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 (Kakeya 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

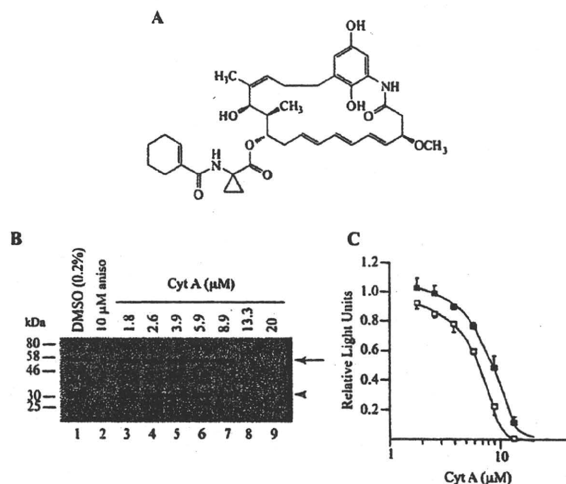

FIGURE 1. Cyt A inhibits eukaryotic translation. (*A*) Chemical structure of Cyt A. (*B*) Cyt A inhibits both cap-dependent and HCV IRES-driven translation in Krebs-2 extracts. In vitro translations were performed in the presence of [^{35}S]methionine and programmed with FF/HCV/Ren. DMSO, anisomycin (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 peptdyl 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)

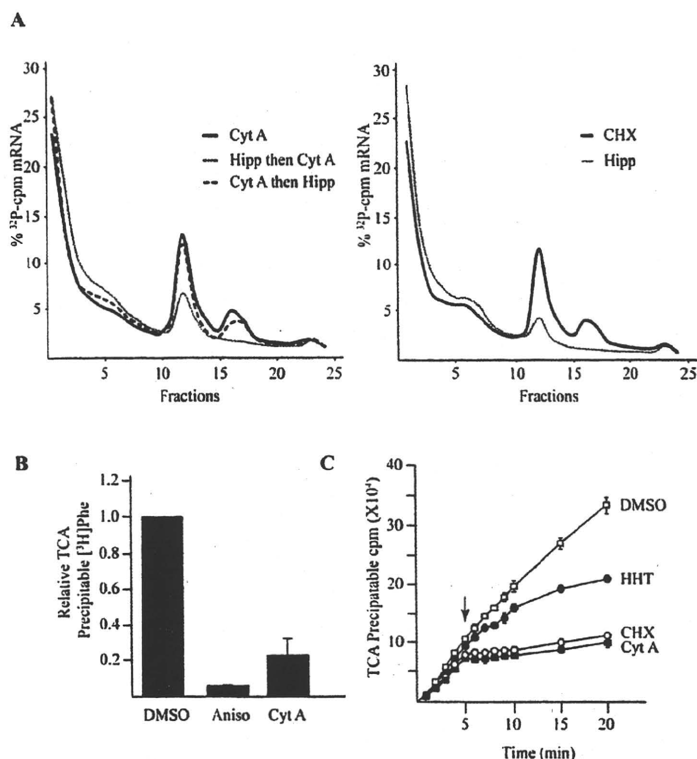

FIGURE 2. Cyt A inhibits translation elongation. (*A*) Cyt A does not inhibit translation initiation. Ribosome bindings were performed in RRL using ^{32}P-labeled CAT RNA. Reactions were separated by centrifugation on 10%–30% glycerol gradients and fractions quantitated by scintillation counting. (*Left*) 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 [^{3}H]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 ~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 [α-^{32}P]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 [α-^{32}P]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

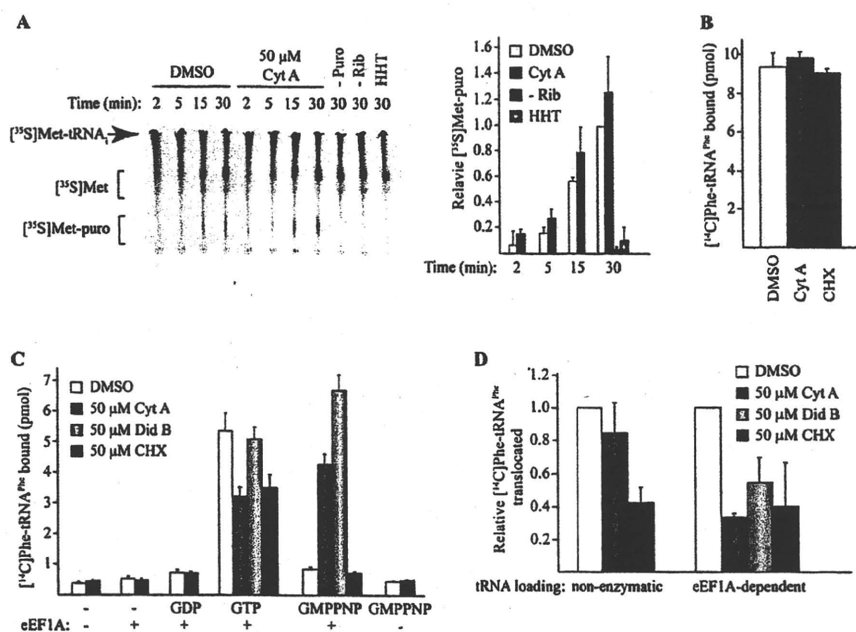

FIGURE 3. The effect of Cyt A on the steps of translation elongation. (*A*) Cyt A does not inhibit peptidyl transferase activity. [^{35}S]Methionine-puromycin formation was monitored in the presence of purified 40S and 60S ribosomes using [^{35}S]Met-tRNA$_i$ and ribosomal high-salt wash from RRL. (*Left*) Aliquots of samples were taken at the indicated time points and separated by TLC. The position of migration of [^{35}S]Met-puro, [^{35}S]Met, and [^{35}S]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}S]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}C]Phe-tRNA^{Phe} binding to 80S ribosomes. Filter binding of [^{14}C]Phe-tRNA^{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}C]Phe-tRNA^{Phe} binding to 80S ribosomes. Filter binding of [^{14}C]Phe-tRNA^{Phe} with purified 80S ribosomes, and 0.4 μg/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}C]Phe-tRNA^{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}C]Phe-tRNA^{Phe} was extracted with ethyl aceteate 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}S]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

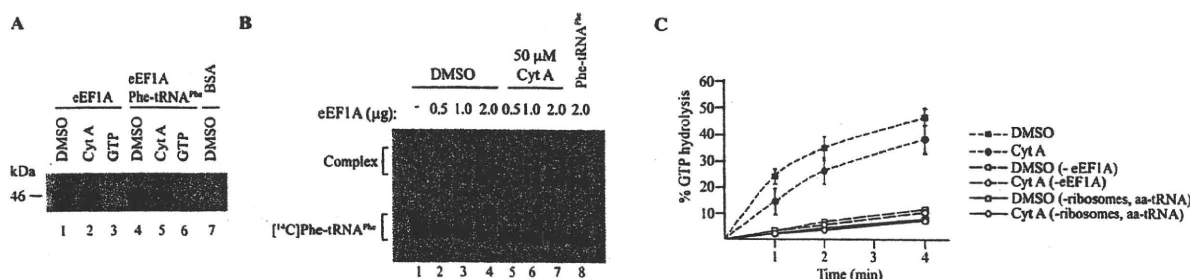

FIGURE 4. Cyt A does not affect ternary formation. (*A*) Cyt A does not inhibit GTP binding to eEF1A. Purified eEF1A (1 μg) was UV cross-linked to [α^{32}P]GTP in the presence (lanes *4–7*) or absence (lanes *1–3*) of Phe-tRNA^{Phe} and 50 μM Cyt A or 1 mM unlabeled GTP. Reactions were treated with RNase A, separated by SDS-PAGE, and visualized by autoradiography. (*B*) Cyt A does not affect [^{14}C]Phe-tRNA^{Phe} binding to eEF1A. Increasing amounts of eEF1A were incubated with [^{14}C]Phe-tRNA^{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 [^{14}C]Phe-tRNA^{Phe} and complexes are indicated to the *left*. (*C*) Cyt A does not affect the GTPase activity of eEF1A. eEF1A and [γ-^{32}P]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

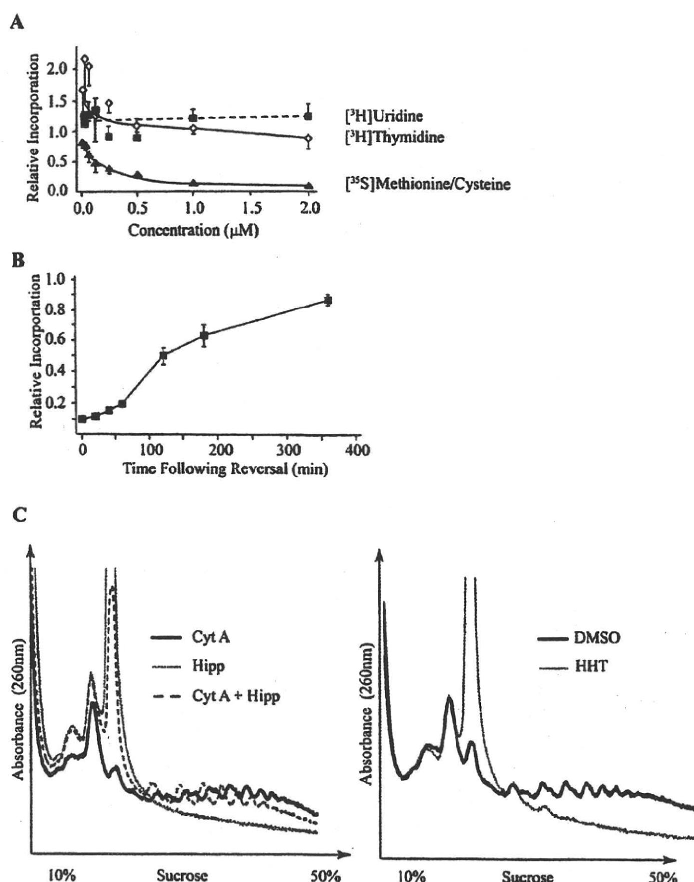

FIGURE 5. Cyt A reversibly inhibits translation in cell culture. (*A*) Consequences of Cyt A exposure on DNA, RNA, and protein synthesis in HeLa cells. Cyt A was added to cell medium for 1 h and [6-^{3}H]thymidine, [5-^{3}H]uridine, or [^{35}S]methionine/cysteine was present during the last 20 min of 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, [^{35}S]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 [^{14}C]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 (Taraboletti 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 H_2O. 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

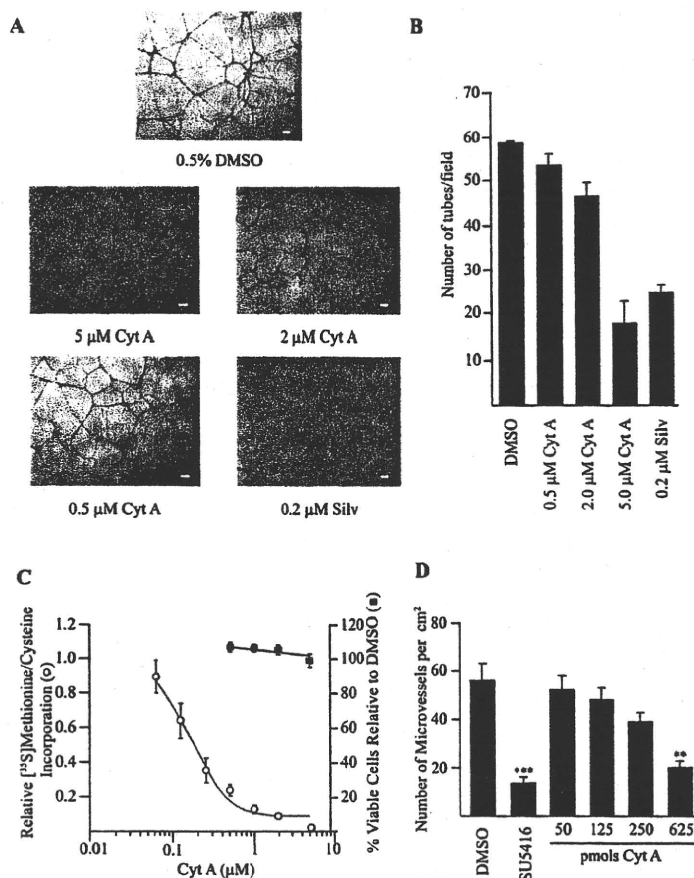

FIGURE 6. Cyt A inhibits angiogenesis. (*A*) Photomicrographs of HUVEC tube formation at different concentrations of Cyt A or silvestrol (Silv). Scale bar, 0.1 mm. (*B*) Quantitation of tube formation in HUVECs. Each well was photographed in seven fields, and the average number of tubes formed was counted. 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 cm^2 area for three samples with the SEM; $^{**}P < 0.01$ (vs. vehicle); $^{***}P < 0.001$ (vs. vehicle).

compound was added for 1 h. [6-^{3}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-^{3}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 μL 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.pA$_{51}$, 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 μg/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 μL) were taken at the indicated times and added to 1.1 μL 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× 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 μM amino acid mix lacking phenylalanine, 40 μM methionine, 0.1 μg/μL poly(U) RNA, 4 μM magnesium acetate, 50 μM potassium acetate, and 50 μCi/mL [^{3}H]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 where visualized by treating cells with DMSO, 2 μM Cyt A, or 0.5 μM HHT for 1 h in a 10-cm^2 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 $MgCl_2$, 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 $MgCl_2$, 1 mM DTT). Samples were centrifuged at 35,000 rpm for 2 h in a SW40 rotor at 4°C. The OD_{260} 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 μg/mL *E. coli* aminoacyl-tRNA synthetases (Sigma) in 50 mM sodium cacodylate (pH 7.4), 15 mM $MgCl_2$, 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]$_7$UAUG[CU]$_{10}$C), 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 μg/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 $MgCl_2$, 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 μg/μL eEF2 and incubated at 37°C for 30 min. The reaction was quenched with 1 M NH_4HCO_3 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 $MgCl_2$) 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 $MgCl_2$, 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 μg/μL 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 En^{3}Hance (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 μL) were taken and reactions stopped in 2 μL of 25 mM EDTA on ice. PEI Cellulose F TLC plates (EMD Chemicals, Inc.) were spotted with a 1.5-μL 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 610*g* for 5 min. Cell pellets were washed in PBS and resuspended in 35 μL 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 μg/mL and 1.75 μL 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 μL 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 × 5-mm sterile filter paper square saturated with either 25 μL 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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REFERENCES

Ahuja D, Vera MD, SirDeshpande BV, Morimoto H, Williams PG, Joullie MM, Toogood PL. 2000. Inhibition of protein synthesis by didemnin B: How EF-1α mediates inhibition of translocation. *Biochemistry* **39:** 4339–4346.

Andersen GR, Nissen P, Nyborg J. 2003. Elongation factors in protein biosynthesis. *Trends Biochem Sci* **28:** 434–441.

Bordeleau ME, Mori A, Oberer M, Lindqvist L, Chard LS, Higa T, Belsham GJ, Wagner G, Tanaka J, Pelletier J. 2006. Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. *Nat Chem Biol* **2:** 213–220.

Cencic R, Carrier M, Galicia-Vazquez G, Bordeleau ME, Sukarieh R, Bourdeau A, Brem B, Teodoro JG, Greger H, Tremblay ML, et al. 2009. Antitumor activity and mechanism of action of the cyclopenta[*b*]benzofuran, silvestrol. *PLoS ONE* **4:** e5223. doi: 10.1371/journal.pone.0005223.

Chan J, Khan SN, Harvey I, Merrick W, Pelletier J. 2004. Eukaryotic protein synthesis inhibitors identified by comparison of cytotoxicity profiles. *RNA* **10:** 528–543.

Chao JR, Wang JM, Lee SF, Peng HW, Lin YH, Chou CH, Li JC, Huang HM, Chou CK, Kuo ML, et al. 1998. mcl-1 is an immediate-early gene activated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathway and is one component of the GM-CSF viability response. *Mol Cell Biol* **18:** 4883–4898.

Crews CM, Collins JL, Lane WS, Snapper ML, Schreiber SL. 1994. GTP-dependent binding of the antiproliferative agent didemnin to elongation factor 1 alpha. *J Biol Chem* **269:** 15411–15414.

Duncan R, Milburn SC, Hershey JW. 1987. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. *J Biol Chem* **262:** 380–388.

Floss HG, Yu TW. 2005. Rifamycin-mode of action, resistance, and biosynthesis. *Chem Rev* **105:** 621–632.

Fraser CS, Berry KE, Hershey JW, Doudna JA. 2007. eIF3j is located in the decoding center of the human 40S ribosomal subunit. *Mol Cell* **26:** 811–819.

Graff JR, Konicek BW, Vincent TM, Lynch RL, Monteith D, Weir SN, Schwier P, Capen A, Goode RL, Dowless MS, et al. 2007. Therapeutic suppression of translation initiation factor eIF4E expression reduces tumor growth without toxicity. *J Clin Invest* **117:** 2638–2648.

Heffron SE, Jurnak F. 2000. Structure of an EF-Tu complex with a thiazolyl peptide antibiotic determined at 2.35 Å resolution: Atomic basis for GE2270A inhibition of EF-Tu. *Biochemistry* **39:** 37–45.

Heys SD, Park KG, McNurlan MA, Calder AG, Buchan V, Blessing K, Eremin O, Garlick PJ. 1991. Measurement of tumour protein synthesis in vivo in human colorectal and breast cancer and its variability in separate biopsies from the same tumour. *Clin Sci* **80:** 587–593.

Isaacs JS, Xu W, Neckers L. 2003. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* **3:** 213–217.

Kahns S, Lund A, Kristensen P, Knudsen CR, Clark BF, Cavallius J, Merrick WC. 1998. The elongation factor 1 A-2 isoform from rabbit: Cloning of the cDNA and characterization of the protein. *Nucleic Acids Res* **26:** 1884–1890.

Kakeya H, Zhang HP, Kobinata K, Onose R, Onozawa C, Kudo T, Osada H. 1997. Cytotrienin A, a novel apoptosis inducer in human leukemia HL-60 cells. *J Antibiot* **50:** 370–372.

Kakeya H, Onose R, Osada H. 1998. Caspase-mediated activation of a 36-kDa myelin basic protein kinase during anticancer drug-induced apoptosis. *Cancer Res* **58:** 4888–4894.

Kubota Y, Kleinman HK, Martin GR, Lawley TJ. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* **107:** 1589–1598.

Le Tourneau C, Raymond E, Faivre S. 2007. Aplidine: A paradigm of how to handle the activity and toxicity of a novel marine anticancer poison. *Curr Pharm Des* **13:** 3427–3439.

Lindqvist L, Pelletier J. 2009. Inhibitors of translation initiation as cancer therapeutics. *Future Med Chem* **1:** 1709–1722.

Lorsch JR, Herschlag D. 1999. Kinetic dissection of fundamental processes of eukaryotic translation initiation in vitro. *EMBO J* **18:** 6705–6717.

Lucas DM, Edwards RB, Lozanski G, West DA, Shin JD, Vargo MA, Davis ME, Rozewski DM, Johnson AJ, Su BN, et al. 2009. The novel plant-derived agent silvestrol has B-cell selective activity in chronic lymphocytic leukemia and acute lymphoblastic leukemia in vitro and in vivo. *Blood* **113:** 4656–4666.

Marco E, Martin-Santamaria S, Cuevas C, Gago F. 2004. Structural basis for the binding of didemnins to human elongation factor eEF1A and rationale for the potent antitumor activity of these marine natural products. *J Med Chem* **47:** 4439–4452.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, et al. 1991.

Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* **83:** 757–766.

Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, Wang X. 2003. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev* **17:** 1475–1486.

Novac O, Guenier AS, Pelletier J. 2004. Inhibitors of protein synthesis identified by a high throughput multiplexed translation screen. *Nucleic Acids Res* **32:** 902–915.

Odom OW, Picking WD, Hardesty B. 1990. Movement of tRNA but not the nascent peptide during peptide bond formation on ribosomes. *Biochemistry* **29:** 10734–10744.

Pelletier J, Peltz SW. 2007. Therapeutic Opportunities in Translation. In *Translational Control in Biology and Medicine*, (ed. MB Mathews et al.), pp. 855–895. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Quintas-Cardama A, Kantarjian H, Garcia-Manero G, O'Brien S, Faderl S, Estrov Z, Giles F, Murgo A, Ladie N, Verstovsek S, et al. 2007. Phase I/II study of subcutaneous homoharringtonine in patients with chronic myeloid leukemia who have failed prior therapy. *Cancer* **109:** 248–255.

Quintas-Cardama A, Kantarjian H, Cortes J. 2009. Homoharringtonine, omacetaxine mepesuccinate, and chronic myeloid leukemia circa 2009. *Cancer* **115:** 5382–5393.

Riboldi E, Musso T, Moroni E, Urbinati C, Bernasconi S, Rusnati M, Adorini L, Presta M, Sozzani S. 2005. Cutting edge: Proangiogenic properties of alternatively activated dendritic cells. *J Immunol* **175:** 2788–2792.

Robert F, Gao HQ, Donia M, Merrick WC, Hamann MT, Pelletier J. 2006. Chlorolissoclimides: New inhibitors of eukaryotic protein synthesis. *RNA* **12:** 717–725.

Robert F, Carrier M, Rawe S, Chen S, Lowe S, Pelletier J. 2009. Altering chemosensitivity by modulating translation elongation. *PLoS ONE* **4:** e5428. doi: 10.1371/journal.pone.0005428.

Rodnina MV, Wintermeyer W. 2001. Fidelity of aminoacyl-tRNA selection on the ribosome: Kinetic and structural mechanisms. *Annu Rev Biochem* **70:** 415–435.

Schmeing TM, Voorhees RM, Kelley AC, Gao YG, Murphy FV IV, Weir JR, Ramakrishnan V. 2009. The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. *Science* **326:** 688–694.

Schneider-Poetsch T, Ju J, Eyler DE, Dang Y, Bhat S, Merrick WC, Green R, Shen B, Liu JO. 2010. Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol* **6:** 209–217.

SirDeshpande BV, Toogood PL. 1995. Mechanism of protein synthesis inhibition by didemnin B in vitro. *Biochemistry* **34:** 9177–9184.

Stanley WM Jr. 1974. Specific aminoacylation of the methionine-specific tRNA's of eukaryotes. *Methods Enzymol* **29:** 530–547.

Taraboletti G, Poli M, Dossi R, Manenti L, Borsotti P, Faircloth GT, Broggini M, D'Incalci M, Ribatti D, Giavazzi R. 2004. Antiangiogenic activity of aplidine, a new agent of marine origin. *Br J Cancer* **90:** 2418–2424.

Thornton S, Anand N, Purcell D, Lee J. 2003. Not just for housekeeping: Protein initiation and elongation factors in cell growth and tumorigenesis. *J Mol Med* **81:** 536–548.

Urdiales JL, Morata P, Nunez De Castro I, Sanchez-Jimenez F. 1996. Antiproliferative effect of dehydrodidemnin B (DDB), a depsipeptide isolated from Mediterranean tunicates. *Cancer Lett* **102:** 31–37.

Watabe M, Kakeya H, Onose R, Osada H. 2000. Activation of MST/Krs and c-Jun N-terminal kinases by different signaling pathways during cytotrienin A-induced apoptosis. *J Biol Chem* **275:** 8766–8771.

Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, Cordon-Cardo C, Pelletier J, Lowe SW. 2004. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* **428:** 332–337.

Wolf H, Chinali G, Parmeggiani A. 1977. Mechanism of the inhibition of protein synthesis by kirromycin. Role of elongation factor Tu and ribosomes. *Eur J Biochem* **75:** 67–75.

Wurmbach P, Nierhaus KH. 1979. Codon-anticodon interaction at the ribosomal P (peptidyl-tRNA) site. *Proc Natl Acad Sci* **76:** 2143–2147.

総合論文

新規有用天然有機化合物の開拓とケミカルバイオロジー研究

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Novel Natural Products Open the Door of Chemical Biology and Medicinal Chemistry

Hideaki Kakeya* and Shinichi Nishimura

Exploitation of novel small molecules from natural sources such as microbial metabolites, medicinal plants, and marine invertebrates has contributed to the discovery of lead molecules for drugs as well as research tools on chemical biology. Chemical biology based on forward/reverse chemical genetics is a new paradigm that accelerates drug development and the functional analysis of genes and proteins. Moreover, novel natural products with unique structural or biological characteristics attract both chemists and biologists, thereby developing the field of chemical biology and medicinal chemistry. We have discovered several novel bioactive microbial metabolites by both *in vivo* cell-based phenotypic screenings and *in vitro* target-oriented screenings, and investigated their modes of action using a chemical genetics or a chemical genomics approach. In this review, we focus on the following topics; i) recent screening technology and the chemical library, ii) overview of bioactive natural products and semi-synthetic derivatives we have discovered, iii) chemical genetics approach for apoptosis signaling pathway, iv) chemical genomics approach for target identification of antifungal agent, and v) perspective.
Key words: natural products, chemical biology, chemical genetics, chemical genomics, medicinal chemistry, system chemotherapy, anti-cancer agent, apoptosis, antifungal agent, angiogenesis

はじめに

人類は有史以来，天然有機化合物が薬として利用できることを学んできた。天然有機化合物の資源は，植物，微生物，海洋無脊椎動物等，多種多様であり，ブロックバスター的化合物は人類の福祉に貢献してきたのみならず，新しい学問領域であるケミカルバイオロジー(化学生物学)の発展にも大きく貢献しつつある(図1)。本稿では，我々の研究成果を中心に，創薬・生命科学の基盤研究としてのスクリーニング技術の動向と，新規有用天然有機化合物の開拓を基盤としたケミカルバイオロジー研究について紹介する。

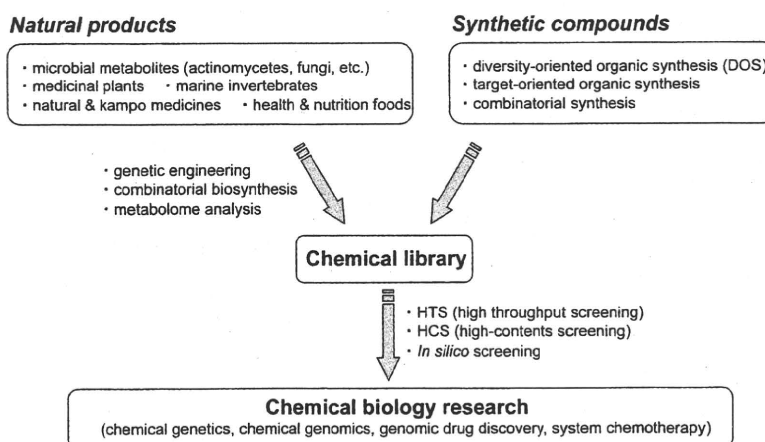

Fig. 1　Chemical library and chemical biology research.

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1. スクリーニング技術の動向

本稿で述べる“スクリーニング”とは，*in vitro* や *in vivo*，あるいは *in silico* において“何らかの基準によって生理活性小分子を選別すること”である。ケミカルバイオロジー研究，および創薬リード化合物創製の成否は，まず，目的とする生物活性を示すリード化合物を迅速に見出せるか否かに大きく依存する。そのためのスクリーニング方法として，ハイスループットスクリーニング(HTS; high throughput screening)やハイコンテンツスクリーニング(HCS; high-contents screening)が重要な戦略となっている。いずれも高度にシステム化された方法で短期間に多数の化合物を生化学的に評価して，新規なリード化合物を迅速に発見する方法論である。一方で，コンピューター科学の発展に伴い，*in silico* スクリーニングも台頭しつつある。

1990年代後半頃から，主として製薬企業を中心にHTSが発展してきた。HTSを成功させるためには，1)化合物ライブラリーの構築，2)評価系のミニチュア化(高密度プレートの利用)，3)HTSに適した評価系の構築，4)スクリーニングロボット等の使用によるオートメーション化，5)データ処理システムの整備，などが基本的には重要である。最近では，HTSの技術を生細胞にも応用したスクリーニング手法，すなわち細胞の形態変化など細胞の表現型や細胞内で起こっている様々なイベント(例えば，細胞内シグナル伝達やタンパク質の細胞内輸送，細胞の形態変化など)を，蛍光タンパク質を用いた多重染色法等で多角的に捉えるHCSも盛んに利用されつつある。さらに，HCSはスクリーニングのみならず，細胞レベルでの化合物のターゲットバリデーションや，リード化合物の最適化研究における化合物のプロファイリングなど多方面で利用されている。現在，HTSおよびHCSで利用されているマイクロプレートは，96穴マイクロプレート(0.25 ml/穴)から，384穴マイクロプレート(0.05 ml/穴)あるいは1,536穴マイクロプレート(0.01 ml/穴)へと変遷しており，アッセイのための総反応量は1/25となり，1日あたりの処理量は10倍以上になっている。評価系のミニチュア化は，アッセイ技術の開発や微量試薬分注器や高感度測定器(検出器)の開発をもたらしている。

2. 天然有機化合物ライブラリーに対する国内外の取り組み

HTSおよびHCSは多種多様な化合物があってこそ威力を発揮する。そのため，創薬の現場ではたくさんの小分子(低分子量化合物)からなる化合物ライブラリーが必要とされている。ただ，化合物が数多くあればよいかというとそれだけではない。類似化合物ばかりのライブラリーでは，質の高いスクリーニングができない。化合物の構造が多様性を持っていることも重要である。実際に，初期のコンビナトリアル合成による化合物ライブラリーでは，化合物の多様性があまり考慮されていなかったが，そのような化合物ライブラリーからは当初期待されたほどの結果が得られていない。しかし，最近では，特定の化学構造に着目したフォーカストライブラリーの構築もなされつつある。一方で，化合物ライブラリーに多様性を持たせる手段として，化学合成の観点から多様性指向型有機合成(diversity-oriented organic synthesis)に重点が置かれつつある[1]。

一方で，複雑かつ多様な構造を有する天然有機化合物の有効利用も重要である。これまでに市販された医薬品の約30%が天然物由来であり，ファーマコフォアを含めると42%が天然有機化合物の情報を利用して製造されているとの報告がある[2]。このことからも国内外の製薬企業の多くは，天然物創薬研究に取り組んできたことがわかる。特に1990年代は製薬企業が南米，アフリカ，東南アジアなどの資源保有国に注目し，新薬開発への投資や共同研究が活発に行われてきた。しかし，2000年

Table 1 Recent development of drugs derived from natural products.

drug	lead ; origin	indications	source[a]
arteether	artemisinin ; plant	antiparasitic	ND
caspofungin	pneumocandin B ; fungi	antifungal	ND
pimecrolimus	ascomycin ; actinomycete	antiallergic	ND
galantamine	galantamine ; plant	anti-Alzheimer's	S
micafungin	FR901379 ; fungi	antifungal	ND
miglustat	1-deoxynojirimycin ; actinomycete	Type I Gaucher disease	S
mycophenolate sodium	mycophenolate ; fungi	immunosuppressant	S
pitavastatin	mevastatin ; fungi	hypolipidemic	S
yondelis/Et-743	Et-743 ; tunicate	anticancer	S

[a] ND; derived from a natural product and modified through semisynthesis. S; synthetic drug.

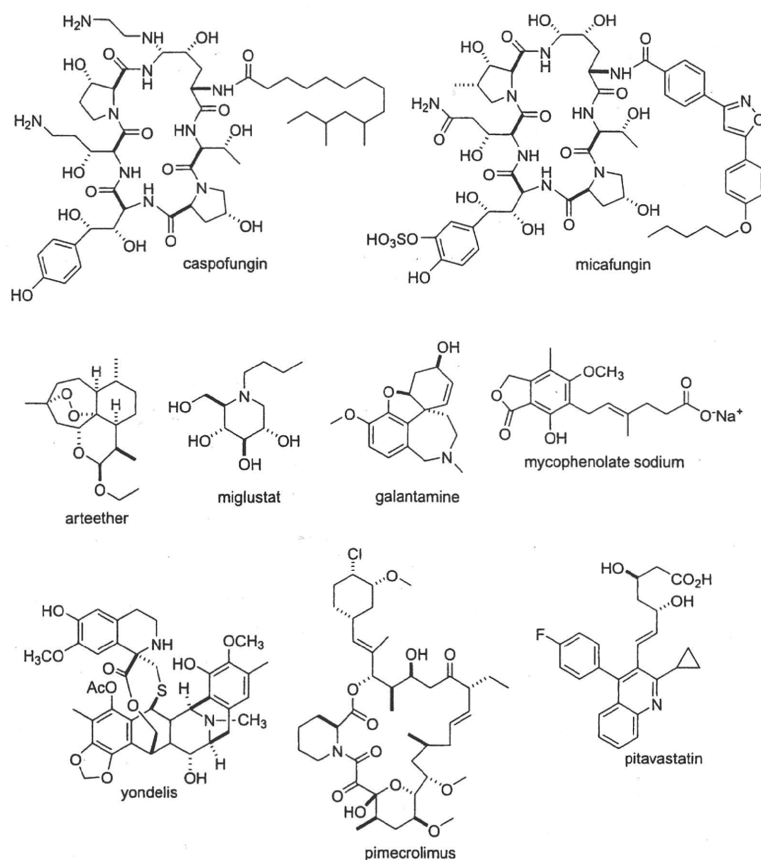


Fig. 2 Chemical structures of drugs described in Table 1.

代に入ると，HTS技術などの進歩に伴い，合成化合物と比較して手間のかかる天然物創薬を縮小・撤退する動きが出てきた。一方で，独自性を発揮するため，天然物の利用を継続・拡大している企業もある。例えば，表1および図2に日米欧で，近年，発売された天然有機化合物由来の医薬品の例を示す[2]。また，天然物創薬関連研究のアウトソーシングも行われており，その役割を担うバイオベンチャーの台頭も著しい。

アメリカでは，国立衛生研究所(NIH)のロードマップの柱として分子スクリーニングネットワークを確立する計画を2004年に発表し，2008年からは2期目に突入している(http://nihroadmap.nih.gov/molecularlibraries/)。この目的を達成させるため，小分子化合物ライブラリーを確立させることがゴールの1つに設定されている。現在，約30万個の化合物が集められているが，天然有機化合物は全体の数%程度である。今後，約100万個の化合物からなる化合物ライブラリーが準備される予定である。欧州では，European Molecular Biology Laboratory (EMBL)–European Bioinformatics Institute(EBI)が約2万個の化合物をカタログ化し，化学構造や生理活性について体系的な整理を行っている(http://www.ebi.ac.uk)。また，韓国では Korea Research Institute of Chemical Technology(KRICT)が

Table 2 Natural products and their target proteins identified by chemical genetics.

compound	target protein	function of target protein
brefeldin A	ARF1-GAP	protein transport
cyclosporin A	cyclophilin	immune system
ECH	pro-caspase-8	protease
ETB	Hsp60	chaperone
FK506 (tacrolimus)	FKBP	immune system
fumagillin	MetAP2	aminopeptidase
geldanamycin	Hsp90	chaperone
lactacystin	20S proteasome	protein degradation
leptomycin B	CRM1/exportin 1	nuclear export
manumycin	farnesyltransferase	prenyltransferase
pironetin	tubulin-α	cytoskeleton
pladienolide	SF3b	splicing
radicicol	Hsp90	chaperone
rapamycin	FKBP	immune system
reveromycin	Ile-tRNA synthetase	protein synthesis
spergualin	Hsp70/Hsc70	immune system
spliceostatin	SF3b	splicing
trapoxin	HDAC	transcription
trichostatin A	HDAC	transcription
wortmannin	PI-3 kinase	lipid kinase

化合物バンクを設立し，25万化合物を目標に整備を進めている(http://www.krict.re.kr/english/index.php)。いずれにおいても，多様性を持たせる観点から天然有機化合物がさらに取り入れられることが期待されている。

日本では，2006年，理化学研究所において微生物代謝産物，天然型合成化合物，天然物誘導体を基盤とする天然化合物バンク(RIKEN Natural Products Depository, NPDepo, http://npd.riken.jp/)が設立された。また，収集した化合物の化学・生化学情報については，天然有機化合物データベース(RIKEN Natural Products Encyclopedia, NPEdia)を整備し公開される予定であり，化合物の保管，寄託・提供の諸手続きを統合するシステムの構築も進められている。

3. 創薬を指向したケミカルバイオロジー研究

ケミカルバイオロジー研究のなかでも最も基本となる概念は，ハーバード大学のS. L. Schreiber博士が最初に提唱した"ケミカルジェネティクス(chemical genetics, 化学遺伝学)である[3]。これまでに，ケミカルバイオロジー研究により，明らかになった天然有機化合物の標的タンパク質の例を表2および図3に示す。

真核細胞の一生は，1個の受精卵から始まり様々な増殖・分化・細胞死決定因子による厳密な制御のもとに「生・死・分化」が決定されている。この厳密な調節機構に異常が生じると，がん・免疫不全・炎症疾患などをはじめとした様々な疾病につながると考えられている。細

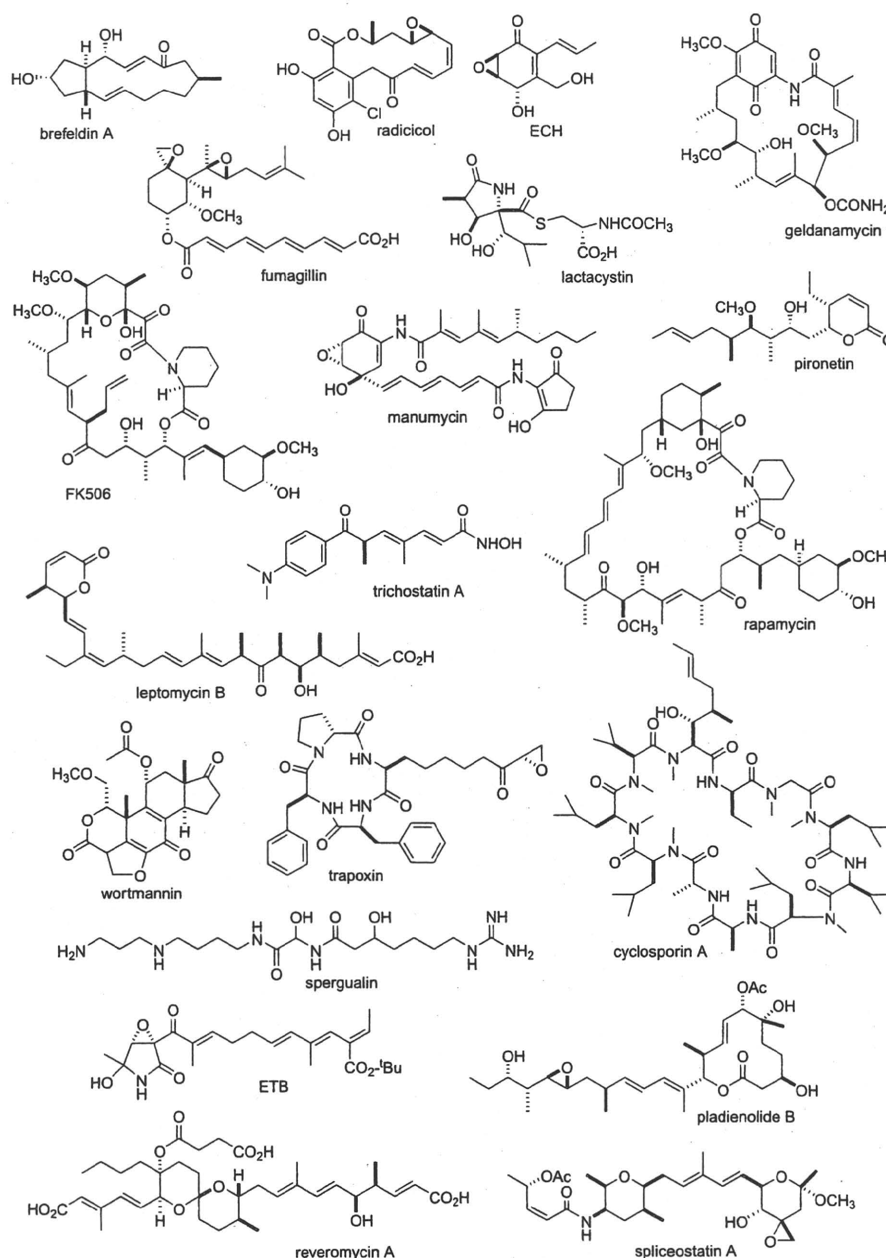


Fig. 3 Chemical structures of natural products described in Table 2.

胞の「生・死・分化」の調節機構の全貌を解明することは，生命科学における究極の課題である。そのためのアプローチとして，生理活性小分子を利用したケミカルバイオロジー的アプローチは，分子遺伝学的アプローチと相補的に，非常に強力かつ有意義なアプローチである[3,4]。ケミカルバイオロジー的アプローチの成功は，標的タンパク質に作用する生理活性小分子の開拓に大きく依存しているといっても過言ではない。我々は，天然物化学を機軸として，微生物や植物等が生産する多くの有用な新規生理活性小分子を開拓し，それらを利用して細胞の「生・死・分化」の調節機構の解明研究に取組んでいる。すなわち，培養細胞系の複雑かつ多様な細胞内情報伝達ネットワークを利用して，細胞死制御活性(細胞死誘導活性/細胞死抑制活性)，神経分化誘導活性，細胞周期阻害活性，血管新生抑制活性等を効率的に検出可能な系を構築し，探索研究・精密構造解析研究の結果，微生物(放線菌，糸状菌など)や植物等が生産する新規生理活性小分子，およびそれらをリード化合物とした新規類縁合成化合物を開拓している(抜粋，表3，図4)。さらに，新規生理活性小分子の生合成機構解析研究，構造活性相関研究を基盤とした機能性分子プローブの設計・創製研究，およびケミカルバイオロジー研究を展開している。これまでに，我々の基礎研究の成果を基盤にして，複数の生理活性小分子が市販化され細胞生物学研究に貢献している。

表3および**図4**に示した化合物群に関する研究の中から抜粋して，アポトーシスを制御する生理活性小分子サイトトリエニンA, MT-21, ETB, ECH, および RKTS-33 & 34 について概説する。

細胞死制御物質の開拓研究

細胞死の1つであるアポトーシスの制御異常は，様々な疾病の原因となりうる[21]。また，アポトーシスは，がんの分子標的治療等において重要な標的であると考えられる。アポトーシスの研究は，1990年代より盛んに研究されているが未解明な点も多い。すなわち，アポトーシスは非常に複雑に制御されていることも明らかとなりつつあるが，一方ではアポトーシス依存性細胞死に加えて，アポトーシス非依存性細胞死の重要性もクローズアップされつつある。このような複雑な生命現象を解析するのに有用なツールとなる生理活性小分子はバイオプローブと呼ばれている[4]。

アポトーシスの研究分野においても，バイオプローブを利用して多くの知見が得られてきた。アポトーシスにおけるバイオプローブは，アポトーシス誘導物質とアポトーシス抑制物質とに大別される。このうちアポトーシス誘導物質は抗がん剤開発の観点などから，数多くの有用なバイオプローブが見出されている。その一方で，アポトーシス抑制物質は，zVAD-fmk に代表されるカスパーゼの基質認識配列に基づく，基質拮抗型のペプチド性阻害剤が主流であり，非ペプチド性のアポトーシス抑制物質は極めて少ない。特に，デスレセプター依存性アポトーシスはがん細胞の除去以外にも，自己免疫疾患や劇症肝炎，慢性関節リウマチなど様々な疾患にも関与しており，デスレセプター依存性アポトーシス抑制剤はこれら疾病の治療薬のリード化合物となりうる。

Table 3 Bioactive natural products and synthetic derivatives discovered by Kakeya, Osada, and co-workers.

name	biological activity	origin	Ref.
epolactaene	neurite differentiation/ apoptosis induction	*Fusarium* sp.	5
tryprostatin A&B	cell cycle arrest (G2/M)	*Aspergillus* sp.	6
spirotryprostatin A&B	cell cycle arrest (G2/M)	*Aspergillus* sp.	7
cyclotryprostatin A-D	cell cycle arrest (G2/M)	*Aspergillus* sp.	8
cytotrienin A	apoptosis induction	*Streptomyces* sp.	9
MT-21	apoptosis induction	synthetic	5, 10
ETB	apoptosis induction	synthetic	11
RK-1009	cell cycle arrest (G2/M)	*Streptomyces* sp.	12
cytoxazone	immunomodulator	*Streptomyces* sp.	13
lucilactaene	cell cycle arrest (G1)	*Fusarium* sp.	14
azaspirene	anti-angiogenesis	*Neosartorya* sp.	15
epoxyquinol A&B	anti-angiogenesis	unidentified fungi	16
epoxytwinol A	anti-angiogenesis	unidentified fungi	17
RK-95113	anti-angiogenesis	*Aspergillus* sp.	18
ECH	apoptosis inhibition	unidentified fungi	19
RKTS-33&34	apoptosis inhibition	synthetic	20

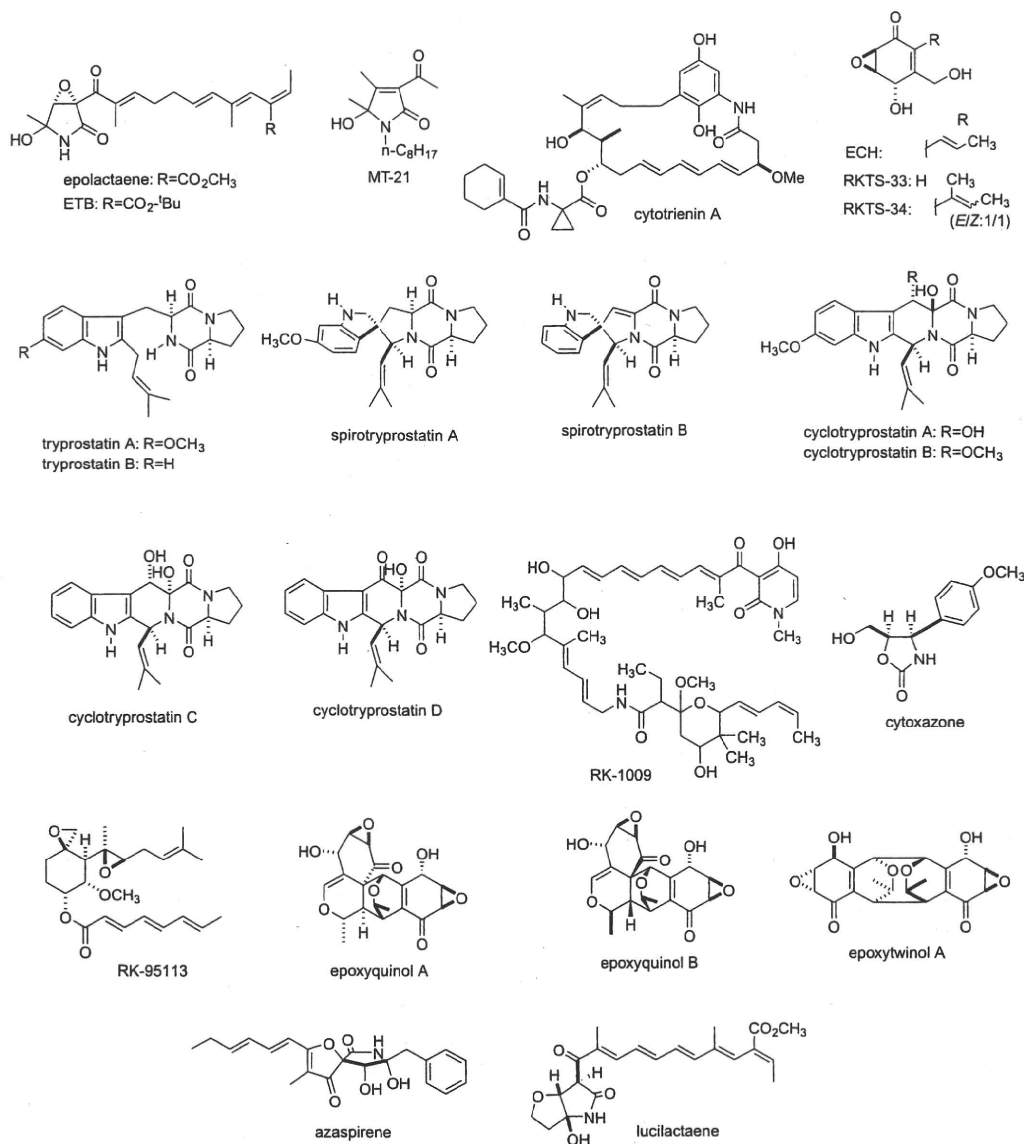


Fig. 4 Chemical structures of natural products and synthetic derivatives described in Table 3.

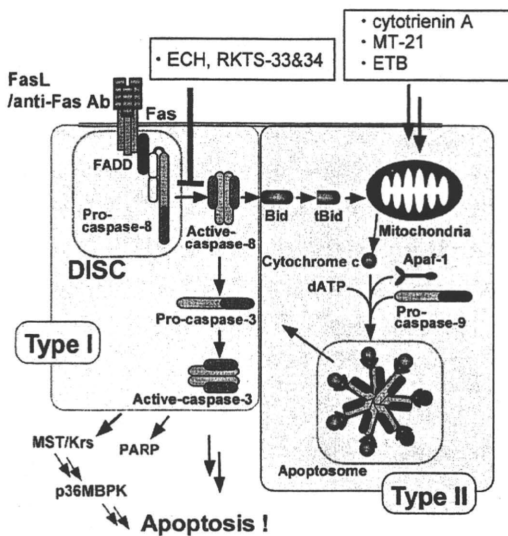


Fig. 5 Signaling pathway for apoptosis.

3.1 アポトーシス誘導剤サイトトリエニンA

ヒト前骨髄性白血病HL-60細胞とヒト正常肺繊維芽WI-38細胞を用いて，HL-60細胞に対して選択的にアポトーシスを誘導する新規物質として放線菌*Streptomyces*属RK 95-74株が生産するアンサマイシン系化合物サイトトリエニンA(cytotrienin A)を見出した[9]。サイトトリエニンAは，放線菌代謝産物では初めてのACC(1-aminocyclopropane-1-carboxylic acid)ユニット構造を含む化合物であることを証明し，各種^{13}C同位体標識前駆体([1-^{13}C]酢酸，[1-^{13}C]プロピオン酸，*L*-[メチル-^{13}C]メチオニン，*L*-[U-^{13}C]メチオニン)のサイトトリエニンAへの取り込み実験の結果，進化的にも非常に興味深い「*L*-メチオニン=>ACC経路」が生産菌内に存在することを明らかにした[9]。なお，高等植物には植物ホルモンであるエチレンの主要な生合成経路として，ACC合成経路の存在が知られているが[22]，放線菌二次

代謝産物以外でも分子内にACC骨格を有する微生物代謝産物は極めて稀であり，放線菌におけるACC合成経路は全く不明であった。

サイトトリエニンAは，HL-60細胞において濃度・時間依存的にアポトーシスに特徴的なクロマチンの凝縮やDNAの断片化を誘導した。一方，正常肺繊維芽WI-38細胞においては1,000倍以上の高濃度でもアポトーシスを誘導しなかった。サイトトリエニンAのアポトーシス誘導は，*N*-アセチルシステインで抑制されたが，その不活性体*N*-アセチルセリンでは抑制されなかったことなどから，ミトコンドリア経由による活性酸素種の関与が示唆された。さらに，サイトトリエニンAは，ミトコンドリア経路を介して，出芽酵母のSte-20キナーゼホモログであるMST/Krsタンパク質を活性化し，アポトーシスを誘導することを明らかにした[9]。すなわち，サイトトリエニンによって活性化されたカスパーゼが，全長のMst 1/Krs 2, Mst 2/Krs 1をカスパーゼ認識部位で切断し，その結果生じた分子量約36 kDaのN末断片(p 36 MBPK; p 36 myelin basic protein kinase)が顕著なキナーゼ活性を有することを明らかにし，それまでその生理的意義の詳細が不明であったMst/Krsタンパク質のアポトーシス誘導等における意義を明らかにした。また，臨床使用されている各種抗がん剤に対する感受性細胞においては，これら抗がん剤によってp 36 MBPKの活性化が認められ，MST/Krsの発現あるいはp 36 MBPKの活性化が抗がん剤感受性マーカーの1つになりうる可能性を示した[9]。さらに，近年，ショウジョウバエやマウス個体においてもSte-20キナーゼホモログのHippoやMst/Krsが増殖，細胞死，組織形成に深く関与していることが示された[23]。

3.2 アポトーシス誘導剤MT-21およびETB

糸状菌*Fusarium*属が生産するエポラクタエンの詳細な構造活性相関研究の過程で，新規アポトーシス誘導剤MT-21，およびETB(epolactaene *tertiary*-butyl ester)を見出した[5,10,11]。現在，MT-21は細胞レベルでチトクロームCの遊離を誘導するANT(adenine nucleotide translocase)阻害剤として広く利用されている[24]。また，ETBは，ミトコンドリア経路を活性化して各種ヒトがん細胞に対して強力なアポトーシス誘導活性を有することを見出した。そこで，構造活性相関研究の知見を基にETB標的分子同定のための分子プローブ(ビオチン化ETB, 図6)を設計・創製し，ETB結合タンパク質の探索・同定を行った。その結果，ETBの細胞内結合タンパク質の1つとして分子シャペロンHsp 60を同定した。リンゴ酸脱水素酵素等を基質とした，Hsp 60の分子シャペロン活性に与えるETBの影響を検討した結果，ETBは顕著にHsp 60の分子シャペロン活性を抑制した。さらに，ETBはHsp 60とCys^{442}を介して共有結合しシャペロン活性を阻害することを明らかにした。Hsp 60のCys^{442}は，大腸菌GroELとのホモロジーモデリングの結果，ATP結合部位近傍に位置することが示唆され，ETBがHsp 60のATPase活性および多量体化にも影響を与えることが示唆された[11]。ETBはHsp 60の機能解明のための強力なツールになるとともに，Hsp 60が新しいがんの分子標的となりうる可能性が期待される。

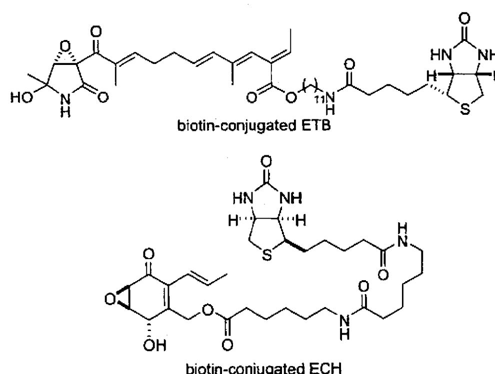


Fig. 6 Chemical structures of biotin-conjugated ETB and ECH.

3.3 アポトーシス抑制剤ECHおよびRKTS-33 & 34

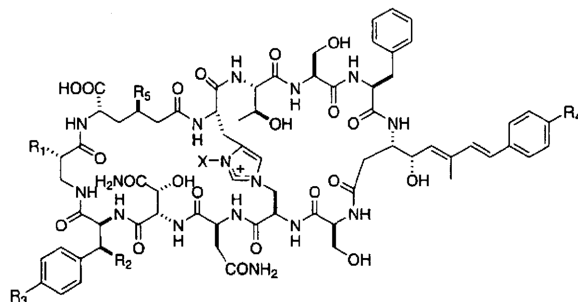
デスレセプター依存性アポトーシス抑制剤として糸状菌(未同定)が生産する非ペプチド系化合物ECH〔(2*R*, 3*R*, 4*S*)-2,3-epoxy-4-hydroxy-5-hydroxymethyl-6-(1*E*)-propenyl-cyclohex-5-en-1-one〕を見出した[19]。ECHの構造活性相関研究の結果，新規合成類縁化合物としてRKTS-33 & 34の開発に成功した[20]。さらに，構造活性相関研究の知見を基にECH標的分子同定のための分子プローブを設計・創製し(ビオチン化ECH, 図6)，ECHの細胞内標的としてpro-caspase-8を同定し，詳細な解析の結果，ECHはDISC(death-inducing signaling complex)の形成には影響を与えず，DISCの形成に引き続くpro-caspase-8の活性化を抑制することを明らかにした[19]。また，ECHおよびRKTS-33 & 34を利用して，Fas/FasLシステムの活性化にはFasのインターナリゼーションが重要であることを明らかにした。さらに，免疫細胞の1つである細胞障害性T細胞(CTL)が標的細胞を殺傷する際に，細胞障害性顆粒の経路に加えて，Fas/FasL経路が重要であることを示した[19,20]。今後，ECHおよびRKTS-33 & 34がFasL/Fasシステムの生理的意義の解析研究に貢献するとともに，創薬リード化合物となることが期待される。

4. ケミカルゲノミクス研究による作用機序解析

上述のETBやECHの標的タンパク質(結合タンパク

質)は，構造活性相関研究に基づいてビオチン基を有する分子プローブを設計・創製し，目的化合物のビオチン標識体と標的タンパク質の物理的相互作用を指標に同定した。ビオチン・アビジンシステムは標的経路の絞り込みでは，なかなか困難であった小分子の標的分子の同定を直接的に行うことができるため，非常に強力な手段である。しかし，天然物の場合，量的制限や化学修飾の限度などがあり，全合成とそれに続く構造活性相関研究がなされていない化合物の場合，強力な活性を有していても標的タンパク質の探索に結びつかないことがしばしばある。一方で，活性のある標識化合物を合成できた場合であっても，標的タンパク質の発現量が少ない，あるいは，形成するべき複合体が生体外では不安定である，といった場合には困難を極める。さらに，標的がタンパク質でない，といった場合にはもはやお手上げである。

Theonellamide 類は，松永らにより報告された 12 残基のアミノ酸からなる海産のペプチドである[25]。Theonellamide A-F は八丈島の海綿 *Theonella* sp.より，theonegramide, theopalauamide はそれぞれ Philippine, Palau および Mozambique で採集された海綿 *Theonella swinhoei* から単離された[26](図 7)。いずれも histidinoalanine 残基によって架橋された特徴的な二環性構造を有しており，異常アミノ酸に富んだ共通のペプチド骨格をもつ。これらの化合物の真の生産者は明らかではな



theonellamide A　R_1 = OH; R_2 = Me; R_3 = Br; R_4 = H; R_5 = OH; X = β-D-Gal
theonellamide B　R_1 = OH; R_2 = Me; R_3 = Br; R_4 = Br; R_5 = OH; X = H
theonellamide C　R_1 = H; R_2 = H; R_3 = H; R_4 = Br; R_5 = OH; X = H
theonellamide D　R_1 = H; R_2 = H; R_3 = Br; R_4 = Br; R_5 = OH; X = β-L-Ara
theonellamide E　R_1 = H; R_2 = H; R_3 = Br; R_4 = Br; R_5 = OH; X = β-D-Gal
theonellamide F　R_1 = H; R_2 = H; R_3 = Br; R_4 = Br; R_5 = OH; X = H
theonegramide　R_1 = OH; R_2 = Me; R_3 = Br; R_4 = H; R_5 = H; X = β-D-Ara
theopalauamide　R_1 = OH; R_2 = Me; R_3 = Br; R_4 = H; R_5 = H; X = β-D-Gal

Fig. 7 Chemical structures of theonellamides and their congeners.

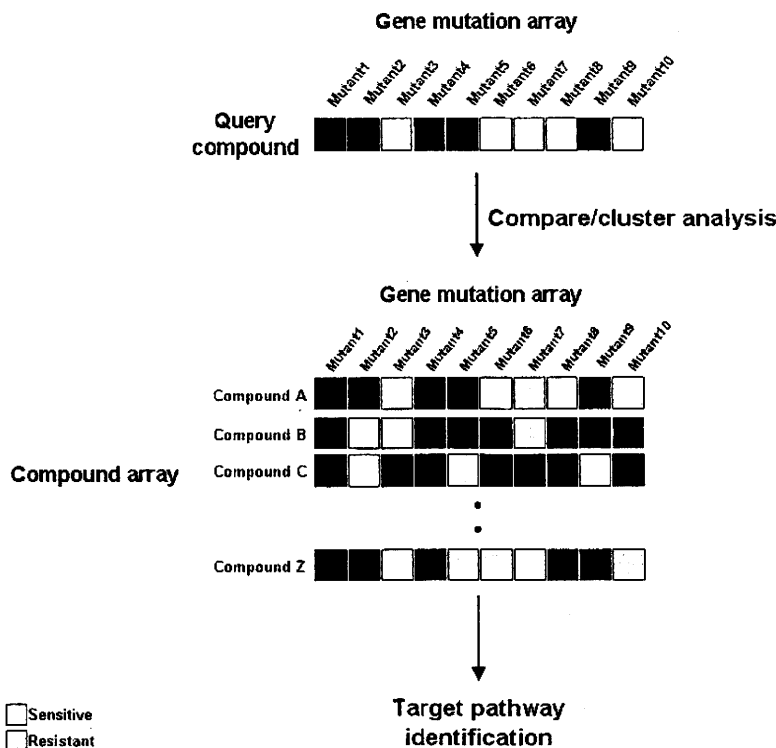


Fig. 8 Chemical genomic profiling for target molecule and pathway identification. Profiling of chemical genetic interactions is of help for identification of target protein and pathway. Once phenotypes of gene mutants for a query compound are defined, modes of action of the compound can be expected from statistical analysis using phenotype compendia. For example, the mutants 6, 8, and 10 are sensitive to the query compound and the mutants 3 and 7 are resistant to the compound. Comparison with the compendia of chemical genomic profiles reveals that the query compound has a similar target pathway or modes of action with that of the compound A.

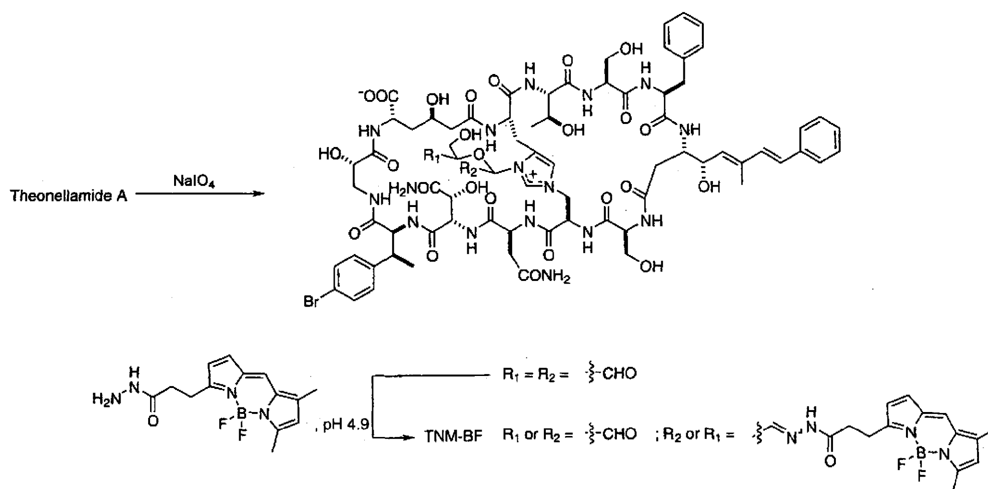


Fig. 9 Synthesis of fluorescently labeled theonellamide, TNM-BF.

いが，theopalauamideは分画実験から海綿に共生する真性細菌が産生している可能性が示唆されている[27]。Theonellamide類は抗真菌活性と弱い細胞毒性を示すことが報告されており，theonellamideと結合するタンパク質も報告されてはいるが[28]，生物活性との関連性は不明であった。

Theonellamide類は抗真菌活性を示し，分裂酵母 *Schizosaccharomyces pombe* に対しても生育阻害を示す。そこで，松山らによって報告された分裂酵母の全遺伝子発現株コレクション[29]を用いて，theonellamide Fの感受性に影響を与えるORF(open reading frame)を探索・検討した[30]。すなわち，クローニングしたORFにタグを融合し，染色体の特定の場所から *nmt1* プロモーターの支配下に発現することができる菌株に対する感受性試験を行い，約5,000のORFのなかから数十の耐性化遺伝子と超感受性化遺伝子とを同定した。これらの遺伝子には，細胞骨格の維持にかかわるものが多く含まれていた。しかし，いずれのORFにコードされるタンパク質もtheonellamide Fと物理的相互作用を示さず，どの遺伝子がtheonellamide Fの一次標的についての直接的な情報を提示しているのか解釈は容易ではなかった。ポストゲノムといわれて久しいが，存在は明らかとなっても機能情報がついていない遺伝子が依然として圧倒的に多いのである。一方，大規模なデータが得られたときのために，いくつかのデータマイニング法が考案されている。特に，クラスター解析は簡便な方法で有用な情報を提示することがあり，重宝されている(図8)。この場合，作用機序の似た化合物をグループ化することが可能である。そこで，作用機序が既知の化合物プロファイルのパターンとtheonellamide Fのプロファイルとを比較したところ，theonellamide Fがポリエン系の抗生物質のプロファイルと弱いながらも類似性を示しており，両者が類似の作用機序あるいは標的分子を有する可能性が示唆された。

クラスター解析の結果を受けて，theonellamideと脂質分子との相互作用を検討した。すなわち，theonellamide Aのガラクトース部分に$NaIO_4$処理による酸化的開裂によってアルデヒド体を作製し，ヒドラジド基を持つBODIPY誘導体と反応させて蛍光誘導体(TNM-BF，図9)を調製した。TNF-BFを用いて脂質との結合試験および細胞内局在観察を行ったところ，TNF-BFは細胞膜中のエルゴステロールを選択的に認識することが示唆された。そして，本稿では詳細は割愛させて頂くが，遺伝学的，生化学的な実験からtheonellamide Fは細胞膜ステロールを特異的に認識し，膜損傷を与え，抗真菌作用を示すことが明らかとなった。Theonellamide類の標的分子は遺伝子に直接コードされていない脂質分子であったわけであるが[30,31]，包括的に化学遺伝学的相互作用を解析するケミカルゲノミクスが，標的分子がタンパク質でない場合にも有効であることを示す好例といえる。

おわりに

我々は引き続き，微生物代謝産物，薬用植物，機能性食品等の天然資源由来の新規生理活性小分子の開拓研究を機軸として，ケミカルバイオロジー研究・ケミカルゲノミクス研究における独創的な研究を展開し，多因子疾患(がん，心疾患，感染症，神経変性疾患，生活習慣病，免疫疾患等)の克服に向けた創薬・生命科学研究を行っている。一方で，微生物や植物によって生産される興味深い化学構造と，生理活性を有する新規生理活性小分子の生合成機構の遺伝子レベル・酵素レベルでの解析研究を展開している。これらの研究は，新規創薬リード化合物の開拓のみならず，二次代謝制御酵素群のグリーンケ

ミストリーを指向したコンビナトリアル生合成研究へと拡がる。いずれの研究もアカデミアにおける利点を活かした"Curiosity-driven Science"でありたいと考えている。

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文　献

1) S. L. Schreiber, *Science*, 287, 1964 (2000)
2) (a) M. S. Butler, *J. Nat. Prod.*, 67, 2141 (2004); (b) D. J. Newman, G. M. Cragg, *J. Nat. Prod.*, 70, 461 (2007); (c) G. M. Cragg, P. G. Grothaus, D. J. Newman, *Chem. Rev.*, 109, 3012 (2009)
3) S. L. Schreiber, *Bioorg. Med. Chem.*, 6, 1127 (1998)
4) *"Bioprobes: Biochemical tools for investigating cell function"* ed. by H. Osada, Springer, Tokyo, 1999
5) (a) H. Kakeya, I. Takahashi, G. Okada, K. Isono, H. Osada, *J. Antibiot.*, 48, 733 (1995); (b) H. Kakeya, C. Onozawa, M. Sato, K. Arai, H. Osada, *J. Med. Chem.*, 40, 391 (1997)
6) (a) C.-B. Cui, H. Kakeya, G. Okada, R. Onose, M. Ubukata, I. Takahashi, K. Isono, H. Osada, *J. Antibiot.*, 48, 1382 (1995); (b) C.-B. Cui, H. Kakeya, G. Okada, R. Onose, H. Osada, *J. Antibiot.*, 49, 527 (1996); (c) C.-B. Cui, H. Kakeya, H. Osada, *J. Antibiot.*, 49, 534 (1996); (d) N. Kato, H. Suzuki, H. Takagi, Y. Asami, H. Kakeya, M. Uramoto, T. Usui, S. Takahashi, Y. Sugimoto, H. Osada, *ChemBioChem.*, 10, 920 (2009)
7) (a) C.-B. Cui, H. Kakeya, H. Osada, *J. Antibiot.*, 49, 832 (1996); (b) C.-B. Cui, H. Kakeya, H. Osada, *Tetrahedron*, 52, 12651 (1996)
8) C.-B. Cui, H. Kakeya, H. Osada, *Tetrahedron*, 53, 59 (1997)
9) (a) H. Kakeya, H.-p. Zhang, K. Kobinata, R. Onose, C. Onozawa, T. Kudo, H. Osada, *J. Antibiot.*, 50, 370 (1997); (b) H.-p. Zhang, H. Kakeya, H. Osada, *Tetrahedron Lett.*, 38, 1789 (1997); (c) H.-p. Zhang, H. Kakeya, H. Osada, *Tetrahedron Lett.*, 39, 6947 (1998); (d) H. Kakeya, R. Onose, H. Osada, *Cancer Res.*, 58, 4888 (1998); (e) M. Watabe, H. Kakeya, R. Onose, H. Osada, *J. Biol. Chem.*, 275, 8766 (2000); (f) Y. Hayashi, M. Shoji, H. Ishikawa, J. Yamaguchi, T. Tamura, H. Imai, Y. Nishigaya, K. Takabe, H. Kakeya, H. Osada, *Angew. Chem. Int. Ed.*, 47, 6657 (2008)
10) M. Watabe, H. Kakeya, H. Osada, *Oncogene*, 18, 5211 (1999)
11) (a) Y. Nagumo, H. Kakeya, J. Yamaguchi, T. Uno, M. Shoji, Y. Hayashi, H. Osada, *Bioorg. Med. Chem. Lett.*, 14, 4425 (2004); (b) Y. Nagumo, H. Kakeya, M. Shoji, Y. Hayashi, N. Dohmae, H. Osada, *Biochem. J.*, 387, 835 (2005)
12) H. Kakeya, M. Morishita, A. Ikeno, K. Kobinata, T. Yano, H. Osada, *J. Antibiot.*, 51, 963 (1998)
13) (a) H. Kakeya, M. Morishita, K. Kobinata, M. Osono, M. Ishizuka, H. Osada, *J. Antibiot.*, 51, 1126 (1998); (b) H. Kakeya, M. Morishita, H. Koshino, T.-i. Morita, K. Kobayashi, H. Osada, *J. Org. Chem.*, 64, 1052 (1999)
14) H. Kakeya, S.-I. Kageyama, L. Nie, R. Onose, G. Okada, T. Beppu, C. J. Norbury, H. Osada, *J. Antibiot.*, 54, 850 (2001)
15) (a) Y. Asami, H. Kakeya, R. Onose, A. Yoshida, H. Matsuzaki, H. Osada, *Org. Lett.*, 4, 2845 (2002); (b) Y. Asami, H. Kakeya, Y. Komi, S. Kojima, K. Beebe, L. Neckers, K. Nishikawa, H. Osada, *Cancer Sci.*, 99, 1853 (2008)
16) (a) H. Kakeya, R. Onose, H. Koshino, A. Yoshida, K. Kobayashi, S.-I. Kageyama, H. Osada, *J. Am. Chem. Soc.*, 124, 3496 (2002); (b) H. Kakeya, R. Onose, A. Yoshida, H. Koshino, H. Osada, *J. Antibiot.*, 55, 829 (2002); (c) H. Kamiyama, T. Usui, H. Sakurai, M. Shoji, Y. Hayashi, H. Kakeya, H. Osada, *Biosci. Biotechnol. Biochem.*, 72, 1894 (2008); (d) H. Kamiyama, T. Usui, M. Uramoto, H. Takagi, M. Shoji, Y. Hayashi, H. Kakeya, H. Osada, *J. Antibiot.*, 61, 94 (2008)
17) H. Kakeya, R. Onose, H. Koshino, H. Osada, *Chem. Commun.*, 2005, 2575
18) Y. Asami, H. Kakeya, G. Okada, M. Toi, H. Osada, *J. Antibiot.*, 59, 724 (2006)
19) (a) Y. Miyake, H. Kakeya, T. Kataoka, H. Osada, *J. Biol. Chem.*, 278, 11213 (2003); (b) T. Mitsui, Y. Miyake, H. Kakeya, H. Osada, *J. Immunol.*, 172, 3428 (2004)
20) (a) H. Kakeya, Y. Miyake, M. Shoji, Satoshi Kishida, Y. Hayashi, T. Kataoka, H. Osada, *Bioorg. Med. Chem. Lett.*, 13, 3743 (2003); (b) T. Mitsui, Y. Miyake, H. Kakeya, Y. Hayashi, H. Osada, T. Kataoka, *Biosci. Bitechnol. Biochem.*, 69, 1923 (2005)
21) D. Hanahan, R. A. Weinberg, *Cell*, 100, 57 (2000)
22) S. F. Yang, N. E. Hoffman, *Ann. Rev. Plant Physiol.*, 35, 155 (1984)
23) (a) R. S. Udan, M. Kango-Singh, R. Nolo, C. Tao, G. Halder, *Nat. Cell Biol.*, 5, 914 (2003); (b) S. Wu, J. Huang, J. Dong, D. Pan, *Cell*, 114, 445 (2003); (c) K. Harvey, N. Tapon, *Nat. Rev. Cancer*, 7, 182 (2007); (d) J. Huang, S. Wu, J. Barrera, K. Matthews, D. Pan, *Cell*, 122, 421 (2005); (e) B. Zhao, X. Wei, W. Li, R. S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z. C. Lai, K. L. Gluan, *Genes. Dev.*, 21, 2747 (2007); (f) J. Dong, G. Feldmann, J. Huang, N. Zhang, S. A. Comerford, M. F. Gayyed, R. A. Anders, A. Maitra, D. Pan, *Cell*, 130, 1120 (2007)
24) (a) M. Watabe, K. Machida, H. Osada, *Cancer Res.*, 60, 5214 (2000); (b) K. Machida, Y. Hayashi, H. Osada, *J. Biol. Chem.*, 277, 31243 (2002)
25) (a) S. Matsunaga, N. Fusetani, K. Hashimoto, M. Walchli. *J. Am. Chem. Soc.*, 111, 2582 (1989); (b) S. Matsunaga, N. Fusetani. *J. Org. Chem.*, 60, 1177 (1995)
26) (a) C. A. Bewley, D. J. Faulkner, *J. Org. Chem.*, 59, 4849 (1994); (b) E. W. Schmidt, C. A. Bewley, D. J Faulkner. *J. Org. Chem.*, 63, 1254 (1998)
27) C. A. Bewley, N. D. Holland, D. J. Faulkner, *Experientia*, 52, 716 (1996)
28) S. Wada, S. Matsunaga, N. Fusetani, S. Watabe, *Mar. Biotechnol. (NY)*, 2, 285 (2000)
29) (a) A. Matsuyama, R. Arai, Y. Yashiroda, A. Shirai, A. Kamata, S. Sekido, Y. Kobayashi, A. Hashimoto, M. Hamamoto, Y. Hiraoka, S. Horinouchi, M. Yoshida, *Nat. Biotechnol.*, 24, 841 (2006); (b) A. Shirai, A. Matsuyama, Y. Yashiroda, A. Hashimoto, Y. Kawamura, R. Arai, Y. Komatsu, S. Horinouchi, M. Yoshida, *J. Biol. Chem.*, 283, 10745 (2008)
30) S. Nishimura, Y. Arita, M. Honda, K. Iwamoto, A. Matsuyama, A. Shirai, H. Kawasaki, H. Kakeya, T. Kobayashi, S. Matsunaga, M. Yoshida, *Nat. chem. Biol.*, accepted.
31) C. H. Ho, L. Magtanong, S. L. Barker, D. Gresham, S. Nishimura, P. Natarajan, J. L. Koh, J. Porter, C. A. Gray, R. J. Andersen, G. Giaever, C. Nislow, B. Andrews, D. Botstein, T. R. Graham, M. Yoshida, C. Boone, *Nat. Biotechnol.*, 27, 369 (2009)