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Stimulators of Translation Identified During a Small Molecule Screening Campaign

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Abstract

In screening a library of natural and synthetic products for eukaryotic translation modulators, we identified two natural products, isohymenialdisine and hymenialdisine, that exhibit stimulatory effects on translation. The characterization of these compounds lead to the insight that mRNA used to program the translation extracts during high throughput assay set-up was leading to phosphorylation of eIF2 α , a potent negative regulatory event that is mediated by one of four kinases. We identified double-stranded RNA-dependent protein kinase (PKR) as the eIF2 α kinase that was being activated by exogenously added mRNA template. Characterization of the mode of action of isohymenialdisine revealed that it directly acts on PKR by inhibiting autophosphorylation, perturbs the PKR-eIF2 α phosphorylation axis, and can be modeled into the PKR ATP binding site. Our results identify a source of false positives for high throughput screening (HTS) campaigns using translation extracts, raising a cautionary note for this type of screen.

Keywords

High Throughput Screens; Translation; PKR; eIF2 α ; Isohymenialdisine; Hymenialdisine

INTRODUCTION

High Throughput Screens (HTS) have been the mainstay of drug discovery programs, chemical biology efforts, and RNA Interference (RNA_i)-based genetic screens. The design and construction of such screens stems from a fundamental understanding of protein function or activity, the desire to obtain a unique tool that can dissect a complex biological

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process, or the need to identify vulnerabilities for a specific therapeutic purpose. Decisions taken during the HTS design process have far-reaching consequences for the nature of molecules that will be identified as well as the spectrum of targets that will be probed – with the results impacting on the subsequent value of the identified molecules as useful reagents or drugs. For example, the dependence on delivery of extraneous factors to an HTS assay may lead to the identification of ‘false-positives’ due to confounding effects on availability or activity of that component. Such events increase the downstream workload and necessitate the implementation of counterscreens and secondary/tertiary confirmatory assays.

Protein synthesis has been extensively probed using translation-competent extracts in several HTS assays, with interests stemming from the desire to identify novel anti-microbial drugs [1; 2], block expression of specific mRNA transcripts [3; 4], identify compounds that target initiation and show anti-neoplastic activity [5; 6; 7; 8], and to correct readthrough nonsense mutations as a means to overcoming nonsense-based genetic defects [9]. In screens designed to identify inhibitors of mammalian translation, extracts from reticulocytes, HeLa cells and Krebs 2 ascites carcinoma have been extensively used [2; 5; 9]. Each system has its peculiarities with reticulocyte lysates being the more efficient extract whereas HeLa and Krebs 2 cell lysate are more cap-dependent and faithfully re-capitulating the cap-poly(A) synergy [10; 11].

The process of translation is suppressed by a number of regulatory kinases that (i) control the phosphorylation status of eukaryotic elongation factor (eEF) 2 to couple translation inhibition (eEF2 phosphorylation) with cellular processes, such as mitosis [12]; (ii) regulate ribosome recruitment to mRNA templates by modulating subunit availability of eukaryotic initiation factor (eIF) 4F [13; 14] – an activity responsible for preparing a ribosome landing pad in the vicinity of the 5' cap structure; (iii) and the phosphorylation status of eIF2 α [15]. During initiation, eIF2 forms a ternary complex with GTP and initiator methionyl-tRNA (Met-tRNA_i^{Met}), which then binds to 40S ribosomal subunits to form 43S pre-initiation complexes (PIC), along with other eIFs. Following recruitment of the PIC to mRNA templates the 43S complex scans the 5'-untranslated regions (UTR) of mRNA templates where, upon recognition of the appropriate AUG start codon, eIF2•GTP is converted to an inactive GDP-bound state. The guanine nucleotide exchange factor eIF2B is then required to recycle eIF2•GDP to eIF2•GTP for a new round of initiation, a process that is inhibited if eIF2 α is phosphorylated [16; 17; 18]. eIF2 is a trimeric complex consisting of α , β and γ subunits and phosphorylation of the α subunit at Ser 51 increases the affinity of eIF2•GDP for eIF2B, leading to sequestration of eIF2•GDP-eIF2B complexes. Since eIF2 is present in excess of eIF2B, small changes in the phosphorylation status of eIF2 leads to a significant inhibitory effect on global protein synthesis [19; 20].

In mammals, four eIF2 α kinases have been identified and extensively characterized: general control non-depressible 2 (GCN2), PKR-like endoplasmic reticulum kinase (PERK), heme-regulated inhibitor (HRI), and double-stranded RNA-dependent protein kinase (PKR). They share a conserved kinase domain but have distinct regulatory domains that respond to different stress signals: GCN2 senses amino acid deprivation and UV irradiation [21; 22], PERK is active in response to accumulation of misfolded proteins in the ER [23], HRI responds to heme deficiency, oxidative stress and heat shock in erythroid tissues [24], and PKR is activated by double-stranded RNA [25; 26]. PKR is a serine/threonine kinase that phosphorylates eIF2 α upon binding to double stranded (ds)RNA of viral, synthetic, or cellular origin. Binding to dsRNA, promotes dimerization of PKR, enables autophosphorylation and activation, which in turn inhibits translation via eIF2 α phosphorylation.

During the course of a recent screening campaign we identified two compounds, isohymenialdisine and hymenialdisine, that had the unusual property of stimulating translation. In characterizing the effect of these compounds, we found that they blocked PKR activation – an event that was unexpectedly caused by the mRNA preparations used to program the *in vitro* translation extracts. Our experiments identify a potential common source of false-positives in HTS screens designed to probe the mammalian process and extend the inhibitory spectrum of isohymenialdisine and hymenialdisine.

MATERIALS AND METHODS

Materials and General Methods

Restriction endonucleases, SP6 and T7 RNA polymerase were purchased from New England Biolabs and T3 RNA polymerase was purchased from Fermentas. [³⁵S]-methionine (>1000 Ci/mmol) and [γ -³²P]-ATP (6000 Ci/mmol) were purchased from PerkinElmer. Cycloheximide (CHX) (Bishop) was resuspended in H₂O, whereas, homoharringtonine (HHT) (Sigma-Aldrich), C16 (PKRi) (Calbiochem) [27], SyK inhibitor (GCN2i) (Calbiochem) [28], GSK PERKi (Toronto Research Chemicals Inc.) [29; 30] and Quercetin (HRIi) (Sigma-Aldrich) [31; 32] were resuspended in 100% DMSO. Antibodies were as follows: anti-phospho-eIF2 α (#3597), anti-eIF2 α (#9722) and anti-eEF2 (#2332) were from Cell Signaling Technology. Preparation of plasmid DNA, restriction digestion, agarose gel electrophoresis of DNA, and bacterial transformations were carried out using standard methods [33].

Isolation of Hymenialdisine and its derivatives from *Stylissa massa*

The sponge sample was collected under contract with Pfizer Research Laboratories (St. Louis). When Pfizer disbanded its natural products program they generously donated their marine invertebrate collection to R.J.A. (UBC). Specimens were collected by hand using SCUBA on a coral reef slope at 50 feet off Manado, Indonesia in November 1994 (N 01° 31'3", E 124° 50'07"). A voucher sample has been deposited at the Netherlands Centre for Biodiversity Naturalis in Leiden, The Netherlands.

Freshly collected sponge was frozen on site and transported frozen. Lyophilized sponge (5 g) was cut into small pieces, immersed in and subsequently extracted repeatedly with MeOH (3 $\times$ 50 mL) at room temperature. The combined methanolic extracts were concentrated *in vacuo*, and the resultant extract was then partitioned between EtOAc (3 $\times$ 5 mL) and H₂O (15 mL). The resulting aqueous extract was then extracted with n-BuOH (3 $\times$ 5 mL). The combined n-BuOH extracts were evaporated to dryness, and the resulting oil was chromatographed on Sephadex LH-20 with MeOH as eluent. The late eluting active fraction was purified via C₁₈ reversed-phase HPLC using a CSC-Inertsil 150A/ODS2, 5 μ m 25 $\times$ 0.94 cm column, with 9:1 (0.05% TFA/H₂O)/MeCN as eluent to give pure samples of hymenialdisine (**1**) (6.6 mg), debromohymenialdisine (**2**) (2.4 mg) and isohymenialdisine (**3**) (3.8 mg). The structures of **1**, **2** and **3** were established by standard 1 and 2D NMR spectroscopic and HRESIMS analyses.

In vitro Transcription and Translation Reactions

In vitro transcriptions were performed as previously described [5; 34]. Plasmids pKS/FF/EMC/Ren, pSP/(CAG)₃₃/FF/HCV/Ren, pGL3/Ren/CrPV/FF and T3LucpA+ were linearized with BamHI and transcribed *in vitro* to generate mRNA transcripts. *In vitro* translations using [³⁵S]-methionine were performed in rabbit reticulocyte lysate (RRL) as recommended by the manufacturer (Promega). For luciferase assays, firefly and renilla luciferase activities were measured on a Berthold Lumat LB9507 luminometer. For gel analysis, translation products were separated on 10% SDS-polyacrylamide gels that were subsequently treated

with EN³HANCE, dried and exposed to Blue Ray film (VWR). *In vitro* translations were performed in Krebs extracts as previously described [5] and in *Escherichia coli* S30 extracts as directed by the manufacturer (Promega).

In vitro Kinase Assays

Plasmids pGEX-5X-1-KD and pET-15b/eIF2 α were transformed into *E. coli* BL21(DE3)/pLysS. GST-PKR-KD and His₆-eIF2 α were expressed and purified as previously described [27; 35]. The eIF2 α kinase assay and PKR autophosphorylation assay were performed as reported with slight modifications [27; 35]. For eIF2 α kinase assays, 0.5 μ g of GST-PKR-KD and His₆-eIF2 α were incubated with 50 μ M ATP in kinase buffer (20 mM Tris-HCl [pH 7.6], 10 mM KCl, 10 mM MgCl₂, and 10% glycerol) at 30°C for 30 min. Phosphorylation of eIF2 α was assessed by Western blotting. For PKR autophosphorylation assays, 0.5 μ g of GST-PKR-KD was incubated with 50 μ M [γ ³²P]-ATP in kinase buffer at 30°C for 30 min. The reaction was quenched with 3x SDS-PAGE loading buffer, boiled and resolved in 10% SDS-polyacrylamide gels. The gel was dried and exposed to Blue Ray film (VWR).

Computational mapping of the PKR structure

Mapping was performed on the PKR kinase domain-eIF2 α -AMP-PNP complex (PDB ID 2A19). Chain A representing eIF2 α , the ligand AMP-PNP, and all crystallographic water molecules were removed prior to calculations. One PKR monomer (Chain B) was globally mapped using the FTMap server (<http://ftmap.bu.edu/>). FTMap places molecular probes, small organic molecules containing various functional groups, on a dense grid defined around the entire protein without any restriction, finds favorable positions using empirical energy functions, further refines the selected poses by energy minimization, clusters the low energy conformations, calculates the partition function for each cluster, and ranks the clusters on the basis of their probabilities [36]. The consensus sites are then defined as those where clusters of different probes overlap; such consensus sites identify the binding hot spots, i.e., regions of interactions that substantially contribute to the binding free energy and hence are of prime importance to ligand binding and to drug design.

Docking of hymenialdisine and isohymenialdisine

Based on the mapping results, a box with 4Å padding was created around the putative binding site. The docking was carried out using the standard settings of AutoDock Vina 1.1.2 [37], and a number of lowest energy binding modes were retained for each ligand. The selection of the most likely pose was based on the atom densities calculated from the mapping results [38]. We considered each retained pose separately, for each atom summed the atomic densities on the grid points within a 1Å radius, and then added these values for all atoms. The poses were ranked on the basis of this overlap measure, and the pose with the best overlap was selected [38]. The selected complexes were minimized using the Charmm potential allowing for complete flexibility [39].

RESULTS

Identification of Hymenialdisine and Isohymenialdisine as Stimulators of Translation In Vitro

During the course of screening natural and synthetic products for inhibitors of protein synthesis using *in vitro* translation assays, we noticed a stimulatory activity during bioassay-guided fractionation of one extract from *Stylissa massa*. Hymenialdisine (**1**), debromohymenialdisine (**2**), and isohymenialdisine (**3**) were purified and individually characterized in RRL programmed with FF/EMC/Ren (Figs. 1A, B). The FF luciferase protein is synthesized in a 5' cap-dependent manner, whereas production of Ren luciferase is

mediated by the ability of the encephalomyocarditis (EMC) internal ribosome entry site (IRES) to directly recruit ribosomes [40]. Anisomycin and hippuristanol, inhibitors of elongation and of initiation respectively [41], blocked production of both firefly (FF) and renilla (Ren) luciferase, as expected (Figs. 1C, D). Titration of hymenialdisine and isohymenialdisine stimulated production of both FF and Ren, with a more pronounced effect observed on EMC-driven Ren production (Figs. 1C, D). Debromohymenialdisine had no effect on the translational output of FF/EMC/Ren (Fig. 1C, compare lanes 7-9 to 1). Neither hymenialdisine nor isohymenialdisine stimulated luciferase enzyme activity *per se* when incubated in the presence of FF or Ren proteins, indicating that the results are not due to specific stimulation of luciferase enzyme activity (U. Shin, data not shown).

To determine if hymenialdisine and isohymenialdisine could also stimulate translation directed from other IRESes, we titrated compounds (Cmpds) **1–3** in RRL that had been programmed with FF/HCV/Ren or Ren/CrPV/FF (Fig. 2A). The hepatitis C virus (HCV) IRES does not require eIF4E, eIF4G, or eIF4A for ribosome recruitment whereas the cricket paralysis virus (CrPV) IRES is capable of recruiting ribosomes in the absence of any canonical initiation factors [40; 42; 43]. In the case of FF/HCV/Ren, hymenialdisine and isohymenialdisine stimulated both cap-dependent (FF) and IRES-mediated (Ren) translation (Fig. 2B). In contrast, the CrPV IRES was refractory to stimulation by any of the compounds used in this study, whereas cap-dependent translation was stimulated by hymenialdisine and isohymenialdisine (Fig. 2C).

To assess if these effects would also be observed in other mammalian translation systems, we tested extracts prepared from Krebs-2 cells programmed with FF/EMC/Ren (Fig. 3A). We observed a stimulatory effect on production of FF and Ren in the presence of isohymenialdisine, but not at similar concentrations of hymenialdisine or debromohymenialdisine. None of the compounds affected translation in *E. coli* coupled transcription-translation extracts when added to a final concentration of 50 μ M (Fig. 3B). Taken together, these results indicate that the effects of isohymenialdisine on increasing the yield of FF and Ren from FF/EMC/Ren is not unique to RRL and extends to Krebs extracts, but not to bacterial translation systems.

To obtain insight into the kinetics of stimulation exerted by Cmpds **1–3**, we documented the kinetics of stimulation upon addition of compounds to actively translating extracts (Fig. 4). Here, RRLs were programmed with FF Luc mRNA (Fig. 4A), compounds added 15 mins after start of the translation reactions, and aliquots removed during the course of translation to monitor the consequences on protein production. In this situation, addition of cycloheximide results in a rapid cessation of translation elongation with little protein production occurring over the ensuing 75 minutes (Fig. 4B). Homoharringtonine (HHT), allows ribosomes to run-off since it has low affinity for translating polysomes but inhibits the first step of elongation [44]. When added to translating RRLs, protein synthesis continues unabated for ~5-7 mins while ribosomes run-off pre-loaded mRNA template, and then stops (Fig. 4C). Addition of vehicle (DMSO) was accompanied by an increase in FF Luc production from 15–45 min followed by a plateauing and little change in FF Luc output beyond 60 min. Similar results were obtained when debromohymenialdisine (**2**) was added to translating RRLs (Fig. 4B). In contrast, addition of isohymenialdisine (**3**) caused a significant increase (~2.5 fold) in FF Luc output that was linear for a much longer period of time than observed from the vehicle control extracts (Fig. 4B). Addition of hymenialdisine (**1**) also stimulated production of FF Luc, but to a lesser degree than obtained with isohymenialdisine (Fig. 4C).

Isohymenialdisine Inhibits PKR-mediated Phosphorylation of eIF2 α

One mechanism that can limit the translational output of mammalian *in vitro* translation extracts is phosphorylation of eIF2 α (see Introduction). Indeed, *in vitro* transcribed RNA using phage polymerases often contains double stranded product due to a snap-back phenomenon which can lead to activation of PKR in translation extracts [45]. To directly test if this was occurring in our system, we probed RRL that had been incubated in the absence or presence of *in vitro* transcribed RNA. The results clearly show phosphorylation of eIF2 α occurs as a function of time upon addition of *in vitro* transcribed RNA (Fig. 4D).

To test if such an event was limiting the translation potential of our translation reactions, we assessed the activity of inhibitors to the four eIF2 α kinases in reticulocyte lysates (Figs. 4E, F). Small molecules targeting PERK (Fig. 4E) [29; 30], HRI (Fig. 4E) [31; 32], and GCN2 (Fig. 4F) [28] had little influence on FF production when added to RRLs programmed with FF-HCV-Ren mRNA. In contrast, addition of the PKR inhibitor, C16 [27], increased FF production to levels that resembled those obtained with isohymenialdisine (Fig. 4F). These results indicate that inhibition of PKR can increase translational output in RRL and phenocopies the effects of isohymenialdisine. To assess if addition of isohymenialdisine (**3**) to RRL would reduce levels of phospho-eIF2 α in RRL, we analyzed aliquots removed 5 and 90 mins following addition of compound to translating RRL extracts (Fig. 4G). The results indicate that isohymenialdisine (**3**), like the PKR inhibitor C16, can reduce phospho-eIF2 α levels in RRL programmed with exogenous mRNA (Fig. 4G). The effect of hymenialdisine was not as pronounced as that of isohymenialdisine, and paralleled the relative potencies of these two compounds for stimulation of translation (Fig 4C). We thus focused our attention on the characterization of isohymenialdisine.

To test if isohymenialdisine (**3**) was capable of inhibiting PKR-mediated phosphorylation of eIF2 α , we expressed and purified recombinant His₆-eIF2 α and the GST-PKR-kinase domain (KD). GST-PKR-KD has been previously used to characterize small molecule inhibitors of PKR [27] and is capable of phosphorylating His₆-eIF2 α *in vitro* (Fig. 5A, compare lanes 4 and 5 to 1-3). PKR_i and isohymenialdisine (**3**) inhibited eIF2 α phosphorylation *in vitro* (Fig. 5A, compare lanes 6-9 to 5). To extend these results and demonstrate a direct effect of isohymenialdisine (**3**) on PKR kinase activity, we incubated GST-PKR-KD with [γ -³²P]-ATP and scored for cross-phosphorylation of PKR. In this scenario, the heterologous GST dimerization domain functionally substitutes for double stranded RNA and enables dimerization and activation of the kinase domain [46]. We found that PKR_i and isohymenialdisine (**3**) inhibited autophosphorylation of PKR-KD (Fig. 5B, compare lanes 4-7 to lanes 2 and 3) consistent with isohymenialdisine being able to directly inhibit PKR activation.

Structure Modeling Predicts Binding of Hymenialdisine and Isohymenialdisine to the PKR ATP pocket

To obtain insight into the interactions between hymenialdisine and isohymenialdisine with PKR, we mapped the kinase domain of PKR for druggable 'hot spots' [36] and docked the two compounds to PKR based on the mapping results [38]. The PKR structure was derived from the PKR kinase domain-eIF2 α -AMP-PNP complex by stripping the eIF2 α and AMP-PNP domains [47]. We found that the main hot spot (i.e., the energetically most important region of the binding site, shown as cyan lines) is in a deep crevice within the ATP site (Fig. 5C). The bound AMP-PNP is superimposed (shown as yellow sticks) for reference and highlights that the main hot spot overlaps with the adenylate ester moiety (see expanded view on 5C). A top-down view of the main hot spot revealed three probe cluster sites: (i) a large consensus site CS1 (cyan, 28 probe clusters), (ii) consensus site CS4 (blue, 7 probe clusters) and (iii) consensus site CS8 (green, 3 probe clusters) (Fig. 5D). Superimposition of

AMP-PNP onto the probe cluster sites showed that the ligand overlaps with the central ring in the major hot spot CS1, but does not occupy two hot spot regions that expand from the ring (Fig. 5E). The best docked pose of hymenialdisine fits better into these additional hot spots (Fig. 5F), in spite of the fact that we mapped the PKR structure from its complex with AMP-PNP. It was shown previously that better fits into hot spots implies better ligand efficiency and higher affinity [48]. In addition, hymenialdisine forms five hydrogen bonds with PKR. Three hydrogen bonds are on the pyrroloazepine double ring, between the N1 atom of the pyrrole ring and the carbonyl oxygen of C369, between the O1 carbonyl oxygen of the azepine ring and the backbone NH group of C369, and between the N2 amide of the azepine ring and the carbonyl oxygen of E367 (Fig. 5F). The other two hydrogen bonds are on the guanidine ring system, between the N5 amino group and the carbonyl oxygen of S418, and between the O2 of the guanidine ring and the side chain of K296 (Fig. 5F). We note that the same atoms of hymenialdisine form hydrogen bonds with CDK2, with the only difference that O2 of the guanidine ring participates in a water-mediated interaction. In contrast, the original ligand in the X-ray structure, AMP-PNP, forms only two hydrogen bonds with PKR (Fig. 5E). The bromine atom of hymenialdisine is predicted to interact with the amide CO group of the Q376 side chain (Fig. 5F). Such halogen bonds are known to be favorable [49], which may explain why debromohymenialdisine is a weaker binder than hymenialdisine. Isohymenialdisine also fits into the binding hot spot very well. Four of the hydrogen bonds are the same as for hymenialdisine (Fig. 5G). Rather than directly interacting, the N5 amino group of the guanidine ring and the carbonyl oxygen of S418 appear to form a water-mediated hydrogen bond, which is not modeled in our calculations and hence not shown in Fig. 5G. The bromine atom is moved into a hydrophobic environment and forms an interaction with the side chain of M366, which is expected to be more favorable than its interaction with the amide CO of Q376. This model data offers a potential mechanism by which isohymenialdisine and hymenialdisine inhibit PKR function.

DISCUSSION

Hymenialdisine has been characterized as an inhibitor of cyclin dependent kinase 1 (CDK1; $IC_{50} = 22$ nM and CDK2; $IC_{50} = 40$ nM), glycogen synthase kinase 3b ($IC_{50} = 10$ nM), and casein kinase 1 ($IC_{50} = 35$ nM) and has been shown to function as a competitive inhibitor of ATP [50]. It is also a potent inhibitor of NF- κ B in U937 cells [51] and of mitogen-activated protein kinase kinase-1 (MEK-1) [52]. Previous studies using affinity chromatography approaches [53] and computation approaches [54] identified a number of additional kinase targets, although not PKR. We find that hymenialdisine also inhibits eIF2 α phosphorylation (Fig. 4G, compare lane 3 to 1 of p-eIF2 α blot). Although little has been reported in the literature on isohymenialdisine, it appears to be more potent at stimulating translation (Figs. 1, 2, and 4) and inhibiting eIF2 α phosphorylation than hymenialdisine (Fig. 4G, compare lane 10 to 8). Our modeling data is consistent with the ability of isohymenialdisine (Fig. 5G) and hymenialdisine (Fig. 5F) to bind to the ATP binding pocket of PKR.

In vitro translation extracts are powerful systems that have been useful in dissecting and characterizing various steps of protein synthesis and in generating proteins for functional studies. Mammalian extracts prepared from RRL, HeLa cells, or Krebs-2 cells have been extensively employed to delineate mRNA structural features required for efficient recruitment of 43S preinitiation complexes to mRNA templates [55]. One major mechanism that limits the translation rates of current mammalian extracts is phosphorylation of eIF2 α [56]. Indeed, heme is typically supplemented to RRL to prevent heme-regulated inhibitor (HRI) from phosphorylating eIF2 α and rapidly inactivating translation initiation [57]. More recently, phosphorylation of eIF2 α in cell-free systems has been characterized by Mikami et al. [58] who attributed eIF2 α phosphorylation to PERK, PKR, and GCN2, and showed that the induced translation inhibition could be overcome by addition of GADD34 (which

stimulated eIF2 α dephosphorylation) or the vaccinia virus K3L eIF2 α pseudosubstrate [58]. As well, extracts prepared from murine embryonic fibroblasts harboring an eIF2 α homozygous knock-in mutation (Ser51 to Ala change that no longer is a substrate for the eIF2 α kinases) have a translational efficiency that is 30-fold higher than extracts made from wild-type cells. These studies document that a current limitation of *in vitro* mammalian translation extracts occurs when eIF2 α phosphorylated.

In the experiments reported herein, we suspected that our *in vitro* transcribed mRNA was responsible for *in vitro* phosphorylation of eIF2 α – an effect that could be blocked by a small molecule inhibitor of PKR (Fig. 4G). This phenomenon has also been reported by Gunnery et al. [45]. Because of this, we were fortunate to detect a translation stimulatory activity in *Stylyssa massa* extracts and could attribute this to isohymenialdisine (and to a lesser extent, hymenialdisine). Consistent with isohymenialdisine blocking eIF2 α phosphorylation is the lack of stimulation by this compound on the CrPV IRES (Fig. 2C) which bypasses the requirement for eIF2 during the initiation process [42]. PKR can also be activated in an RNA-independent manner by heterodimerizing with PACT (Protein Activator of the Interferon-induced Protein Kinase R) [59]. Although we cannot formally exclude an effect of isohymenialdisine on this interaction our *in vitro* kinase studies with recombinant PKR indicates a direct effect on PKR activity (Fig. 5B).

There is much interest in targeting the translation process and consequently, robust *in vitro* functional assays have been developed for high throughput screens in search for inhibitors of cap-dependent translation and HCV IRES-mediated translation [5; 60]. Such assays rely on programming extracts with exogenous mRNA, which our results have shown can result in unwanted activation of PKR. This could be prevented by supplementing the lysates with a PKR inhibitor, more rigorous purification of the mRNA template, or use of extracts prepared from eIF2 α ^{S51A} cells, making the assays more sensitive by improving the signal/background ratio. This would also avoid the time consuming pursuit of compounds that affect translation by modulating eIF2 α phosphorylation.

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Figure 1.

Effects of Hymenialdisine, Debromohymenialdisine, Isohymenialdisine on translation reactions in rabbit reticulocyte lysates (RRL). **(A)** Chemical structures of Hymenialdisine (1), Debromohymenialdisine (2), and Isohymenialdisine (3). **(B)** Schematic diagram of pKS/FF/EMC/Ren. The plasmid was linearized with BamHI and used in *in vitro* transcriptions to generate mRNA. **(C)** Representative autoradiogram demonstrating the effects of Cmpds 1 - 3 on translation of FF/EMC/Ren mRNA. RRL was programmed with FF/EMC/Ren mRNA (24 ng/ul) in the presence of [³⁵S]-methionine and the indicated amounts of compound. Following incubation at 30°C for 1 hr, samples were fractionated by electrophoreses in 10% SDS-polyacrylamide gels. Gels were treated with EN³HANCE, dried and exposed to Blue Ray film (VWR). The position of migration of firefly (FF) and renilla (Ren) luciferase is denoted. **(D)** Hymenialdisine and Isohymenialdisine stimulate production of firefly and renilla luciferase from FF/EMC/Ren mRNA. Summary of translations performed in RRL. The relative luciferase values are set relative to vehicle (0.25% DMSO) controls (which were set at 1). On average, the firefly and renilla luciferase values in the vehicle controls were ~400,000 and 1,500,000 RLU. n=3; Bars denote the error of the mean.

Figure 2.

Effects of Hymenialdisine, Dehymenialdisine, and Isohymenialdisine on translation from the HCV and CrPV IRESes in RRL. **(A)** Schematic diagram of bicistronic reporters harboring the HCV or CrPV IRESes. Each plasmid was linearized with BamHI for *in vitro* transcription reactions. **(B)** Effects of Cmpds **1** – **3** on translation from FF/HCV/Ren mRNA in RRL. *Top*: Representative autoradiogram demonstrating the relative translation efficiencies of FF/HCV/Ren mRNA. Following *in vitro* translations, samples were fractionated in 10% SDS-polyacrylamide gels. Gels were treated with EN³HANCE, dried and exposed to Blue Ray film (VWR). The position of migration of FF and Ren luciferase is denoted by arrows to the right. *Bottom*: Quantitation of effects of Cmpds **1** – **3** on translation from FF/HCV/Ren mRNA in RRL. Luciferase activities were normalized to the activity obtained in vehicle (0.25% DMSO) controls. On average, the firefly and renilla luciferase values in the vehicle controls were ~2,000,000 (FF) and ~150,000 (Ren) RLU for FF/HCV/Ren. n=3; Bars denote the error of the mean. **(C)** Effects of Cmpds **1** – **3** on translation from Ren/CrPV/FF mRNA in RRL. *Top*: Representative autorad demonstrating the relative translation efficiencies of Ren/CrPV/FF mRNA. Following *in vitro* translations, samples were fractionated in 10% SDS-polyacrylamide gels. Gels were treated with EN³HANCE, dried and exposed to Blue Ray film (VWR). The position of migration of FF and Ren luciferase is denoted by arrows to the right. *Bottom*: Quantitation of effects of Cmpds **1** – **3** on translation from Ren/CrPV/FF mRNA in RRL. Luciferase activities were normalized to the activity obtained in vehicle (0.25% DMSO) controls. On average, the luciferase values in the vehicle controls were ~200,000 (Ren) and ~800,000 (FF) RLU for Ren/CrPV/FF. n=3; Bars denote the error of the mean.

Figure 3.

Effects of Cmpds **1 - 3** on translation in Krebs and *E. coli* S30 extracts. **(A)** Effect of compounds on translation in Krebs extracts programmed with FF/EMC/Ren mRNA (24 ng/ul). Luciferase values were normalized to vehicle (0.25% DMSO) controls. On average, the firefly and renilla luciferase values in the vehicle controls were ~70,000 and ~2,500,000 RLUs. n=3; Bars denote the error of the mean. **(B)** Effect of compounds (50 μ M) on coupled transcription-translation reactions in *E. coli* S30 extracts. Extracts were programmed with pBESTluc DNA (50 ng/ul) and the obtained firefly luciferase activity normalized to vehicle (0.25% DMSO) controls. On average, the firefly luciferase values in the vehicle controls were ~500,000 RLUs. n=3; Bars denote the error of the mean.

Figure 4.

Effect of adding Compds **1 - 3** to actively translating extracts. **(A)** Schematic diagram of T3LucpA+. The template was linearized with BamHI and used for *in vitro* transcription reactions. **(B-C)** Translations in RRL were initiated by addition of T3LucpA+ mRNA (4 ng/ul). Aliquots for luciferase activity were removed at various time points. Compounds were added at the times indicated by a downward arrow (15 min). Hymenialdisine and derivatives were tested at final concentration of 25 μ M. DMSO was present at 0.25%. **(D)** Western blot documenting eIF2 α phosphorylation status from RRL lacking or containing exogenously supplied FF/HCV/Ren mRNA (4 ng/ul). Samples for analysis were removed at the indicated times and probed with antibodies directed to the proteins shown to the right of the blots. All probings were performed sequentially on the membrane. **(E-F)** Inhibition of PKR prolongs translational output in RRL. Translation extracts were pre-incubated with the indicated compounds (5 μ M) for 20 min, at which point translations were initiated by the addition of FF/HCV/Ren mRNA (4 ng/ul). Aliquots were taken at the indicated times and cap-dependent firefly luciferase activity measured. **(G)** Western blot illustrating phosphorylation status of eIF2 α from RRL programmed with FF/HCV/Ren mRNA (4 ng/ul) in the presence of the indicated compounds at 5 μ M. Samples were taken 5 and 90 mins after the start of translation. All probings were performed sequentially on the membrane.

Figure 5.

Inhibitory effect of Isohymenialdisine on GST-PKR-KD-mediated eIF2α phosphorylation and GST-PKR-KD autophosphorylation. **(A)** Western blot probing the phosphorylation status of recombinant eIF2α following pre-incubation with GST-PKR-KD and the indicated small molecules. All probeds were performed sequentially on the membrane. **(B)** Autoradiogram demonstrating the autophosphorylation activity of GST-PKR-KD in the presence of the indicated small molecules. Kinase reactions were performed as described in the Materials and Methods. **(C)** Location of the largest hot spot of PKR. Results are shown for the PKR structure bound to eIF2α•AMP-PNP (PDB code 2A19). Expanded view shows the position of the probe cluster site (cyan lines) relative to AMP-PNP (shown as yellow sticks). **(D)** Detailed top-down view of the three consensus probe clusters that define the major binding hot spot (CS1 cyan, CS4 blue, and CS8 green). PKR side chains close to the hot spot are also shown (red – oxygen, blue – nitrogen). **(E)** Overlay of AMP-PNP (shown in stick representation, carbon atoms colored yellow) with the probe clusters. Hydrogen bonds are shown as black dashed lines. **(F)** Overlay of hymenialdisine (in stick representation, carbon and polar hydrogen atoms shown in white) with the probe clusters, showing the five hydrogen bonds formed. **(G)** Overlay of isohymenialdisine with the probe clusters.