

Herein, we demonstrate that hippuristanol interacts with amino acids within and adjacent to motifs V and VI of eIF4AI, two regions implicated in RNA, ATP, and interdomain contacts [23,25]. Hippuristanol inhibits the RNA-dependent ATPase activity and RNA binding ability of eIF4A, but does not prevent binding of ATP to eIF4A [20]. The hippuristanol-binding site on
eIF4A-CTD is adjacent to, or overlapping with, the NTD- and ATP-interacting surfaces. Since hippuristanol does not bind to the NTD, ATP would still be able to bind to eIF4A-NTD (its main binding site) in the presence of hippuristanol. The amino acid corresponding to Thr<sup>329</sup> of eIF4AI (Fig. S1) in Vasa and eIF4AIII is implicated in RNA binding via interaction with a phosphate residue on the RNA backbone. We therefore speculate that either: (i) hippuristanol interferes with proper interdomain interaction, which in turn abolishes RNA binding or (ii) affects alignment of Thr<sup>329</sup> with its target phosphate on RNA. We favor the former possibility as this mechanism of action has been documented by Nakamura and colleagues, who identified an RNA aptamer that inhibits eIF4A activity by also interfering with interdomain interaction [27].

Our results extend previous studies implicating eIF4A’s helicase activity and eIF4A:eIF4G interaction as being essential for translation (Fig. 4). eIF4AI<sup>G/T</sup>, but not eIF4AI<sup>D/T</sup>, was capable of rescuing hippuristanol-induced translation inhibition (Fig. 4), implying that eIF4AI<sup>G/T</sup> can assemble into the eIF4F complex and does not rescue translation as a free subunit. Consistent with this interpretation, rescue of translation by eIF4AI<sup>G/T</sup> is inhibited by the cap analog (m<sup>7</sup>GDP) [data not shown]. Although eIF4A plays an accessory role in promoting 48S complex formation on unstructured mRNA templates, it is required for 48S complex formation and translation of mRNAs containing weakly structured (−13.6 kcal/mol) hairpins [28]. eIF4A has been proposed to cycle through the eIF4F complex during initiation [12] and mutants of eIF4A have been previously described which appear to act in a dominant-negative manner to trap eIF4F in an inactive state, thus inhibiting translation [10,11]. The inverse relationship between 5′ secondary structure and sensitivity to inhibition by a dominant-negative mutant of eIF4A [11] is consistent with the idea that eIF4A functions to unwind secondary structure in the 5′ UTR during initiation to create a ribosome landing pad. However, not all potential activities of DEAD box proteins are necessarily involved in their function. Case in point is eIF4AIII where ATP hydrolysis is not required for its participation in NMD [29]. Therefore, we directly tested the requirement for eIF4A’s helicase activity in translation using a hippuristanol-resistant helicase-defective mutant and found it to be essential for eIF4A’s participation in initiation (Fig. 4). We also find that eIF4AI and eIF4AII are interchangeable in their ability...
Figure 4. Functional requirements for eIF4A activity in translation. (A) Schematic representation of the reporter construct used in these studies is shown on top. (B) Left Panel: Rescue of hippuristanol-induced translation inhibition by eIF4A<sup>IG/T</sup>. In vitro translations in RRL programmed with capped FF/HCV/Ren mRNA (8 μg/ml) and containing vehicle (0.1% DMSO) or 5 μM hippuristanol (in 0.1% DMSO) were supplemented with 0.5 μg recombinant protein. Protein synthesis was assessed by using <sup>35</sup>S-methionine incorporation as well as by monitoring luciferase assays. Protein products were separated by SDS-PAGE and visualized by autoradiography. The arrow indicates the position of migration of the firefly luciferase, whereas the arrowhead denotes the position of migration of Renilla luciferase. Right panel: Relative luciferase activity obtained in the presence of recombinant eIF4A. Firefly RLU readings obtained in the presence of recombinant eIF4A and hippuristanol were standardized to Renilla RLU values and set relative to the values obtained in the presence of vehicle (DMSO). The average of 3–8 experiments is shown with the standard deviations denoted. (C) eIF4AI and eIF4AII are functionally interchangeable. In vitro translations in RRL containing vehicle (DMSO) or 5 μM hippuristanol were supplemented with 0.8 μg recombinant eIF4A where indicated, and programmed with capped FF/HCV/Ren mRNA (8 μg/ml). Left panel: Protein products were separated by SDS-PAGE and visualized by autoradiography. The arrow indicates the position of migration of the firefly luciferase, whereas the arrowhead denotes the position of migration of Renilla luciferase. Right panel: Relative luciferase activity obtained in the presence of recombinant eIF4A and hippuristanol was standardized to Renilla Luciferase levels and set relative to the values obtained in the presence of vehicle (DMSO). The average of 3–8 experiments is shown with the standard deviations denoted. (D) Translational rescue by eIF4A<sup>IG/T</sup> is selective for hippuristanol. In vitro translations in RRL containing vehicle (DMSO), 5 μM hippuristanol, or 0.4 μM pateamine were supplemented with 0.8 μg recombinant eIF4A where indicated, and programmed with capped FF/HCV/Ren mRNA (8 μg/ml). Protein products were separated by SDS-PAGE and visualized by autoradiography. The arrow indicates the position of migration of the firefly luciferase, whereas the arrowhead denotes the position of migration of Renilla luciferase. The figure is a representative display of one of two experiments.

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Figure 5. Hippuristanol targets eIF4A in vivo. (A) Inhibition of Renilla luciferase reporter in a yeast in vitro translation extract. Hippuristanol was added to a S. cerevisiae cytosolic translation extract programmed with 0.12 μg/ml capped Renilla luciferase mRNA. At various points following initiation of the translation reaction, aliquots were removed and the relative luciferase units (RLU) determined. (B) Haploinsufficiency for Tif1/2p leads to increased sensitivity to hippuristanol in vivo. Haploid wild type cells (strain CWO4) or an isogenic strain carrying the temperature-sensitive tif1V79A allele (strain SS13-3A/pSSC120) were cultivated in rich medium (YPD) at 27°C to an O.D.600 of 0.2, at which point hippuristanol (1 μM or 10 μM final concentration) or solvent (DMSO, 0.1%) was added and the growth of different cultures was monitored for several hours by measuring the O.D.600. (C) Serial dilutions of different haploid yeast strains were plated on YPD-plates containing the indicated concentrations of hippuristanol and incubated for 2–3 days at 27°C: wt (CWO4), wild type strain CWO4; 4A-ts, strain SS13-3A/pSSC120 carrying the tif1V79A allele; Δtif1, a BY4741-derivative strain carrying a tif1::kanX deletion; Δtif2, a BY4741-derivative strain carrying a tif2::kanX deletion; wt (BY4741), wild type strain BY4741. doi:10.1371/journal.pone.0001583.g005

Figure 6. In vivo rescue of hippuristanol-induced translation inhibition by eIF4AIG/ΔT. (A) Rescue of translation in Xenopus oocytes by eIF4AIG/ΔT. The percent rescue was determined by normalizing the Firefly luciferase values to Renilla luciferase [to standardize for small variations in sample injection volumes], followed by dividing by the ratio obtained from the vehicle-treated samples (which was set at 100%). The data presented is the average of 9 independent sets of injections with the standard deviations denoted. (B) Western blot of extracts prepared from oocyte extracts. The equivalent of one oocyte was separated on a 10% SDS-PAGE, transferred to Immobilon-P, and probed with α-His6 (to detect recombinant His6-eIF4A) or α-tubulin antibodies. doi:10.1371/journal.pone.0001583.g006
to support translation in vitro. Previous reports documented that both eIF4AI and eIF4AII can associate with the eIF4F complex [12,13], and our studies extend these results by demonstrating that an eIF4A mutant lacking this function cannot participate in the translation process. The ability to generate hippuristanol-sensitive alleles of various DDX family members is a powerful tool with which to assign function and undertake structure-function studies.

### Materials and Methods

#### NMR Spectroscopy and modeling

NMR spectra were recorded at 298 K on a Varian Inova 600 and Inova 500 instruments equipped with cryogenic probes. Samples for NMR measurements typically contained 0.4–1 mM protein in buffer containing 20 mM Tris-HCl, 300 mM NaCl, 5 mM DTT, 1 mM EDTA, 0.01% NaN₃, 0.2 mM AEBSF, and 10% D₂O. The spectra were processed with NMRPipe [30] and analyzed with XEASY [31]. Sequential resonance assignments for eIF4AI-CTD and its complex with hippuristanol were obtained from standard triple-resonance NMR experiments (HNCA, HN(CA)CB, HN(CO)CA, HN(COCA)CB, HNCO, HN(CA)CO) on uniformly 15N-labeled samples with 70% deuteration. 15N-edited NOESY-HSQC and TOCSY-HSQC experiments were recorded on uniformly 15N-labeled samples. Intramolecular NOEs between eIF4AI-CTD and hippuristanol were measured using uniformly 15N/13C/2H-labeled CTD (52 mIC) complexed with 100 μM hippuristanol. 15N-dispersed NOE spectra were recorded in H₂O, and exhibited intramolecular NOE cross peaks between peptide HN and hippuristanol in an otherwise empty spectral region as described previously [32].

Homology modeling of the eIF4AI-CTD was previously described [24]. The model of full-length eIF4AI was constructed by manually superimposing the structure of human eIF4A-NTD (PDB# 2G9N) and the homology model of eIF4AI-CTD with the structure of eIF4AIII from the exon-junction complex (PDB# 2HYI) [22].

#### Recombinant DNA Constructs

Site-directed mutagenesis was performed using a PCR based strategy. Oligonucleotides harboring the mutant sequence(s) were used in amplification reactions with either upstream or downstream oligonucleotides spanning unique restriction sites (Table S1). Following gel purification of PCR products, these were combined and used in amplification reactions in the absence of primer for 5 cycles, at which point primers targeting the ends of the fragments were added and amplifications continued for 30 cycles. DNA fragments were cloned into expression plasmids using convenient restriction sites and all clones used in this study were sequenced to ensure the absence of secondary undesired mutations. The murine eIF4AI cDNA was subcloned from pET3b/eIF4AI into pET15b using NdeI and BamHI and inserted into the same sites in pET15b to amplify the full length mutant. The product was digested with DraIII and BamHI and inserted into pET15b/eIF4AII as template and into the primer pairs: (i) DraIII Forward and T Reverse and (ii) T Forward and BamHI Reverse (Table S1). The products were combined and cloned as described for pET15b/eIF4AIIG. For pET15b/eIF4AIIP, two PCR products were produced using pET15b/eIF4AI as template and into the primer pairs: (i) DraIII Forward and T Reverse and (ii) T Forward and BamHI Reverse (Table S1). The products were combined and cloned as described for pET15b/eIF4AIIG. Plasmid pET15b/eIF4AIIP was used as template for two PCR reactions, using either (i) primers DraIII Forward and T Reverse and (ii) T Forward and BamHI Reverse. The products were combined and cloned as described for pET15b/eIF4AIIG. Plasmid pET15b/eIF4AIIP was used as template for two PCR reactions, using either (i) primers DraIII Forward and T Reverse and (ii) T Forward and BamHI Reverse. The products were combined and cloned as described for pET15b/eIF4AIIG.

For pET15b/eIF4AIQuad/IG/T, two PCR products were produced using pET15b/eIF4AIQuad/IG/T as template and the primer pairs: (i) primers D265R/E268K and 4A1220–1238AS and (ii) D265R/E268K reverse and pET15b Oligo (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers pET15b Oligo and 4A1220–1238AS were used to amplify the full length mutant. The product was digested with NdeI and BamHI and inserted into the same sites in pET15b to create pET15b/eIF4AIQuad/IG/T. An additional round of mutagenesis was undertaken using this as template and the primer pairs: (i) D296A/T298K and pET15b Oligo, and (ii) D296A/T298K reverse and pET15b Oligo (Table S1). Following gel purification, the fragments were used to extend off of each other during 5 rounds of PCR amplification, after which the primers pET15b Oligo and 4A1220–1238AS were used to amplify the full length mutant. The product was digested with NdeI and BamHI and inserted into the same sites in pET15b to create pET15b/eIF4AIQuad/IG/T. Two PCR products were produced using pET15b/eIF4AIQuad/IG/T as template and the primer pairs: (i) AIGForward and 4A1220–1238AS and (ii) AIGReverse and pET15b Oligo (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers pET15b Oligo and 4A1220–1238AS were used to amplify the full length mutant. The product was digested with NdeI and BamHI and inserted into the same sites in pET15b to create pET15b/eIF4AIQuad/IG/T. Two PCR products were produced using pET15b/eIF4AIQuad/IG/T as template and the primer pairs: (i) AIGForward and 4A1220–1238AS and (ii) AIGReverse and pET15b Oligo (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers pET15b Oligo and 4A1220–1238AS were used to amplify the full length mutant. The product was digested with NdeI and BamHI and inserted into the same sites in pET15b to create pET15b/eIF4AIQuad/IG/T. Two PCR products were produced using pET15b/eIF4AIQuad/IG/T as template and the primer pairs: (i) AIGForward and 4A1220–1238AS and (ii) AIGReverse and pET15b Oligo (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers pET15b Oligo and 4A1220–1238AS were used to amplify the full length mutant. The product was digested with NdeI and BamHI and inserted into the same sites in pET15b to create pET15b/eIF4AIQuad/IG/T. Two PCR products were produced using pET15b/eIF4AIQuad/IG/T as template and the primer pairs: (i) AIGForward and 4A1220–1238AS and (ii) AIGReverse and pET15b Oligo (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers pET15b Oligo and 4A1220–1238AS were used to amplify the full length mutant.
For pET28a/eIF4AIIP\textsuperscript{III,IV}, two PCR products were produced using pET28a/eIF4AI as template and the primer pairs: (i) Primer D and Primer F and (ii) Primer E and Primer A (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers Primer A and Primer D were used to amplify the full length mutant. The product was digested with BamHI and inserted into the same site in pET28a to generate pET28a/eIF4AIIP\textsuperscript{III,IV}. The T mutation was introduced (Fig. S1) using pET28a/eIF4AIIP as template and the primer pairs: (i) Primer D and Primer C and (ii) Primer B and Primer A (Table S1). Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers Primer A and Primer D were used to amplify the full length mutant. The product was digested with BamHI and inserted into the same site in pET28a. For pET28a/eIF4AIIP\textsuperscript{III,IV,QQV}, two PCR products containing the TLLQQV mutation were produced from pET28a/eIF4AIIP: one with the primers Primer D and 4AIII(TLLQQV)AS and the other with 4AIII(TLLQQV)S and Primer A. Following gel purification, the fragments were used to extend off of each during 5 rounds of PCR amplification, after which the primers Primer A and Primer D were used to amplify the full length mutant. The product was digested with BamHI and inserted into the same site in pET28a.

**Purification of Hippuristanol**

Hippuristanol was extracted from the gorgonian *I. hippuris* as previously described [20].

**Recombinant Protein Expression and Purification**

Recombinant His\textsubscript{6}-eIF4AII and His\textsubscript{6}-eIF4AI were expressed in *E. coli* BL21 (DE3) codon+. Bacteria were grown to an OD\textsubscript{600} of 0.6 and induced with 1mM IPTG. Growth was continued an additional 3h at 37°C. Wild-type and mutant eIF4AI and eIF4AII proteins were resuspended in sonication buffer (20 mM Tris\textsubscript{7.5}, 10% glycerol, 0.1 mM EDTA, 200 mM KCl, 0.1% Triton X-100 and 3.4 mM β-mercaptoethanol). After sonication (9 pulses of 20 sec) and clarification by centrifugation (twice at 27,000 × g for 30 min), the lysate was loaded on a Ni\textsubscript{2+}-NTA agarose column as previously described [38]. Briefly, collagenased E. Coli was treated with RNAse A, separated on a 10% SDS-PAGE, and the proteins were resuspended in sonication buffer (20 mM Tris\textsubscript{7.5}, 10% glycerol, 0.1 mM EDTA, 200 mM KCl, 0.1% Triton X-100 and 3.4 mM β-mercaptoethanol). After sonication (9 pulses of 20 sec) and clarification by centrifugation (twice at 27,000 × g for 30 min), the lysate was loaded on a Ni\textsubscript{2+}-NTA agarose column as previously described [38]. Briefly, collagenased E. Coli was treated with RNAse A, separated on a 10% SDS-PAGE, and the proteins were resuspended in sonication buffer (20 mM Tris\textsubscript{7.5}, 10% glycerol, 0.1 mM EDTA, 200 mM KCl, 0.1% Triton X-100 and 3.4 mM β-mercaptoethanol). After sonication (9 pulses of 20 sec) and clarification by centrifugation (twice at 27,000 × g for 30 min), the lysate was loaded on a Ni\textsubscript{2+}-NTA agarose column as previously described [38].

**ATPase, RNA binding, and Helicase Assays**

ATPase assays were performed as described by Herschlag and Lorsch, using their “Condition B”[34]. Briefly, 0.1 μg or 1 μg protein (indicated in figure legend) was incubated with 2.5 μM poly (U) and 1 μM γ\textsuperscript{32}P-ATP (10 Ci/mmol) (0.01 μCi) at 25°C (except where specifically indicated) and time points taken at the indicated intervals by removing 2 μL aliquots and diluting into 2 μL of 25 mM EDTA. Inorganic phosphate and γ\textsuperscript{32}P-ATP were separated by TLC as described previously [34]. Results were quantitated using a Fuji BAS 2000 phosphomager with a Fuji imaging screen. The fraction of P\textsubscript{i} at t = 0 was typically 1–3% and subtracted as background.

For Tif1/2p, 0.5 μg Tif1/2p and 1 μg yeast eIF4G (aa342-893) was incubated with 12 nM [35S]RNA and 1 mM ATP at 25°C for 1h. The reaction was stopped with 50 mM EDTA and the formation of free phosphate determined as ammonium molybdate complex photometrically with malachite green (at A\textsubscript{650}). Calibration curves were prepared with increasing concentrations of Na\textsubscript{2}PO\textsubscript{4} (5–100 μM) (BioAssays Systems).

Chemical cross-linking was performed with 0.5–1 μg recombinant protein and oxidized \textsuperscript{32}P-labelled CAT mRNA (~18,000 cpm/μg) in the presence of 0.9 mM ATP with 10 μM hippuristanol or vehicle (DMSO) for 10 min at 30°C [20,35], after which time sodium cyanoborohydride was added, and the incubation continued overnight at 4°C. The samples were then treated with RNase A, separated on a 10% SDS-PAGE, and visualized by autoradiography.

RNA helicase assays were performed as previously described [7,36]. Briefly, 0.4 μM recombinant His\textsubscript{6}-eIF4AI or His\textsubscript{6}-eIF4AII was incubated with 2 μM RNA-1/11 duplex in the presence of 1 mM ATP for 15 minutes at 35°C. Reactions were resolved on native 12% polyacrylamide gels, which were dried and exposed to X-Omat (Kodak) film at −70°C.

**Rescue of hippuristanol-induced translation inhibition**

The plasmid pKS/FF/HCV/Ren was linearized with BamHI and transcribed with T3 RNA polymerase to generate FF/HCV/Ren mRNA [20]. *In vitro* translations were performed in rabbit reticulocyte lysates following the manufacturer's instructions (Promega). Extracts were programmed with FF/HCV/Ren mRNA (8 μg/ml) and translations performed at a final concentration of 135 mM KCl. eIF4A rescue experiments were performed by the addition of 0.5 or 0.8 μg recombinant eIF4A (0.9–1.4 μM) to vehicle- or hippuristanol-treated extracts. Firefly luciferase activities were measured on a Berthold Lumat LB 9507 luminometer. Reactions performed in the presence of [\textsuperscript{35}S]methionine were separated on a 10% SDS-polyacrylamide gel which was treated with EN\textsuperscript{2}Hance, dried, and exposed to X-Omat (Kodak) film at −70°C. For *in vitro* translation reactions in yeast, wild type strain BY4741 was used to prepare an *in vitro* translation extract and assays were performed as previously reported [37].

*Translation in Xenopus* oocytes were performed essentially as previously described [38]. Briefly, collagenased *Xenopus* oocytes were sorted and incubated for 4 h at 16°C in 5 μM hippuristanol or 0.05% DMSO. Each oocyte was then injected with 50 nL of 0.94 mg/mL recombinant His\textsubscript{6}-eIF4AII or recombinant His\textsubscript{6}-eIF4AII (i) or buffer alone immediately followed by 10 nL of 0.02 μM in vitro transcription FF/HCV/Ren mRNA. Oocytes were then incubated for 4h at room temperature in fresh compound dilutions. Three oocytes were homogenized in 150 μL Passive Lysis Buffer (Promega). The cell lysates were cleared by centrifugation at 14,000 × g for 5 min. Ten microliters of the lysate was read per sample using the Dual Luciferase Assay system (Promega). Values were normalized to Renilla activity and the percent rescue...
determined as average value of compound challenged samples divided by the average value of DMSO challenged samples.

**Time resolved fluorescence energy transfer (TR-FRET).** Recombinant His6-eIF4AI protein (20 nM) and GST-eIF4GI517–606 (40 nM) or GST-eIF4GI648–983 (200 nM) were incubated with Eu-W1024 labeled anti-6xHis antibody (1 nM) [Perkin Elmer] and anti-GST IgG antibody conjugated to SureLight-Allophycocyanin (100 nM) [Perkin Elmer] in TR-FRET buffer (20 mM Hepes pH 7.5, 10 mM KCl, 1 mM DTT, 0.015% Tween 20, 1 μg/ml IgG). Reactions were performed at room temperature for 3 hrs. FRET signal was monitored using an Analyst HT reader (LJL Biosystems) [39]. Data collection using the “Criterion Host v.2.0.1” software (LJL Biosystems) involved setting the Z height at 1 mm and utilizing 1 excitation filter (330/80) and 2 emission filters (620/7.5 and 665/7.5). A dichroic filter with a wavelength of 400 nm was used. For the measurement at 620 nm we employed 100 readings per well, with 10 ms between reading, integration time of 1000 μs, a delay time of 200 μs and 1000 μs integration time for the fluorescence emission recording. The parameters for the measurement at 665 nm were the same as for 620 nm, except for an integration time of 150 μs and a delay time of 50 μs. Due to the time delay, only the longer-lived FRET signal is detected, eliminating short-lived background fluorescence. The 665/620 ratio was calculated and normalized to the negative control reaction (containing His6-eIF4EWT3A which does not interact with eIF4G) or 4E-BP) to yield the S/B ratio. The 665 nm emissions are due to APC FRET and the 620 nm emissions are due to Eu-W1024 fluorescence.

**Supporting Information**

**Table S1**

Found at: doi:10.1371/journal.pone.0001583.s001 (0.05 MB DOC)

**Figure S1**

Amino acid alignment of the hippuristanol binding site among murine eIF4AI, murine eIF4AI1, and human eIF4AI1 alleles used in this study. Direct protein-hippuristanol NOEs are highlighted in yellow, whereas those within 5Å of the motif are indicated. Amino acids corresponding to the hippuristanol binding site in S. cerevisiae Ded1p is also shown.

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**Figure S2**

Amino acid alignment of the hippuristanol binding site among DDX family members. Alignments shown are for members of the murine (A) or human (B) DEAD box family members. The Entrez Protein IDs are provided in parenthesis for each member. Direct protein-hippuristanol NOEs are highlighted in yellow, whereas those within 5Å of the motif are in grey. The position of the first and last amino acid of the motif is indicated. Amino acids corresponding to the hippuristanol binding site in S. cerevisiae Ded1p is also shown.

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**Figure S3**

RNA-dependent ATPase activity of eIF4A1G/T, eIF4A1G1, and eIF4A1T mutant alleles. ATP hydrolysis was monitored using 1 μg recombinant protein. Each value represents the average of two measurements with the error of the mean presented. [Note in this assay, the protein preparation was different and not as active as the preparation used in Fig. 3A.]

Found at: doi:10.1371/journal.pone.0001583.s004 (10.00 MB TIF)

**Figure S4**

Characterization of eIF4AI and eIF4AI1 hippuristanol-resistant mutants. (A) Crosslinking of recombinant proteins to RNA in the presence of hippuristanol. 32P-labelled CAT RNA was cross-linked to 0.5–1 μg of the indicated recombinant protein in the presence or absence of hippuristanol, separated by SDS-PAGE, and visualized by autoradiography. (B) The helicase activities of the eIF4A1G/T and eIF4AI1G1/T mutants are resistant to hippuristanol. Helicase assays were performed with recombinant protein (0.4 μM) and duplexed RNA as described in the Materials and Methods. Reactions were resolved on a native 12% acrylamide gel, which was dried, and exposed to BioMax XAR film (Kodak) film at −70°C. The position of migration of duplexed (ds) and single-stranded (ss) RNA are denoted to the right.

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**Figure S5**

Characterization of eIF4A mutants. (A) RNA-dependent ATPase activity of eIF4AIWT/G/T and eIF4AI1Quad/G/T mutants. ATP hydrolysis was monitored using 1 μg recombinant protein. Each value represents the average of two measurements with the error of the mean presented. In this experiment, the protein preparations were different and not as active as the preparations used in Fig. 3A. (B) The helicase activity of eIF4AIWT/G/T is impaired. Recombinant protein (0.4 μM) was incubated with duplexed RNA as described in Materials and Methods. Reactions were resolved on a native 12% acrylamide gel and visualized by autoradiography. The migration of duplexed and ssRNA are determined by the incubation of duplexed RNA alone at 35°C (lane 1) or boiling for 5 minutes (lane 2), respectively. (C) Crosslinking of eIF4AI1Quad/G/T and eIF4AIWT/G/T to RNA in the presence of hippuristanol. 32P-labelled CAT RNA was cross-linked to 1 μg of the indicated recombinant protein in the presence or absence of hippuristanol, separated by SDS-PAGE, and visualized by autoradiography. (D) Helicase activity of eIF4AIQuad/G/T is not impaired and resistant to hippuristanol. Helicase assays were performed with recombinant protein (0.4 μM) and duplexed RNA as described in the Materials and Methods. Reactions were resolved on a native 12% acrylamide gel, which was dried, and exposed to BioMax XAR film (Kodak) film at −70°C. The position of migration of duplexed (ds) and single-stranded (ss) RNA are denoted to the right.

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**Figure S6**

The interaction of eIF4AIQuad/G/T with eIF4G1 is impaired. (A) Schematic representation of the various functional domains of eIF4G1. Protein and RNA binding sites on eIF4G1 are indicated. The numbers below eIF4G1 refer to the amino acid location of each binding site. A schematic of the recombinant eIF4G1 fragments utilized and the regions they span are shown in grey boxes. (B) TR-FRET analysis of the interaction between eIF4AI, eIF4AIWT/G/T, eIF4AI1Quad/G/T with eIF4G1 fragments. GST-eIF4G1 fragments were incubated with recombinant His6-eIF4A1 protein, as well as with Eu-W1024 labeled anti-6xHis antibody and anti-GST IgG antibody conjugated to SureLight-Allophycocyanin. The FRET signal (expressed as the signal to background ratio (S/B)) was measured using an Analyst HT reader (LJL Biosystems) to represent the average of 4 experiments with the standard error of the mean shown. The signal obtained with eIF4AI and eIF4AI1WT/G,060 was equivalent to the background signal (S/B = 1).

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Author Contributions
Conceived and designed the experiments: FF GW MA JP LL MO RC MB.
Performed the experiments: FF MA JP LL MO MR RC MB EV. Analyzed
the data: GW MA JP LL MO MR RC AM. Contributed reagents/
materials/analysis tools: JT. Wrote the paper: JP LL.

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