Inhibition of translation by cytotrienin A—a member of the ansamycin family

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ABSTRACT

The ansamycins are a diverse and often physiologically active group of compounds that include geldanamycin and rifamycin, inhibitors of heat shock protein 90 and prokaryotic DNA-dependent RNA synthesis, respectively. Cytotrienin A is an ansamycin-type small molecule with potent antiproliferative and proapoptotic properties. Here, we report that this compound inhibits eukaryotic protein synthesis by targeting translation elongation and interfering with eukaryotic elongation factor 1A function. We also find that cytotrienin A prevents HUVEC tube formation and diminishes microvessel formation in the chorioallantoic membrane assay. These results provide a molecular understanding into cytotrienin A's previously reported properties as an anticancer apoptosis-inducing drug.

Keywords: cytotrienin A; eEF1A; translation inhibitor; protein synthesis

INTRODUCTION

There is much interest in identifying and characterizing novel inhibitors of eukaryotic protein synthesis, both as tools to characterize the translation machinery and as drugs that can curtail malignant cell proliferation (Pelletier and Peltz 2007). There are several observations that suggest a high therapeutic index can be achieved by inhibiting translation in cancers. One vulnerability of cancer cells is at the level of ribosome recruitment, where mRNAs must compete with each other for limiting amounts of translation initiation factors (Duncan et al. 1987). Translation of mRNAs that are “weak” competitors for eukaryotic initiation factors (eIFs) are therefore more sensitive to small changes in the levels of these factors. Since several of the “weak” mRNAs characterized to date encode for antiapoptotic or prosurvival factors, their selective down-regulation prefer-

entially curtails growth of tumor cells in preclinical cancer models (Graff et al. 2007; Cencic et al. 2009; Lucas et al. 2009). In addition, translation initiation inhibitors have been shown to exert antiangiogenic activities, a property that may contribute to their anti-cancer activity (Graff et al. 2007; Cencic et al. 2009). Additionally, the rapid reduction in levels of pro-oncogenic and pro-survival proteins having short half-lives (Chao et al. 1998; Nijhawan et al. 2003) occurs upon translation inhibition, and this can impair the growth of transformed cells. Higher translation rates also occur in human tumors and appear to be required to maintain their oncogenic state (Heys et al. 1991; Wendel et al. 2004). These latter two points may explain why some inhibitors of translation elongation show efficacy in preclinical cancer models as well as in the clinic (Quintas-Cardama et al. 2009; Robert et al. 2009).

The first step of translation elongation is catalyzed by eukaryotic elongation factor (eEF) 1A, which delivers the aminoacyl-tRNA (aa-tRNA) to the ribosomal A site, followed by GTP hydrolysis (provided that the proper codon–anticodon interaction occurs). There are two isoforms of eEF1A, eEF1A1 and eEF1A2, which are encoded by separate genes and show 95% identity. Both isoforms are thought to

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be functionally redundant for translation, although they are differentially expressed (Kahns et al. 1998) and both have been shown capable of acting as oncogenes in the appropriate setting (Thornton et al. 2003).

Here, we describe the characterization of a novel modulator of eEF1A from the ansamycin family. Cytotrienin A (Cyt A) is a natural product produced by *Streptomyces* sp., which has been previously reported to induce apoptosis in leukemia cell lines by activating c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and p36 myelin basic protein (MBP) kinase (Takeya et al. 1998; Watabe et al. 2000). Here, we report that Cyt A inhibits translation elongation by interfering with eEF1A function. Our results provide molecular insight into Cyt A's previously reported properties as an anti-cancer compound.

RESULTS

Cytotrienin A inhibits translation elongation

During the course of a high-throughput screen to identify translation inhibitors (Novac et al. 2004), Cyt A (Fig. 1A) was identified as a "hit" that inhibited both cap-dependent (Firefly [FF] luciferase) and hepatitis C virus (HCV)-driven (*Renilla* [Ren] luciferase) translation in Krebs-2 extracts

FIGURE 1. Cyt A inhibits eukaryotic translation. (A) Chemical structure of Cyt A. (B) Cyt A inhibits both cap-dependent and HCV IRES-driven translation in Krebs-2 extracts. In vitro translations were performed in the presence of [35 S]methionine and programmed with FF/HCV/Ren. DMSO, anisomycin (aniso), or Cyt A (lanes 3–9) were added to Krebs-2 extracts at the indicated concentrations. Proteins were separated by SDS-PAGE and visualized by autoradiography. The arrow and arrowhead denote Firefly and *Renilla* luciferase, respectively. (C) Luciferase activity from translations performed in Krebs-2 extracts programmed with FF/HCV/Ren shown in B. Light units were set relative to the values obtained in the presence of vehicle (DMSO). The average of three measurements is shown with the SEM represented by error bars.

(Fig. 1B,C) and in rabbit reticulocyte lysate (RRL) (data not shown). Cyt A was also active in wheat-germ extracts, but did not significantly inhibit prokaryotic translation in *E. coli* S30 extracts at 50 μM (data not shown). These results indicate that Cyt A inhibits both cap-dependent and IRES-dependent translation. To determine whether the initiation phase of translation was affected by Cyt A, we performed ribosome binding experiments to assess the effects of Cyt A on 80S complex formation (Fig. 2A). Cyt A was able to stabilize 80S complexes to a similar degree as cycloheximide (CHX) (Fig. 2A, left and right panels, respectively). As well, addition of the initiation inhibitor hippuristanol, followed by addition of Cyt A to the binding reactions, caused a decrease in 80S complex formation (Fig. 2A, left), similar to if Hipp alone was present in the binding reactions (Fig. 2A, right). However, if Cyt A was present in the extract prior to the addition of Hipp, 80S complexes were trapped to the same efficiency as observed for Cyt A (Fig. 2A, left). Consistent with these results, Cyt A inhibited the translation of poly(Phe) from poly(U) RNA (Fig. 2B). Taken together, these experiments strongly suggest that Cyt A targets translation elongation.

The elongation inhibitors homoharringtonine (HHT) and bruceantin (Bru) inhibit only newly initiated ribosomes during the first step of elongation and allow translating ribosomes to run-off mRNA templates (Pelletier and Peltz 2007; Robert et al. 2009). To determine whether Cyt A showed similar properties, we performed in vitro translation reactions in the presence of [35 S]methionine, where compound was added 5 min after the start of translation (Fig. 2C). A kinetic analysis was performed to quantitate the amount of product synthesized. Inhibition of translation by HHT is delayed by several minutes following its addition to a translating extract as polysomes run-off mRNA templates due to the reduced affinity of HHT for actively translating ribosomes (Fig. 2C; Chan et al. 2004). Addition of Cyt A immediately inhibited protein synthesis in a manner similar to CHX. These results indicate that Cyt A affects translating ribosomes and does not allow polysome run-off.

Cyt A modulates eEF1A-dependent aa-tRNA binding to the ribosome

To better understand the mechanism by which Cyt A inhibits elongation, we analyzed its effects on tRNA binding to the ribosome, peptide bond formation, and translocation. We first tested whether Cyt A could inhibit the peptidyl transferase activity of the ribosome by monitoring the formation of [35 S]methionyl-puromycin. Cyt A did not inhibit peptidyl transferase activity under these conditions, unlike the known peptidyl transferase inhibitor HHT (Fig. 3A).

We next assessed whether Cyt A could affect binding of aa-tRNA to ribosomes in eEF1A-independent [high poly(U)

FIGURE 2. Cyt A inhibits translation elongation. (A) Cyt A does not inhibit translation initiation. Ribosome bindings were performed in RRL using ^{32}P -labeled CAT RNA. Reactions were separated by centrifugation on 10%–30% glycerol gradients and fractions quantitated by scintillation counting. (Left) Ribosome bindings were performed in the presence of 50 μM Cyt A alone, preincubated with 50 μM hippuristanol (Hipp), followed by addition of 50 μM Cyt A, or preincubated with 50 μM Cyt A, followed by the addition of 50 μM Hipp. (Right) Ribosome bindings were performed in the presence of 0.6 mM CHX or 50 μM Hipp. Both panels are part of the same experiment, but were separated for clarity. (B) Cyt A inhibits translation elongation. In vitro translations in RRL supplemented with ^3H phenylalanine and programmed with poly(U) RNA. Polypeptides were TCA precipitated and quantitated by scintillation counting. Counts were set relative to DMSO levels. The average of four measurements is shown with the SEM. (C) Cyt A does not permit ribosome run-off. In vitro translation reactions of Krebs-2 extracts were allowed to proceed in the absence of compound for 5 min, after which time DMSO, HHT (200 μM), CHX (50 μM), or Cyt A (20 μM) were added. Aliquots were taken at the indicated times, TCA precipitated, and quantitated by scintillation counting. The average of three measurements is shown with the SEM. The downward arrow indicates the point of addition of compound or vehicle.

RNA concentration] or eEF1A-dependent [low poly(U) RNA concentration] reconstituted systems. The ability of ^{14}C Phe-tRNA^{Phe} to bind ribosomes was not affected by Cyt A when binding was eEF1A independent (Fig. 3B), indicating that Cyt A does not compete with ^{14}C Phe-tRNA^{Phe} for the ribosome. Under eEF1A-dependent conditions, the levels of ribosome-bound ^{14}C Phe-tRNA^{Phe} in the presence of GDP or GMPPNP were similar to those binding reactions lacking eEF1A in DMSO controls (Fig. 3C). [Also note that tRNA binding without eEF1A in this experiment is much lower than in the experiment presented in Fig. 3B, due to a 1000-fold decrease in poly(U) RNA template.] In the presence of GTP, the amount of ^{14}C Phe-tRNA^{Phe} bound to ribosomes

increased significantly (Fig. 3C). Under this condition, both Cyt A and CHX decreased ^{14}C Phe-tRNA^{Phe} binding by $\sim 40\%$, while Did B had no significant effect. The low amount of ^{14}C Phe-tRNA^{Phe} binding to ribosomes observed in the presence of GMPPNP was increased when either Cyt A or Did B was present in the reactions (Fig. 3C). One interpretation of this result is that Cyt A stabilizes the ternary complex on the ribosome (see Discussion).

eEF2-dependent translocation is inhibited by Cyt A only when aa-tRNA is delivered in an eEF1A-dependent manner

The ability of Cyt A to affect eEF2-dependent translocation was also investigated. After either nonenzymatic (as in Fig. 3B) or eEF1A-dependent aa-tRNA binding to the ribosome (with GTP, as in Fig. 3C), translocation was initiated by the addition of puromycin and eEF2. Under these conditions, CHX inhibited translocation regardless of whether ^{14}C Phe-tRNA^{Phe} binding was eEF1A dependent or eEF1A independent, whereas Cyt A inhibited translocation only when charged tRNA was loaded in an eEF1A-dependent manner (Fig. 3D). Did B served as a positive control in the eEF1A-dependent translocation assay and was found to inhibit this reaction (Fig. 3D).

Cyt A inhibits neither ternary complex formation nor the GTPase activity of eEF1A

The inhibitory effect in the presence of GTP and stimulatory effect in the presence of GMPPNP of Cyt A on tRNA binding could result from improper ternary complex formation (eEF1A:GTP:aa-tRNA). To determine whether Cyt A affects the ability of eEF1A to bind to GTP, we performed a UV cross-linking experiment with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in the presence or absence of Phe-tRNA^{Phe} (Fig. 4A). We observed no significant change in the efficiency of GTP cross-linking to eEF1A in the presence of Cyt A (Fig. 4A, cf. lanes 2 and 5 with 1 and 4, respectively). Excess GTP competed for the radiolabeled $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in this assay (cf. lane 3 and 6 with 1 and 4, respectively). As well, Cyt A did not prevent eEF1A: ^{14}C Phe-tRNA^{Phe} complex formation, as assessed by electrophoretic mobility shift assay (EMSA) (Fig. 4B). We investigated

FIGURE 3. The effect of Cyt A on the steps of translation elongation. (A) Cyt A does not inhibit peptidyl transferase activity. $[^{35}\text{S}]\text{Methionine-puromycin}$ formation was monitored in the presence of purified 40S and 60S ribosomes using $[^{35}\text{S}]\text{Met-tRNA}_i$ and ribosomal high-salt wash from RRL. (Left) Aliquots of samples were taken at the indicated time points and separated by TLC. The position of migration of $[^{35}\text{S}]\text{Met-puro}$, $[^{35}\text{S}]\text{Met}$, and $[^{35}\text{S}]\text{Met-tRNA}_i$ is indicated to the left. The addition of 50 μM Cyt A, 40 μM HHT, or the absence of puromycin (–Puro) or ribosomes (–Rib) is indicated at top. (Right) Quantitation of $[^{35}\text{S}]\text{Met-puro}$ production. The average of four experiments relative to the DMSO control at 30 min is shown. Note that values obtained from the reaction in the absence of puromycin were subtracted as background. The SEM is represented using error bars. (B) Cyt A does not inhibit eEF1A-independent $[^{14}\text{C}]\text{Phe-tRNA}^{\text{Phe}}$ binding to 80S ribosomes. Filter binding of $[^{14}\text{C}]\text{Phe-tRNA}^{\text{Phe}}$ was performed with purified 80S ribosomes, 0.4 mg/mL poly(U) RNA, and either DMSO, 50 μM Cyt A, or 50 μM CHX. The average of four experiments is shown with the SEM indicated by error bars. (C) Cyt A modulates eEF1A-dependent $[^{14}\text{C}]\text{Phe-tRNA}^{\text{Phe}}$ binding to 80S ribosomes. Filter binding of $[^{14}\text{C}]\text{Phe-tRNA}^{\text{Phe}}$ with purified 80S ribosomes, and 0.4 $\mu\text{g/mL}$ poly(U) RNA in the presence of either DMSO, 50 μM Cyt A, 50 μM DidB, or 50 μM CHX. The presence of eEF1A and nucleotide is indicated. The average of three to six measurements is shown with SEM indicated by error bars. (D) eEF2-dependent translocation of $[^{14}\text{C}]\text{Phe-tRNA}^{\text{Phe}}$ is inhibited by Cyt A only when aminoacyl-tRNA is loaded in an eEF1A-dependent manner. Following nonenzymatic or eEF1A-dependent tRNA binding (as described in B and C with GTP, respectively), eEF2 was added to the reaction with puromycin. The amount of puromycin-active $[^{14}\text{C}]\text{Phe-tRNA}^{\text{Phe}}$ was extracted with ethyl acetate and quantitated by scintillation counting. tRNA already bound to the P-site was subtracted from these values (see Materials and Methods) and set relative to the DMSO control. The average of two to four experiments is shown with the SD.

whether Cyt A affects the GTPase activity of eEF1A and found no evidence to this effect (Fig. 4C). We conclude that Cyt A does not interfere with ternary complex formation.

Cellular protein synthesis is inhibited by Cyt A

$[^{35}\text{S}]\text{Methionine/cysteine}$ labeling of HeLa cells was inhibited by Cyt A, whereas DNA and RNA synthesis was not dramatically affected (Fig. 5A). Inhibition of translation was reversible and showed almost complete recovery by 6 h after removal of the compound (Fig. 5B). The polysome profile of cells exposed to Cyt A for 1 h showed a similar to slight increase in polysomes compared with those isolated from cells exposed to vehicle (DMSO) (Fig. 5C). When hippuristanol was added during the last 30 min of Cyt A treatment, polysomes were still present, unlike what was observed when cells were exposed to only hippuristanol

(Fig. 5C, left). Cells treated with HHT, which is known to allow ribosome run-off, showed an absence of polysomes (Fig. 5C, right). This data is consistent with Cyt A causing stalling of translating ribosomes and allowing their accumulation on mRNA templates.

Antiangiogenic properties of Cyt A

Inhibition of translation has been shown to impair angiogenesis and has been suggested as a mechanism by which they function as anti-cancer therapeutics (Taraboletti et al. 2004; Graff et al. 2007; Cencic et al. 2009). We therefore tested whether Cyt A might have similar properties. To examine this, we utilized a HUVEC tube formation assay, which has been previously used to mimic some aspects of angiogenesis (Kubota et al. 1988; Graff et al. 2007; Cencic et al. 2009). The inhibition of tube formation with Cyt A was dose dependent (Fig. 6A,B) at concentrations where

FIGURE 4. Cyt A does not affect ternary formation. (A) Cyt A does not inhibit GTP binding to eEF1A. Purified eEF1A (1 μg) was UV cross-linked to $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in the presence (lanes 4–7) or absence (lanes 1–3) of Phe-tRNA^{Phe} and 50 μM Cyt A or 1 mM unlabeled GTP. Reactions were treated with RNase A, separated by SDS-PAGE, and visualized by autoradiography. (B) Cyt A does not affect $[\text{C}^{14}]\text{Phe-tRNA}^{\text{Phe}}$ binding to eEF1A. Increasing amounts of eEF1A were incubated with $[\text{C}^{14}]\text{Phe-tRNA}^{\text{Phe}}$ in the presence of DMSO, 50 μM Cyt A, or unlabeled Phe-tRNA^{Phe} competitor. EMSAs were performed on 6% polyacrylamide gels and visualized by autoradiography. The position of migration of free $[\text{C}^{14}]\text{Phe-tRNA}^{\text{Phe}}$ and complexes are indicated to the left. (C) Cyt A does not affect the GTPase activity of eEF1A. eEF1A and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ were incubated with 40S and 60S ribosomes and Phe-tRNA^{Phe} in the presence of 50 μM Cyt A or DMSO. GTPase activity was also measured in the absence of eEF1A or without ribosomes or Phe-tRNA^{Phe}. The average of three to four measurements is shown with the SEM represented as error bars.

general translation was inhibited by >90%, (Fig. 6C, open circles) similar to effects observed with silvestrol (Silv), a previously reported translation initiation inhibitor with antiangiogenic properties (Fig. 6A; data not shown) (Cencic et al. 2009). Importantly, cells remained viable under these conditions (Fig. 6C, squares). We also tested the ability of Cyt A to inhibit angiogenesis in the more physiological chorioallantoic membrane (CAM) assay. Cyt A inhibited new vessel growth in a dose-dependent manner (Fig. 6D), similar to the inhibitor of VEGF receptor tyrosine kinase Semaxanib (SU5416) (Riboldi et al. 2005).

DISCUSSION

Ansamycins form a diverse family of compounds exerting a number of physiological effects on mammalian and viral systems (Isaacs et al. 2003; Floss and Yu 2005). In this study, we identified a member of this family as an inhibitor of eukaryotic translation elongation. Other ansamycins such as rifabutin and 17-AAG did not inhibit protein synthesis in vitro in Krebs-2 extracts at 50 μM (data not shown), indicating that this is not a general property of this group of compounds.

Increasing evidence links deregulated protein synthesis and cancer growth (Lindqvist and Pelletier 2009). Indeed, two inhibitors of elongation (HHT and a derivative of Did B) have advanced to clinical trials (Le Tourneau et al. 2007; Quintas-Cardama et al. 2007). In addition, we have previously shown that inhibitors of elongation can sensitize select tumors to the pro-apoptotic properties of the clinical agent doxorubicin (Robert et al. 2009). Inhibition of translation could, in principle, suppress drug resistance by curtailing the synthesis of antiapoptotic proteins and/or drug transporters. Leukemic cell lines have been previously shown to be more sensitive to Cyt A-induced apoptosis compared with other tumor cell lines, supporting a potential therapeutic use of Cyt A in blood cancer treatment

(Watabe et al. 2000). Here, we show that Cyt A inhibits protein synthesis in cell lines that were previously shown to be resistant to Cyt A-induced apoptosis (Fig. 5A) as well as in nontransformed HUVECs (Fig. 6C). Indeed HeLa, HUVEC, and Jurkat (a leukemia cell line previously shown to undergo apoptosis after a 24-h exposure to Cyt A [IC_{50} = 13.87 nM]; Watabe et al. 2000) cells all had very similar IC_{50} s with respect to translation inhibition (data not shown). These results suggest that the differential sensitivity of different cell lines to the apoptotic response is not due to a difference in sensitivity to Cyt A-induced protein synthesis inhibition but may depend on intrinsic factors that link the apoptotic response to the translation apparatus. We demonstrate that translation inhibition occurs well before apoptosis can be detected and, therefore, must precede the apoptotic response (Fig. 6C). The fact that Cyt A induces apoptosis more readily in leukemia is consistent with reports that B-cell and leukemia-cell lines also are more sensitive to the translation initiation inhibitor silvestrol compared with other cell types (Monks et al. 1991; Lucas et al. 2009).

Translation elongation can be inhibited in an eEF1A-dependent manner also by interfering with ternary complex formation (eEF1A:GTP:aminoacyl-tRNA). Indeed, several antibiotics target this step, including GE2770A and pulvomycin (Heffron and Jurnak 2000; Andersen et al. 2003). This mechanism is in contrast to that of Cyt A (Fig. 4A,B). Pulvomycin is known to increase the GTPase activity of EF-Tu, the bacterial homolog of eEF1A (Andersen et al. 2003), while both Did B and Cyt A do not alter GTPase activity of eEF1A to any significant extent (Fig. 4C; Crews et al. 1994; Ahuja et al. 2000). Therefore, the mechanism of action of Cyt A does not seem to be reminiscent of these EF-Tu-targeting inhibitors.

Cyt A stalled polyribosomes on mRNA templates and inhibited translating ribosomes, similar to what has been reported for the translation elongation inhibitors CHX and

FIGURE 5. Cyt A reversibly inhibits translation in cell culture. (A) Consequences of Cyt A exposure on DNA, RNA, and protein synthesis in HeLa cells. Cyt A was added to cell medium for 1 h and [⁶⁻³H]thymidine, [⁵⁻³H]uridine, or [³⁵S]methionine/cysteine was present during the last 20 min of incubation. Counts from TCA-precipitated material were normalized to total protein content and set relative to the DMSO control. The average of four data points is shown with the SEM indicated by error bars. (B) Inhibition of translation by Cyt A is reversible. HeLa cells were incubated in 2 μM Cyt A for 1 h, after which fresh medium lacking Cyt A was added. Twenty minutes before lysis, [³⁵S]methionine/cysteine was added. Normalization was performed to total protein concentration and set relative to the DMSO control. The average of four measurements is shown with the SEM represented by error bars. (C) Cyt A does not allow ribosome run-off in cell culture. Polysome formation in HeLa cells exposed to 2 μM Cyt A for 1 h and/or 5 μM Hipp for 30 min or 0.5 μM HHT for 1 h. Panels are from the same experiment and were separated for clarity.

Did B (Fig. 5C; Urdiales et al. 1996; Schneider-Poetsch et al. 2010). In the eEF1A-dependent aa-tRNA-binding experiment (Fig. 3C), the amount of [¹⁴C]Phe-tRNA^{Phe} bound to ribosomes was significantly reduced in the presence of GMPPNP compared with GTP (Fig. 3C). We believe this may be due to the large dilution (~100-fold) that occurs during processing of the samples for filter binding, allowing dissociation of the ternary complex from the ribosome. This is consistent with the finding that only after GTP hydrolysis is the charged tRNA locked in the A site (Rodnina and Wintermeyer 2001). Hence, one interpretation of our results is that in the presence of GMPPNP, the aa-tRNA is lost from the ribosome. However, this is not observed if Did B or Cyt A are present

(Fig. 3C), suggesting that these compounds stabilize the aa-tRNA:ribosome interaction, perhaps by blocking release of eEF1A. Both Cyt A and Did B inhibited translocation when aa-tRNA was loaded in an eEF1A-dependent manner (Fig. 3D; SirDeshpande and Toogood 1995), which would be consistent with this model, since eEF2 and the ternary complex share binding sites on the ribosome (Marco et al. 2004). Indeed, this mode of action has been suggested for Did B previously and is the mechanism of action of the antibiotic kirromycin (Wolf et al. 1977; Ahuja et al. 2000; Andersen et al. 2003; Schmeing et al. 2009). It remains to be determined whether Cyt A binds directly to the ribosome and/or to eEF1A.

It has recently been suggested that tumor reduction caused by eIF4F inhibition may partially be caused by inhibiting angiogenesis (Graff et al. 2007; Cencic et al. 2009). Here, we show that Cyt A can also inhibit angiogenesis as Cyt A-inhibited HUVEC tube formation (Fig. 6A,B) as well as microvessel development in the CAM assay (Fig. 6D) in a manner similar to Did B (Tarabotti et al. 2004). These results suggest that Cyt A merits further study, not only for hematological cancers, but also for solid tumors requiring angiogenesis for optimal growth.

MATERIALS AND METHODS

Materials

Cyt A was prepared as previously described and stored in 100% DMSO (Kakeya et al. 1997). Didemnin B (Did B) (NCI-Developmental Therapeutics Program), homoharringtonine (HHT) (Sigma-Aldrich), and cycloheximide (CHX) (Bioshop) were stored in 100% DMSO, whereas anisomycin (Sigma) was resuspended in H₂O. Hippuristanol was purified as previously described (Bordeleau et al. 2006). All compounds were stored at -80°C.

Cell culture experiments

HeLa cells were grown in DMEM containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. HUVEC cells (Lonza Walkersville, Inc.) were grown in EMB-2 medium supplemented with EGM-2.

For thymidine labeling of DNA, cells were serum starved for 48 h, followed by the addition of serum for 7 h, at which point

FIGURE 6. Cyt A inhibits angiogenesis. (A) Photomicrographs of HUVEC tube formation at different concentrations of Cyt A or silvestrol (Silv). Scale bar, 0.1 mm. (B) Quantitation of tube formation in HUVECs. Each well was photographed in seven fields, and the average number of tubes formed was counted. The average of four experiments is shown. Error bars represent the SEM. (C) Cyt A inhibits protein synthesis without inducing apoptosis in HUVECs. Following a 24-h exposure to Cyt A or DMSO, HUVECs were labeled for 20 min with [35 S]methionine/cysteine or monitored for apoptosis. For the translation assays, TCA-precipitable material was normalized to total protein content and set relative to the DMSO control. The average of four measurements is shown with the SEM represented by error bars. Cell viability was judged by the relative percent of Annexin-FITC or propidium iodide staining compared with DMSO controls. The average of five data points is shown with the SEM represented by error bars. (D) Cyt A inhibits angiogenesis in the CAM assay. Values presented represent the average number of vessels per cm² area for three samples with the SEM; ** P < 0.01 (vs. vehicle); *** P < 0.001 (vs. vehicle).

compound was added for 1 h. [$6\text{-}^3\text{H}$]thymidine (10 Ci/mmol) (Perkin Elmer) was present for the last 20 min of the reaction. For RNA labeling, cells were not serum starved and [$5\text{-}^3\text{H}$]uridine (26.3 Ci/mmol) (Perkin Elmer) was present during the last 20 min of a 1-h compound treatment. Cells were washed in PBS and lysed in RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Radioactive incorporation was measured by TCA precipitation (5% TCA) onto GF/C filters (preblocked with 5% TCA and 0.1 M inorganic pyrophosphate). Filters were washed with 5 mL of cold 1% TCA, followed by 5 mL 100% ethanol, and quantified by scintillation counting. Counts were standardized to total protein content that had been determined using the D_c protein assay (Bio-Rad).

To monitor protein synthesis, cells were seeded into a 24-well dish and exposed to compound for 1 h with labeling performed during the last 20 min using [35 S]Easy Tag Express Protein Labeling mix (1175 Ci/mmol) (Perkin Elmer). Cells were lysed in RIPA buffer and an aliquot processed for TCA precipitation as described above.

HUVEC tube formation assays were performed as published previously (Cencic et al. 2009). HUVECs were seeded at 100,000 cell/well in the presence of compound on top of 300 μ L of solidified BD Matrigel Matrix (BD Biosciences) in a 24-well dish. After 24 h, pictures were taken using a Nikon Eclipse TE300 microscope.

In vitro translation assays

In vitro translations were performed as previously reported (Novac et al. 2004). Translations were performed using a capped bicistronic mRNA reporter FF/HCV/Ren transcribed from pSP/(CAG)₃₃/FF/HCV/Ren.pA₅₁, in which firefly (FF) luciferase protein is produced by cap-dependent translation and *Renilla* (Ren) luciferase protein is generated by Hepatitis C virus (HCV) IRES-mediated initiation. Translation extracts were programmed with 8 μ g/mL mRNA.

Experiments analyzing the consequences of Cyt A on actively translating ribosomes were performed in Krebs-2 extracts in the absence of in vitro-transcribed RNA, but in the presence of [35 S]methionine (Perkin Elmer), with compound being added 5 min after translation had been initiated. Aliquots (10 μ L) were taken at the indicated times and added to 1.1 μ L of 0.5 mM cycloheximide (CHX) and placed on dry ice to stop the reaction. Reactions were spotted onto 3 MM Whatman paper that had been preblocked with 50 $\times$ amino acid mix (GIBCO). Filters were incubated in 10% TCA + 0.1% methionine on ice for 20

min, boiled in 5% TCA for 15 min, washed with 100% ethanol, dried, and the radioactivity quantitated by scintillation counting.

In vitro translation of poly(Phe) was performed in RRL using 50% RRL (Promega), 40 μ M amino acid mix lacking phenylalanine, 40 μ M methionine, 0.1 μ g/ μ L poly(U) RNA, 4 μ M magnesium acetate, 50 μ M potassium acetate, and 50 μ Ci/mL [^3H]phenylalanine (Perkin Elmer). Following a 1-h incubation at 30°C, reactions were processed for TCA precipitation as described above.

Ribosome-binding assays and polysome profiling

Ribosome-binding assays were performed essentially as described previously (Robert et al. 2006). Briefly, compound was preincubated

with RRL at a final KCl concentration of 150 mM for 5 min, after which ^{32}P -labeled CAT mRNA was included. When a second compound was added, it was delivered 3 min after addition of RNA and reactions allowed to proceed at 30°C for 10 min. Reactions were centrifuged through a 10%–30% glycerol gradient at 39,000 rpm for 3.5 h in a SW40 rotor. Fractions (0.5 mL) were collected and quantitated by Cherenkov counting.

Polysome profiles of HeLa cells were visualized by treating cells with DMSO, 2 μM Cyt A, or 0.5 μM HHT for 1 h in a 10-cm² dish. Hippuristanol (5 μM) was added during the remaining 30 min. Cells were then washed in PBS containing 0.1 mg/mL CHX, scraped, and lysed in hypotonic lysis buffer (5 mM Tris-HCl at pH 7.5, 2.5 mM MgCl₂, 1.5 mM KCl, 0.1 mg/mL CHX, 2 mM DTT). The lysate was supplemented with 0.5% Triton X-100 and 0.5% sodium deoxycholate, centrifuged briefly (12,000g for 2 min), and the supernatant loaded onto 10%–50% sucrose gradients (20 mM HEPES-KOH at pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT). Samples were centrifuged at 35,000 rpm for 2 h in a SW40 rotor at 4°C. The OD₂₆₀ was monitored with a UA-6 UV detector (ISCO) using a Brandel tube piercer. Data was recorded using InstaCal Version 5.70 and TracerDaq Version 1.9.0.0 (Measurement Computing Corporation).

Peptidyl transferase assays

The peptidyl transferase assay was performed as previously described (Lorsch and Herschlag 1999). Briefly, [^{35}S]methionyl-tRNA_i was generated by incubating 0.25 mg/mL total calf liver tRNA (Novogen) with 10 mM ATP, 10 mM CTP, 0.25 mg/mL leucovorin, 1 mCi/mL [^{35}S]methionine, and 0.875 $\mu\text{g/mL}$ *E. coli* aminoacyl-tRNA synthetases (Sigma) in 50 mM sodium cacodylate (pH 7.4), 15 mM MgCl₂, and 7 mM 2-mercaptoethanol at 37°C for 30 min (Stanley 1974). Charged tRNA was purified by phenol/chloroform extraction, exclusion chromatography on a Sephadex G-50 spin-column, and ethanol precipitation.

Purified 40S and 60S ribosomes (0.06 μM) (Fraser et al. 2007), 0.5 mM GTP, 1 μM model RNA (GGAA[UC]₇UAUG[CU]₁₀C), 2 nM labeled [^{35}S]methionyl-tRNA_i, and a high-salt wash of ribosomes (Lorsch and Herschlag 1999) were incubated with 50 μM Cyt A. Reactions were subsequently started by the addition of 0.4 mM puromycin at 26°C. Aliquots were stopped in 0.4 M sodium acetate, spotted on cation-exchange IONEX-25 SA-Na TLC plates (Macherey-Nagel) (prerun in distilled water and dried), and developed in 2 M ammonium acetate and 10% acetonitrile. Experiments were visualized by phosphorimaging (Typhoon Trio, Amersham).

tRNA-binding and translocation assays

tRNA-binding and translocation assays were performed essentially as described (SirDeshpande and Toogood 1995; Robert et al. 2006). [^{14}C]Phe-tRNA^{Phe} was prepared by charging 0.2 mg/mL yeast tRNA^{Phe} (Sigma) with 3.75 mM ATP, 0.06 mM [^{14}C]phenylalanine in 50 mM Tris-HCl at pH 7.5, 20 mM Mg(OAc)₂, and 120 mM KCl using 10% (v/v) yeast S100 as the source of tRNA synthetase. Charged tRNA was purified via phenol/chloroform extraction, passed through a Sephadex G-50 spin-column, followed by ethanol precipitation (Odom et al. 1990).

For eEF1A-dependent assays, reactions were performed with 1.77 μM salt-washed 80S ribosomes, (0.4 $\mu\text{g/mL}$) poly(U) RNA,

and 0.2 μM [^{14}C]Phe-tRNA^{Phe} with 4.65 μg of eEF1A. Either 0.15 mM GMP-PMP, GDP, or GTP was added in HEPES buffer (20 mM HEPES at pH 7.5, 10 mM MgCl₂, 100 mM KCl, 1 mM DTT) and reactions (100 μL) were incubated at 37°C for 30 min. Aliquots (6% of the total reaction) were taken, diluted in 0.8 mL of HEPES buffer and filtered through Type HA nitrocellulose filters (Millipore). Amino acyl-tRNA binding was quantitated by scintillation counting and values obtained without ribosomes were subtracted to remove background. The remaining reaction volume (of samples containing GTP) was used to perform translocation assays. Additional GTP (1 mM) was added to 15% of the samples in the presence or absence of 0.5 mM puromycin and/or 0.05 $\mu\text{g/mL}$ eEF2 and incubated at 37°C for 30 min. The reaction was quenched with 1 M NH₄HCO₃ and extracted with ethyl acetate. Ninety percent of the organic layer was used for quantitation by scintillation counting. A puromycin assay was performed on 10% of the original reaction to determine the amount of aminoacyl-tRNA already bound to the P-site (Wurmbach and Nierhaus 1979), which was normalized and deducted from the values obtained above to determine the total amount of tRNA translocated.

Nonenzymatic tRNA-binding reactions were performed essentially as described for eIF1A, except higher amounts of poly(U) RNA (0.4 mg/mL) were used, and the reaction was performed in the absence of both GTP (or its analogs) and eEF1A. Reactions were carried out in Tris reaction buffer (50 mM Tris-HCl at pH 7.5, 60 mM KCl, 20 mM MgCl₂) containing 50 μM of compound. Translocation assays were performed as described above, except that they were carried out in Tris reaction buffer.

eEF1A enzymatic assays

GTP cross-linking to eEF1A was performed in 20- μL reactions containing 1 μg of eEF1A and 2.5 μCi of [α - ^{32}P]GTP (3000 Ci/mmol) (Perkin Elmer) with or without 0.8 μg of Phe-tRNA^{Phe} (Sigma) in GTPase buffer (25 mM HEPES at pH 7.5, 125 mM KCl, 8.5 mM MgCl₂, 1 mM DTT). Reactions were incubated at 37°C for 15 min in the presence of 50 μM Cyt A, 1 mM cold GTP competitor, or DMSO, and cross-linked using a 254-nm germicidal UV lamp at 4°C for 15 min. Reactions were digested with 0.5 $\mu\text{g/mL}$ RNase A for 10 min at 37°C, separated by SDS-PAGE, and visualized by autoradiography. Negative controls contained 1 μg of BSA instead of eEF1A.

Electrophoretic mobility shift assays were performed in 10- μL reactions in GTPase buffer using 0.5–2 μg of eEF1A and 1 mM GTP. Reactions were preincubated at room temperature for 10 min, after which time 20,000 cpm of [^{14}C]Phe-tRNA^{Phe} was added, and the incubation continued for an additional 15 min. Equivalent molar amounts of unlabeled Phe-tRNA^{Phe} were used as competitor. Reactions were analyzed on 6% native polyacrylamide (29:1 acrylamide:bisacrylamide) gels and electrophoresis performed in 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were then treated with En³Hance (Perkin Elmer), washed in water, dried, and visualized by autoradiography.

GTPase assays (20 μL) were performed in GTPase buffer containing 0.5 μg of eEF1A and 1 μCi of [γ - ^{32}P]GTP (6000 Ci/mmol) (Perkin Elmer) incubated with or without 0.8 μg of unlabeled Phe-tRNA^{Phe}, 16.8 pmol 40S, and 60S ribosomal subunits, and 31.4 pmol poly(U) RNA at 25°C. Control reactions were also performed without eEF1A or using only eEF1A (without tRNA, ribosomes or

RNA). Aliquots (2 μ L) were taken and reactions stopped in 2 μ L of 25 mM EDTA on ice. PEI Cellulose F TLC plates (EMD Chemicals, Inc.) were spotted with a 1.5- μ L sample and developed using 0.3 M NaH_2PO_4 /1 M LiCl_2 . TLCs were quantitated using phosphorimaging on a Typhoon Trio (Amersham).

Viability assays

Viability assays were performed using Annexin-FITC and propidium iodide (PI) staining. HUVECs were treated with compound for 24 h in a 24-well plate. Cells were washed in PBS and trypsinized. Cells, PBS washes, and cell culture medium were collected together and centrifuged at 610g for 5 min. Cell pellets were washed in PBS and resuspended in 35 μ L Annexin V binding buffer (10 mM HEPES-NaOH at pH 7.5, 140 mM NaCl, 2.5 mM CaCl_2). PI (Sigma) to a final concentration of 5 μ g/mL and 1.75 μ L FITC Annexin V (BD Biosciences Pharmingen) were added to reactions and incubated at RT for 20 min in the dark. Samples were diluted by the addition of 200 μ L of Annexin V binding buffer and analyzed on a Guava Easy Cyte Plus (Millipore). Each experiment included unstained, PI-only, and Annexin V-only controls.

Chorioallantoic membrane (CAM) assay

The CAM assay was performed by Links Biosciences, LLC. Fertilized eggs were placed in an egg incubator at 37°C and 50% humidity. After 6 d, the egg shell was cracked and gently opened. A 5 $\times$ 5-mm sterile filter paper square saturated with either 25 μ L of compound (50, 125, 250, 625 pmol), 4.2 nmol SU5416 (Sugen, Inc.), or vehicle (2% DMSO in PBS) was placed in areas between vessels. After 48 h, the CAMs were isolated and fixed in methanol/acetone. Representative images were collected by photography to permit quantitative analysis of vessel density.

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