The resorcylic acid lactone hypothemycin selectively inhibits the mitogen-activated protein kinase kinase-extracellular signal-regulated kinase pathway in cells

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The resorcylic acid lactone hypothemycin has been shown to inactivate protein kinases by binding to a cysteine conserved in 46 protein kinases, including mitogen-activated protein kinase kinase (MEK), extracellular signal-regulated kinase (ERK) and platelet-derived growth factor receptor (PDGFR). We assessed the selectivity of hypothemycin in cellular contexts. Hypothemycin normalized the morphology and inhibited anchorage-independent growth of Ki-ras transformed normal rat kidney (NRK) cells with selectivity and potency comparable to or greater than that of the MEK inhibitor U0126. In Ki-ras-transformed and phorbol-12-myristate-13-acetate (PMA)-treated NRK cells, hypothemycin blocked ERK activation but showed a minimal effect on autophosphorylation of protein kinase D1 (PKD1), another kinase containing the conserved cysteine. Hypothemycin potently inhibited PDGFR autophosphorylation and activation of the MEK-ERK pathway in platelet-derived growth factor (PDGF)-treated NRK cells. However, the phosphoinositide-3-kinase (PI3K) pathway was only modestly attenuated. Hypothemycin also inhibited growth factor- and anchorage-independent growth of human cancer cell lines with a constitutively active MEK-ERK pathway. Although hypothemycin has the potential to inactivate various protein kinases, the results indicate that in intracellular environments, hypothemycin can inhibit the MEK-ERK axis with sufficient selectivity to normalize transformed phenotypes of cells dependent on this pathway.

Key words hypothemycin; resorcylic acid lactone; protein kinase; signal transduction

Resorcylic acid lactones (RALs) constitute a family of polyketide mycotoxins with a variety of biological activities. Representative examples are the heat shock protein 90 inhibitor radicicol and the estrogen agonist zearalenone. Hypothemycin (Fig. 1), a RAL containing a cis-enone moiety that initially did not reveal any particularly interesting activity, was later shown to inhibit ras-transformation and T cell activation. Hypothemycin and other closely related cis-enone RALs gained attention as compounds that irreversibly inhibit certain protein kinases such as mitogen-activated protein kinase kinase (MEK), extracellular signal-regulated kinase (ERK) and TAK1, but not RAF, protein kinase C (PKC) or protein kinase A (PKA). From their structures and irreversible mode of action, cis-enone RALs were predicted to form stable Michael addition products with cysteine residues of protein kinases. Bioinformatics studies revealed that a cysteine residue that immediately precedes the highly conserved Asp-Phe-Gly (DFG) motif is the RAL binding site. This cysteine is conserved in 9% (46/4640) of the protein kinases in the human kinome. Although different kinases contain the RAL target cysteine, cis-enone RALs have been isolated as selective inhibitors of MEK.

Fig. 1. Structure of Hypothemycin

ras-induced transformation was associated with accelerated cyclin D1 degradation, and a MEK inhibitor caused a loss of cyclin D expression in BRAF mutants. Here, we compared the effects of hypothemycin and MEK inhibitors on signaling pathways in cells. Our results show that even though the RAL target cysteine is not confined to the components of the MEK-ERK pathway, in cellular settings hypothemycin preferentially blocks the MEK-ERK axis with sufficient selectivity to impair transformed phenotypes of cancer cells requiring this pathway.

MATERIALS AND METHODS

Materials Hypothemycin and 7′,8′-dihydrohypothemycin were isolated from culture broth of Hypomyces subiculatus TAMU 117. U0126 was purchased from Promega (Madison, WI, U.S.A.) and PD98059 was obtained from Calbiochem (San Diego, CA, U.S.A.). Rat platelet-derived growth factor (PDGF)-BB is a product of R&D Systems (Minneapolis, MN, U.S.A.). Anti-phosphotyrosine antibody
4G10 was purchased from Millipore (Billerica, MA, U.S.A.). Phospho-Akt (Ser473), phospho-PKD/PKCα (Ser 916), phospho-ERK1/2 (Thr202/Tyr204), phospho-p90RSK (Thr359/S363), phospho-S6 ribosomal protein (Ser 235/236) and eukaryotic initiation factor 4E antibodies were acquired from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.).

**Measurement of Anchorage- and Growth Factor-Independent Growth**

Anchorage-independent growth was measured on poly-2-(hydroxyethyl methacrylate) (polyHEMA)-coated plates as described previously. Cells were inoculated in polyHEMA-coated culture plastic 96-well plates in a volume of 135 μl at a density of 1000 cells per well for Ki-ras transformed normal rat kidney (NRK) cells (Ki-ras/NRK) and 5000 per well for HCT116 and HT29. For measurement of growth in serum-free medium, cells were suspended in Dulbecco’s Modified Eagle Medium : Nutrient Mixture F-12, 1 : 1 supplemented with 1 mg/ml fatty acid-free bovine serum albumin and 5.5 μg/ml transferrin, and seeded in wells of collagen-coated 96-well plates in a volume of 135 μl at a density of 5000 cells per well. Compounds dissolved in 15 μl of medium were added to the wells, and the cells were cultured. After 4 d, 15 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in phosphate buffered saline) was added and further incubated for 4 h. The resulting MTT formazan was solubilized with 100 μl of sodium dodecyl sulfate (SDS) solution (20% in 10 mM HCl), and the absorbance was measured after 24 h at 570 nm and a reference wavelength of 690 nm.

**Immunoblotting Analysis**

Ki-ras/NRK cells were grown in 24-well plates to approximately 80% confluency and treated with inhibitors for 24 h. Cells were fixed for 10 min with 10% cold trichloroacetic acid and lysed with 9 M urea, 2% Triton X-100 and 2% lithium dodecyl sulfate. Lysates were neutralized with 2 M Tris and passed through a syringe to reduce viscosity. Proteins were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. To observe effects on phorbol 12-myristate 13-acetate (PMA)- and PDGF-signaling, NRK cells were seeded in 96-well plates in a volume of 100 μl at a density of 10000 cells per well, cultured for 2 d, and then serum starved for 24 h. Cells were treated with inhibitors for 3 h and stimulated with 100 nM PMA for 30 min or 50 ng/ml rat PDGF-BB for 10 min and then processed for immunoblotting as described above.

To observe multiple signaling pathways downstream of PDGFR simultaneously, a cocktail was used that contained antibodies against phosphorylated forms of the components of the PKC pathway (phospho-PKD), the phosphoinositide-3-kinase (PI3K)-AKT pathway (phospho-AKT, phospho-S6R) and the MEK-ERK pathway (phospho-ERK and phospho-p90RSK).

**RESULTS**

**Hypothemycin Normalizes Growth Properties of Ki-ras-Transformed Rat Fibroblasts**

We have previously reported that the MEK inhibitor U0126 normalizes growth properties of Ki-ras-transformed rat fibroblasts (Ki-ras/NRK). Hypothemycin also has been shown to reverse ras-mediated transformation. We compared the effects of hypothemycin with those of two MEK inhibitors, U0126 and PD98059; we focused on the morphology and anchorage-independent growth of Ki-ras/NRK cells. Treatment of Ki-ras/NRK with 2 μM hypothemycin caused distinct morphological reversion to a flat, normal appearance (Fig. 2). U0126 and PD98059 also induced cell flattening, but higher concentrations were required. The morphology of Ki-ras/NRK cells did not change when treated with 7,8-dihydrohypothemycin, indicating that the cis-enone system is necessary for morphology-reversing activity.

We next examined the effects of hypothemycin and MEK inhibitors on anchorage-independent growth of Ki-ras/NRK cells using polyHEMA-coated 96-well plates. Hypothemycin suppressed the growth of Ki-ras/NRK cells on the nonadhesive polyHEMA surface at concentrations lower than those required on normal tissue culture plastic (Fig. 3). U0126 and PD98059 also inhibited anchorage-independent growth of Ki-ras/NRK cells, but at higher concentrations. The results indicate that hypothemycin reverses Ki-ras-induced pheno-

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Fig. 2. Morphological Reversion of Ki-ras/NRK Cells Induced by MEK Inhibitors

Cells were treated for 24 h with the indicated concentrations of inhibitors. HTM, hypothemycin; d-HTM, 7,8-dihydrohypothemycin.
types with potency greater than or at least comparable to that of U0126 and PD98059.

**Hypothemycin Preferentially Inhibits the MEK-ERK Pathway in Rat Fibroblasts**
cis-Enone RALs including hypothemycin have been reported to irreversibly inhibit a subset of protein kinases such as MEK and TAK1.6,8) The irreversible inhibition was shown to be a result of Michael addition to a cysteine residue that immediately precedes the conserved DFG-motif.9) This cysteine is conserved in 46 of the 510 identified protein kinases in the human kinome. We investigated whether hypothemycin can discriminate among the possible targets in cellular contexts. Because previous reports raised the possibility that hypothemycin selectively blocked the MEK-ERK pathway, we compared the phosphorylation of ERK, which is mediated by MEK, and autophosphorylation of PKD1 (PKC\(\mu\)), another protein kinase containing the conserved cysteine.

Cell lysates were prepared from hypothemycin-treated Ki-ras/NRK cells shown in Fig. 2 and analyzed by immunoblotting. Phosphorylation of ERK was completely inhibited in hypothemycin-treated cells with normalized morphology (Fig. 4a). U0126 and PD98059 also reduced ERK phosphorylation, but 7,8'-dihydrohypothemycin had no effect. Schirmer et al. demonstrated 94% inhibition of PKD with 2 \(\mu\)M hypothemycin in an in vitro assay.9) However, hypothemycin at the same concentration did not have any effect on autophosphorylation of PKD1 in Ki-ras/NRK cells. Likewise, hypothemycin inhibited PMA-induced ERK phosphorylation in NRK cells (97% at 4 \(\mu\)M and 81% at 2 \(\mu\)M), with a modest effect on autophosphorylation of PKD1 (Fig. 4b). The results demonstrate that in cells, hypothemycin does not inhibit every protein kinase with the RAL-target cysteine and in vitro inhibition does not always predict inhibition in a cellular environment.

PDGFR\(\alpha\) and PDGFR\(\beta\) are two of the seven tyrosine kinases that contain the RAL target cysteine.9) NRK cells were stimulated with PDGF-BB in the presence of various concentrations of hypothemycin and the cell lysates were subjected to immunoblotting using an anti-phosphotyrosine antibody. As expected, hypothemycin inhibited the PDGF-induced tyrosine phosphorylation in NRK cells (Fig. 5, top). The cell lysates were also immunoblotted with a cocktail that contains antibodies against phosphorylated forms of the components of the PKC pathway (PKD), the PI3K pathway (AKT and S6 ribosomal protein), and the MEK-ERK pathway (ERK and p90RSK) to examine the effects of hypothemycin on signaling pathways downstream of PDGFR.

Inhibition of the downstream targets was not completely parallel with the reduction in PDGFR autophosphorylation (Fig. 5, bottom). Surprisingly, although ligand-stimulated phosphorylation of PDGFR appeared to be fully blocked by 10 \(\mu\)M hypothemycin, phosphorylation of S6 ribosomal pro-
tein only decreased 38%. At 4 μM hypothemycin, PDGFR autophosphorylation was still inhibited about 85%, but the phosphorylation of S6 ribosomal protein was no longer impaired. Phosphorylation of AKT and PKD1 also displayed hypothemycin resistance to some extent and substantial phosphorylation was observed after 10 μM treatment.

On the other hand, components of the MEK-ERK pathway appeared to be more susceptible. The PDGF-stimulated phosphorylation of ERK and p90RSK was inhibited by 8 μM hypothemycin to levels indistinguishable from that of the unstimulated control. The phosphorylation of p90RSK was the most sensitive, and apparent inhibition was observed at 2 μM hypothemycin. These results demonstrate that hypothemycin inhibits PDGFR activation, but the inhibitory effect decreases as signals are transduced downstream, and signaling pathways that diverge from PDGFR are not affected uniformly.

Hypothemycin Impairs the Transformed Growth Properties of Human Cancer Cells with a Constitutively Active MEK-ERK Pathway

Cancer cell lines with mutations that activate the MEK-ERK pathway, the BRAF V600E mutation in particular, have been shown to be exceedingly sensitive to hypothemycin.9,11,13) We tested whether hypothemycin could normalize the transformed phenotypes of human cancer cell lines that harbor MEK-ERK activating mutations. We chose two colon cancer cell lines, HT29, a BRAF V600E mutant, and HCT116, a KRAS G13D mutant, and examined the effects of hypothemycin on their growth factor-independent and anchorage-independence, the two major in vitro parameters of transformation. If hypothemycin is a selective inhibitor of the MEK-ERK pathway in cells, it should show enhanced activity against HT29 and HCT116 cells deprived of growth factors or anchorage.

As shown in Fig. 6a, hypothemycin inhibited the growth of HT29 and HCT116 cells in serum-free defined medium at concentrations lower than those required in serum-supplemented medium. The IC₅₀ of hypothemycin in serum-free medium and serum-supplemented medium were 0.078 μM and 0.90 μM, respectively, for HT29 cells, and 0.83 μM and >2 μM, respectively, for HCT116 cells. The MEK inhibitor U0126 also selectively inhibited the growth factor-independent growth of HT29 and HCT116 cells (Fig. 6b), but the cytotoxic anti-cancer agents paclitaxel (Fig. 6c) or doxorubicin (not shown) did not display any enhanced toxicity against the two cell lines in serum-free medium. The growth of cancer cells without MEK-ERK activating mutations was inhibited at similar concentrations of hypothemycin in the two media. For example, the IC₅₀ of the MCF7 breast cancer cell line in serum-free and serum-supplemented medium were 5.3 μM and 5.4 μM, respectively.

Hypothemycin also selectively inhibited the anchorage-independent growth of HT29 and HCT116 cells on polyHEMA over anchorage-dependent growth on plastic (Fig. 7a), with an efficacy comparable to or greater than that of U0126 (Fig. 7b). Hypothemycin did not show preference for polyHEMA to tissue culture plastic in cells without a constitutively activated MEK-ERK pathway, and cytoxic anti-cancer reagents.
Hypothemycin has been shown to potently inhibit the growth of cancer cell lines that harbor MEK-ERK pathway activating mutations.9,11,13 To further confirm the kinase selectivity of hypothemycin in cells, we examined whether hypothemycin can reverse the transformed phenotypes of human colon cancer cell lines dependent on the MEK-ERK pathway. Hypothemycin displayed enhanced activity against HT29 (a BRAF V600E mutant) and HCT116 (a KRAS G13D mutant) cells deprived of growth factors or anchorage. We obtained analogous results with another BRAF V600E mutant Colo205 and another Ki-ras mutant DLD1 (not shown). Our results suggest that in cells, hypothemycin inhibits the MEK-ERK axis with sufficient selectivity to impair the transformed growth properties of cancer cells requiring this pathway.

Hypothemycin interferes with the MEK-ERK pathway on multiple levels. This is an important feature that likely contributes to its selectivity in cells. As shown in Fig. 5, signaling pathways can be remarkably resilient. Inhibition upstream does not ensure inhibition downstream because even near complete inhibition at the level of the receptor may be restored in the process of signal transduction. Both MEK-ERK and PI3K pathways were activated by PDGF in NRK cells. Somewhat unexpectedly, phosphorylation levels of the downstream components did not decrease in proportion to PDGFR inhibition. However, the phosphorylation level of the downstream effector S6 ribosomal protein was above 60%. This indicates that the PI3K pathway can amplify a weak, almost undetectable residual activation signal and attenuate the inhibition observed at the level of the receptor. The MEK-ERK pathway was clearly more sensitive to inhibition by hypothemycin than the PI3K pathway. This is most likely because hypothemycin inhibits not only PDGFR, but also MEK, ERK and p90RSK. In contrast, none of the major components downstream of PDGFR and upstream of S6 ribosomal proteins in the PI3K pathway (PI3K, PDK1, AKT, mTOR and p70S6K) are targets of RALs.

Several cis-enone RALs such as hypothemycin, LL-Z1640-2, L-783,277, Ro 09-2210, radicicol A, and 4-O-demethylhypothemycin have been reported to inhibit protein kinases.2,5—8,14,15 Whether these compounds differ in selectivity has not been thoroughly investigated. The Ki of hypothemycin for reversible binding to protein kinases varied as much as 10000-fold.9 It is possible that slight differences in structure could cause significant differences in the inhibitory spectrum. Further modifications may lead to enhanced selectivity and broaden the application range of this new type of PDGFRs in the screen of a 124 kinase panel.9 However, PKD1 was not inhibited in any of the cellular contexts we tested. These results demonstrate that effects of protein kinase inhibitors in cells cannot always be extrapolated from in vitro assays.

RALs containing a cis-enone moiety have been reported to inhibit certain protein kinases.2,5—8,14,15 Because the inhibition was time dependent and irreversible, these compounds were speculated to bind reversibly to targets and then form Michael addition products with protein thiols. Schirmer et al. recognized that a cysteine residue adjacent the catalytically important DFG-motif is the cis-enone RAL binding site.9) Although this cysteine is contained in 46 human protein kinases, hypothemycin has been isolated as a potent and selective inhibitor of MEK. Here, we investigated the effects of hypothemycin in cells.

Hypothemycin normalized morphology of Ki-ras/NRK cells and selectively inhibited their anchorage-independent growth on polyHEMA over anchorage-dependent growth on plastic. We have tested various compounds for growth inhibition of Ki-ras-transformed cells on polyHEMA, and only MEK-inhibitors have shown noteworthy selectivity. It thus seems reasonable to suppose that hypothemycin mainly targeted the MEK-ERK pathway in Ki-ras/NRK cells. As expected, hypothemycin potently inhibited ERK phosphorylation in Ki-ras/NRK and PMA-stimulated NRK cells, but did not inhibit the autophosphorylation of PKD1, another kinase containing the target cysteine. Autophosphorylation of PKD1 in PDGF-stimulated NRK cells was also resistant to hypothemycin. PKD1 was more susceptible to hypothemycin than hypothemycin in cells.

Fig. 7. Hypothemycin (a) and U0126 (b) Selectively Inhibit Anchorage-Independent Growth of HT29 and HCT116 Cells

Cells were grown in polyHEMA-coated (closed circles, anchorage-independent growth) or uncoated (open circles, anchorage-dependent growth) tissue culture plates with inhibitors and quantified using the MTT assay as described under Materials and Methods. Each plot represents the mean of triplicate wells; bars, SD. HTM, hypothemycin.

did not show any surface selectivity in any of the cells tested (not shown). The results imply that hypothemycin blocks the MEK-ERK pathway with selectivity sufficient to impair the transformed properties of HT29 and HCT116 cells.

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kinase inhibitor that binds covalently to kinases.

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