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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 led to the insight that mRNA used to program the translation extracts during high-throughput assay setup was leading to phosphorylation of eIF2α, a potent negative regulatory event that is mediated by one of four kinases. We identified double-stranded RNA-dependent protein kinase (PKR) as the eIF2α 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–eIF2α phosphorylation axis, and can be modeled into the PKR ATP binding site. Our results identify a source of "false positives" for high-throughput screen campaigns using translation extracts, raising a cautionary note for this type of screen.
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High-throughput screens (HTSs)[1] 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 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 affecting 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 probed extensively using translation-competent extracts in several HTS assays, with interests stemming from the desire to identify novel antimicrobial drugs [1,2], block expression of specific mRNA transcripts [3,4], identify compounds that target initiation and show antineoplastic activity [5–8], and correct read-through nonsense mutations as a means of 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 used extensively [2,5,9]. Each system has its peculiarities, with reticulocyte lysates being the more efficient extract and HeLa and Krebs 2 cell lysates being more cap dependent and faithfully recapitulating 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

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[1] *Abbreviations used:* HTS, high-throughput screen; RNA_i, RNA interference; eEF, eukaryotic elongation factor; eIF, eukaryotic initiation factor; PIC, pre-initiation complex; GCN2, general control nondepressible 2; PERK, PKR-like endoplasmic reticulum kinase; HRI, heme-regulated inhibitor; PKR, double-stranded RNA-dependent protein kinase; KD, kinase domain; dsRNA, double-stranded RNA; CHX, cycloheximide; HHT, homoharringtonine; PKRi, C16; RRL, rabbit reticulocyte lysate; Luc, luciferase; FF, firefly; Ren, renilla; SDS, sodium dodecyl sulfate; EMC, encephalomyocarditis; IRES, internal ribosome entry site; HCV, hepatitis C virus; CrPV, cricket paralysis virus; CDK, cyclin-dependent kinase.

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the vicinity of the 5′ cap structure; (iii) and regulate the phosphorylation status of eIF2α [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 (PICs) along with other eIFs. Following recruitment of the PICs to mRNA templates, the 43S complex scans the 5′-untranslated regions (UTRs) of mRNA templates where, on 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 eIF2α is phosphorylated [16–18]. eIF2 is a trimeric complex consisting of α, β, and γ subunits, and phosphorylation of the α subunit at Ser51 increases the affinity of eIF2·GDP for eIF2B, leading to sequestration of eIF2·GDP–eIF2B complexes. Because eIF2 is present in excess of eIF2B, small changes in the phosphorylation status of eIF2 lead to a significant inhibitory effect on global protein synthesis [19,20].

In mammals, four eIF2α kinases have been identified and characterized extensively: general control nondepressible 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 (KD) but have distinct regulatory domains that respond to different stress signals; GCN2 senses amino acid deprivation and ultraviolet (UV) irradiation [21,22], PERK is active in response to accumulation of misfolded proteins in the endoplasmic reticulum [23], HRI responds to heme deficiency, oxidative stress, and heat shock in erythroid tissues [24], and PKR is activated by double-stranded RNA (dsRNA) [25,26]. PKR is a serine/threonine kinase that phosphorylates eIF2α on binding to dsRNA of viral, synthetic, or cellular origin. Binding to dsRNA promotes dimerization of PKR and enables autophosphorylation and activation, which in turn inhibits translation via eIF2α 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 HTSs 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. [^{35}S]Methionine (>1000 Ci/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were purchased from PerkinElmer. Cycloheximide (CHX, Bishop) was resuspended in H_2O, whereas homoharringtonine (HHT, Sigma–Aldrich), C16 (PKRi, Calbiochem) [27], SyK inhibitor (GCN2i, Calbiochem) [28], GSK PERKi (Toronto Research Chemicals) [29,30], and quercetin (HRIi, Sigma–Aldrich) [31,32] were resuspended in 100% dimethyl sulfoxide (DMSO). Antibodies were as follows: anti-phospho-eIF2α (cat. no. 3597), anti-eIF2α (cat. no. 9722), and anti-eEF2 (cat. no. 2332) were obtained 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 *Stylissa massa* sponge sample was collected under contract with Pfizer Research Laboratories (St. Louis, MO, USA). When Pfizer disbanded its natural products program, it generously donated its marine invertebrate collection to one of the current authors (R.J.A.). 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 (Leiden, The Netherlands).

Freshly collected sponge was frozen on-site and transported frozen. Lyophilized sponge (5 g) was cut into small pieces and immersed in and subsequently extracted repeatedly with MeOH (3 × 50 ml) at room temperature. The combined methanolic extracts were concentrated in vacuo, and the resultant extract was then partitioned between EtOAc (3 × 5 ml) and H_2O (15 ml). The resulting aqueous extract was then extracted with *n*-BuOH (3 × 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 C_{18} reversed-phase high-performance liquid chromatography (HPLC) using a CSC-Inertsil 150A/ODS2 column (5 μm, 25 × 0.94 cm) with 9:1 (0.05% trifluoroacetic acid [TFA]/H_2O)/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 one- and two-dimensional NMR spectroscopic and HRE SIMS analyses.

In vitro transcription and translation reactions

In vitro transcriptions were performed as described previously [5,34]. Plasmids pKS/FF/EMC/Ren, pSP/$(CAG)_{33}$/FF/HCV/Ren, pGL3/Ren/CrPV/FF, and T3LucpA+ were linearized with *Bam*HI and transcribed in vitro to generate mRNA transcripts. In vitro translations using [^{35}S]methionine were performed in rabbit reticulocyte lysate (RRL) as recommended by the manufacturer (Promega). For luciferase (Luc) assays, firefly (FF) and renilla (Ren) luciferase activities were measured on a Berthold Lumat LB9507 luminometer. For gel analysis, translation products were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels that were subsequently treated with EN^{3}HANCE, dried, and exposed to Blue Ray film (VWR). In vitro translations were performed in Krebs extracts as described previously [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/eIF2α were transformed into *E. coli* BL21(DE3)/pLysS. GST–PKR–KD and His_6–eIF2α were expressed and purified as described previously [27,35]. The eIF2α kinase assay and PKR autophosphorylation assay were performed as reported with slight modifications [27,35]. For eIF2α kinase assays, 0.5 μg of GST–PKR–KD and His_6–eIF2α was incubated with 50 μM ATP in kinase buffer (20 mM Tris–HCl [pH 7.6], 10 mM KCl, 10 mM $MgCl_2$, and 10% glycerol) at 30 °C for 30 min. Phosphorylation of eIF2α was assessed by Western blotting. For PKR autophosphorylation assays, 0.5 μg of GST–PKR–KD was incubated with 50 μM ATP in kinase buffer at 30 °C for 30 min. The reaction was quenched with 3 × SDS–PAGE (polyacrylamide gel electrophoresis) loading buffer, boiled, and resolved in 10% SDS–polyacrylamide gels. The gel was dried and exposed to Blue Ray film (VWR).

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