Jpn. J. Cancer Res. **90**, 1139–1145, October 1999

Antitumor Efficacy of Hypothemycin, A New Ras-signaling Inhibitor

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We have devised a new drug screening assay to discover anti-cancer drugs which inhibit Ras-mediated cellular signals, by utilizing a Ras-responsive element (RRE)-driven reporter gene system. We found that hypothemycin, an anti-bacterial, reduces RRE-dependent transcription. Treatment of tumor cells with hypothemycin resulted in reduced expression of Ras-inducible genes, including *MMP* (matrix metalloproteinase)-*1*, *MMP-9*, transforming growth factor-β (*TGF-β*), and vascular endothelial growth factor (*VEGF*), but not that of the constitutively expressed gene, *MMP-2*. The results of zymography demonstrated that hypothemycin reduced the production of MMP-9 and MMP-3, another Ras-inducible MMP, in the culture medium. Hypothemycin selectively inhibits anchorage-independent growth of Ras-transformed cells in comparison with anchorage-dependent growth. These findings suggest that hypothemycin inhibits Ras-mediated cellular signaling. Daily treatment of tumor-bearing mice with hypothemycin resulted in significant inhibition of tumor growth. Since MMP-1, MMP-3 and MMP-9 play important roles in tumor invasion and TGF-β and VEGF are involved in tumor angiogenesis, hypothemycin is considered to be an example of a new class of antitumor drugs, whose antitumor efficacy can be at least partly attributed to inhibition of Ras-inducible genes.

Key words: Reporter gene-based assay — Ras-inhibitor — Hypothemycin — Matrix metalloproteinases — Vascular endothelial growth factor

The *ras* gene is frequently found to be mutated in pancreas, colon, and lung tumors with high incidences of 90%, 50%, and 30%, respectively, indicating that mutated Ras contributes to the development of these carcinomas.[1, 2] Ras and Ras-derived cellular signals have been considered as promising targets for novel anticancer drugs.[3, 4]

As farnesylation is required for the oncogene activity of Ras, numerous FTase inhibitors have been developed and show antitumor activities *in vitro* and *in vivo*.[5] Some FTase inhibitors have been studied in clinical trials.[6] However, they were not very effective against tumors carrying mutated K-Ras4B,[7] which has been most frequently found in pancreas, colon and lung tumors.[1] This insensitivity was explained by compensatory geranylgeranylation of K-Ras4B.[8] It was also suggested that the antitumor effect of FTase inhibitors was dependent on the inhibition of prenylation of RhoB, not Ras.[9]

The downstream molecules of Ras, such as MEK, MAPK, Rho, and PI3K, have been extensively studied as target molecules for blocking Ras-derived signals.[4, 10] The inhibitors of these molecules showed anti-Ras activities and antitumor activity in *in vivo* experiments.[4]

We and others have searched for anti-Ras inhibitors which can re-normalize the transformed morphology of cultured cells carrying activated Ras and other oncogenes.[4, 11] We have discovered trichostatin A,[12] depudecin,[13] trapoxin A,[14] and oxamflatin,[15] although the mechanisms by which these molecules reverse the transformation-induced morphological change have not been clarified to date. Unfortunately they show limited antitumor efficacy *in vivo*.

Transcriptional activation of cellular genes by mutated Ras is known to be responsible for the malignant phenotype of certain tumors. These genes include *MMP-1*, *MMP-3*, *MMP-9*, *TGF-β*, and *VEGF*, which play pivotal roles in invasion, angiogenesis, and metastasis.[16, 17] Transcriptional activation of these genes was mostly mediated by RRE or an RRE-like element,[18, 19] which had been originally identified as the *cis*-element responsible for transcriptional activation by Ras in the polyoma virus enhancer.[20] We constructed an RRE-driven luciferase reporter gene expression plasmid and transfected it into *v*-K-*ras* transformed NIH3T3 (DT) cells.[21] Utilizing this cell line, we established an assay system to search for inhibitors of Ras-mediated cellular signals. We found that hypothemycin, a known product of *Hypomyces trichothecoides* with anti-bacterial activity,[22] can selectively inhibit RRE-dependent transcriptional activation.

Hypothemycin reduced the transcription of several Ras-inducible genes, such as *MMP-1*, *MMP-9*, *TGF-β*, and

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The abbreviations used are: FTase, farnesyl-protein transferase; MEK, MAP kinase/ERK kinase kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidyl-inositol-3 kinase; MMP, matrix metalloproteinase; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; RRE, Ras responsive element; CPT, camptothecin; NVL-3, NIH VL30 clone 3; CAT, chloramphenicol acetyltransferase; IR, inhibition rate; IC_{50}, 50% inhibition concentration; CDDP, *cis*-diammine dichloroplatinum.

VEGF, in mouse colon carcinoma cells and selectively inhibited anchorage-independent growth of Ras-transformed cells in soft agar culture. Hypothemycin also suppressed the growth of three different mouse and human tumor cells which were transplanted into the backs of mice. Our results suggest that hypothemycin exhibits antitumor activity by down regulation of the Ras-inducible genes.

MATERIALS AND METHODS

Materials Hypothemycin (7′-dehydro-4′,5′-dihydroxy-1′:2′-epoxyzearalenone 4-methyl ester) was purified from the culture broth of *H. trichothecoides* in our laboratory. CPT was purchased from Sigma Chem. Co. (St. Louis, MO).

Plasmid construction The pGV-P luciferase reporter plasmid was purchased from Toyo Ink (Tokyo). An oligonucleotide of RRE (CAGGATATGACTCT, nucleotide sequence from the mouse *NVL-3* gene[23]) was chemically synthesized, and three copies were inserted upstream of the SV40 minimal promoter of pGV-P, giving pRRE3-luc.

Cells and culture condition NIH3T3 cells were provided by Dr. K. C. Robbins (National Institute of Health, Bethesda, MD). DT cells (NIH3T3 subline transformed by *v*-K-*ras* gene) were provided by Dr. M. Noda (Kyoto Univ., Kyoto).[21] Two stable cell lines designated DT-C and DT-R were established by transfecting the reporter plasmid into DT cells. DT-C cells and DT-R cells had a minimal-SV40-promoter-driven luciferase transcription unit (pGV-P) and an RRE-regulated luciferase transcription unit (pRRE3-luc), respectively. Human lung squamous carcinoma Ma44 cells and murine colon adenocarcinoma Colon 26 cells were obtained from Dr. T. Komiya (Kinki Univ., Osaka) and Dr. T. Tsuruo (Tokyo Univ., Tokyo), respectively. Human colon adenocarcinoma HCT116 was purchased from the American Tissue Culture Collection (Rockville, MD). All cell lines were maintained in Dulbecco's modified minimal essential medium containing 60 μg/ml kanamycin and supplemented with 10% (v/v) fetal calf serum (Hyclone, Logan, UT) under humidified air with 5% CO_2 at 37°C.

Luciferase-reporter gene assay For transient transfection assay, 2×10^5 cells were seeded in 6-cm-diameter culture dishes and incubated overnight. Plasmids were transfected into the cells with Lipofectamine reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. After a further 24 h incubation, the cells were lysed with lysis buffer PGC-50 (Toyo Ink, Tokyo). Aliquots of sample were mixed with ATP-luciferin solution (Pickagene Luminescence Kit PGL2000) (Toyo Ink) and the luminescence was measured with a luminometer, Lumat LB9501 (Berthold, Bad Wildbad, Germany). In each transfection, SV40-promoter-driven CAT expression vector was co-transfected and the amount of CAT was quantified by CAT ELISA (Boehringer Mannheim, Mannheim, Germany) to allow correction for transfection efficiency.

For large-scale screening, 2500 cells/well of DT-C or DT-R cells were seeded into 96-well multi-plates and incubated overnight. Test compounds were added to each well at various concentrations in a 3-fold dilution series. After a further 24 h incubation, the cells were lysed with lysis buffer PGC-50. The samples were transferred to opaque 96-well multi-plates (Microlite 1 (Dynateck, Cantilly, VA)) for luminescence measurement. ATP-luciferin solution (50 ml/well) was added and the luminescence was measured with a luminometer CT9000D (Dia-Iatron, Tokyo).

Northern analysis RNA extraction and northern analysis were performed as described previously.[24] The blots were stained with 0.02% methylene blue-0.3 *M* sodium acetate solution to verify the amount loaded on the gels. Quantitative analysis was performed with a bio-imaging analyser, BAS-2000II (Fuji Photo Film, Tokyo).

Zymography Zymography was performed as described previously.[24] Gelatin and α-casein were used as substrates for MMP-2, -9 and MMP-3 assays, respectively.

Colony-forming assay The colony-forming assay was carried out as described elsewhere.[15] Briefly, DT cells were pretreated with hypothemycin for 24 h. These pretreated cells were further cultured either in liquid medium or soft agar medium containing 0.4% agarose in the presence of hypothemycin. As a control, pretreated cells were cultured without hypothemycin. After 8 days of culture, the colonies were stained with crystal violet and the number of colonies was counted.

***In vivo* therapeutic experiments** Antitumor efficacy was evaluated as described previously.[25] In all experiments, 8 mice were employed per group. On day 0, 1×10^6 Colon 26 cells, Ma44 cells, or 1×10^7 HCT116 cells were transplanted i.d. into the backs of the mice. Six-week-old female BALB/c mice (Japan SLC, Shizuoka) were used for the Colon 26 experiment, and 6-week-old female BALB/c-*nu*/*nu* mice (Clea Japan, Tokyo) for the Ma44 and HCT116 experiments. Hypothemycin was suspended in vehicle (0.4% Tween 80, 0.5% carboxymethylcellulose, and 0.9% benzyl alcohol) and was injected i.p. once a day from day 1 to day 14 (Colon 26 and Ma44 experiments) or from day 5 to day 18 (HCT116 experiment). All studies were performed under the guidelines and with the approval of the Shionogi Animal Care and Use Committee. Tumor volume and body weight were scored throughout each experiment. The growth-inhibitory effect was estimated from the IR (%) = (1 − tumor volume of hypothemycin-treated mouse / tumor volume of vehicle-treated mouse) × 100). The statistical significance of differences was evaluated with Dunnet's test.[26]

RESULTS

Ras-dependent transcriptional activation via RRE The luciferase reporter gene expression vector, pGV-P or pRRE3-luc, and the human activated K-Ras expression vector, pEF-H-K-ras, or mock vector, pEF-BOS, were co-transfected into NIH3T3 cells. Co-expression of active c-K-Ras resulted in 5-fold enhancement of the luciferase activity in pRRE3-luc-transfected cells, although it failed to enhance the luciferase activity in pGV-P-transfected cells (Fig. 1). These results demonstrated that activated Ras selectively enhanced the RRE-dependent transcription.

Suppression of RRE-dependent transcription by hypothemycin We transfected pGV-P and pRRE3-luc into activated K-Ras-transformed NIH3T3 cells, DT cells, and obtained two stable cell lines, DT-C and DT-R cells, respectively. In large-scale screening utilizing these two cell lines, we found that hypothemycin selectively suppressed the luciferase transcription in DT-R cells in comparison with DT-C cells. The IC_{50} values of transcriptional suppression by hypothemycin were 0.11±0.01 μg/ml and 1.56±0.24 μg/ml in DT-R cells and DT-C cells, respectively (Fig. 2a). Cytotoxic anticancer reagents, for example, the topoisomerase I inhibitor CPT, did not show selective inhibition of RRE-dependent reporter gene expression (Fig. 2b). The inhibitory activity of hypothemycin on RRE-dependent transcription was then confirmed in a transient transfection experiment using the parent cell line, DT. As shown in Fig. 3, RRE-dependent activation of transcription of the reporter gene was concentration-dependently suppressed by hypothemycin in DT cells.

Reduction of transcripts of Ras-inducible genes by hypothemycin We examined whether hypothemycin would reduce the transcripts of Ras-inducible genes by northern analysis. Colon 26, a murine colon adenocarcinoma cell line, was used in this study. We found that codon 12 of the *c*-K-*ras* gene in Colon 26 cells was mutated from GGT (Gly) to GTT (Val) (data not shown). RRE-dependent activation of transcription was detected in Colon 26 cells as well as in DT cells in the transient luciferase-reporter gene assay (data not shown). Northern analysis showed that the transcripts of three Ras-inducible genes, mouse retrotransposon *NVL-3*, *MMP-1*, and *VEGF*, were clearly reduced in the cells treated even with 0.1 μg/ml hypothemycin (Fig. 4). Although it seemed that reduction of transcripts was modest with the other two Ras-inducible genes, *MMP-9* and *TGF-β*, quantitative analysis revealed that the treatment with 0.3 μg/ml hypothemycin reduced the transcripts to 48% and 78%, respectively, while it caused no significant degradation of 28S ribosomal RNA. On the other hand, the transcript of *MMP-2*, a constitutively expressed gene,[27] did not decrease, but

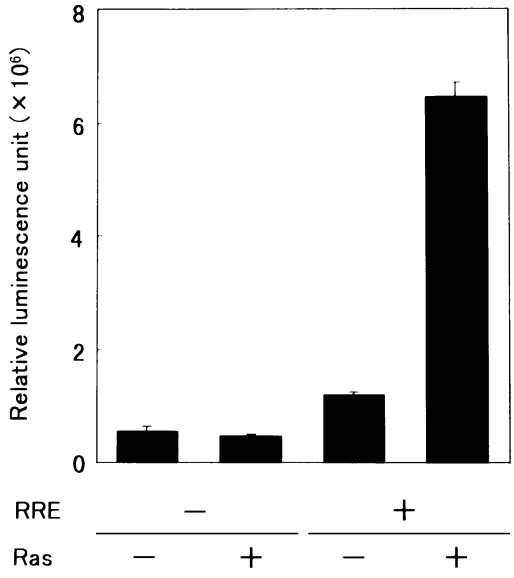

Fig. 1. RRE activation by Ras. pGV-P or pRRE3-luc was transfected into NIH3T3 cells. pEF-BOS or pEF-H-K-ras was co-transfected. Mean values of relative luminescence units from three independent transfections and the SD are shown.

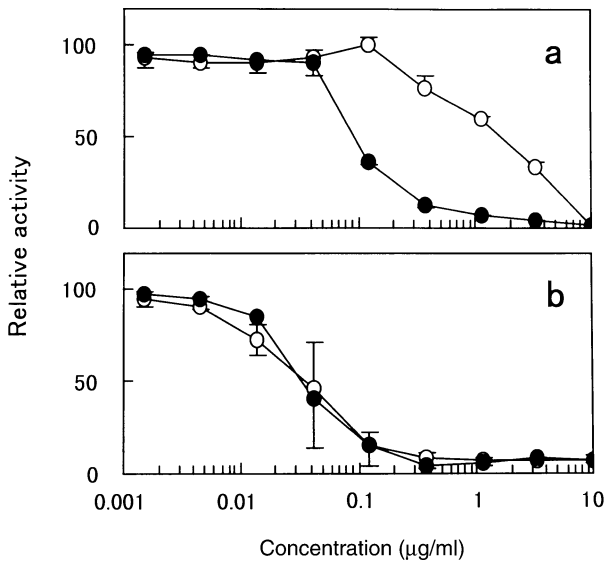

Fig. 2. Selective inhibition of RRE-mediated transcription by hypothemycin. Relative luciferase activity of DT-C cells (○) or DT-R cells (●) was calculated as a percentage of the relative luminescence units of each cell line with no drug added. The abscissa shows the concentration of hypothemycin (a) or CPT (b). Mean values of triplicate experiments and the SD are shown.

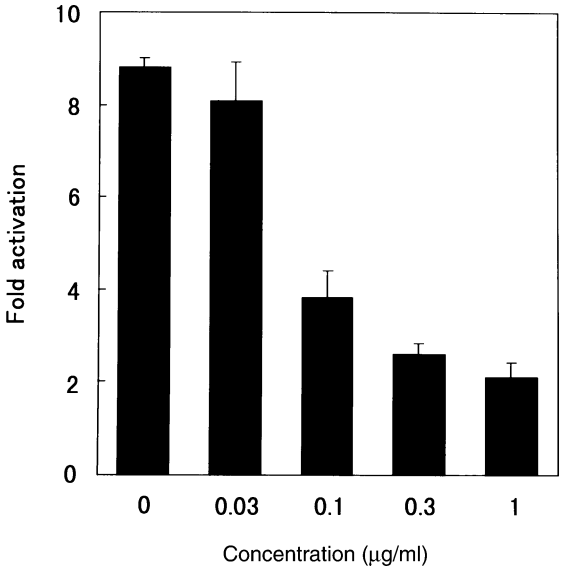

Fig. 3. Inhibition of RRE activation by hypothemycin in transient transfection experiments. Activation was calculated as the ratio of the relative luminescence units of pRRE3-luc-transfected DT cells compared to pGV-P-transfected DT cells. Mean values from three independent transfections and the SD are shown. Abscissa shows the concentration of hypothemycin.

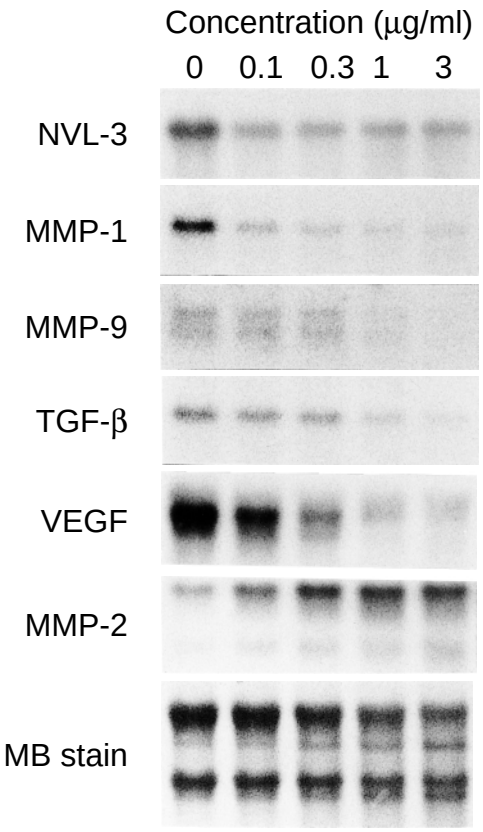

Fig. 4. Reduction of Ras-inducible gene transcription by hypothemycin. Murine colon adenocarcinoma cells, Colon 26, were treated with 0, 0.1, 0.3, 1, or 3 μg/ml of hypothemycin for 24 h. Total RNA (5 μg) from cells under each condition was electrophoresed and blotted. Radiolabeled cDNA probes are indicated at the left side. The lowest row shows the methylene blue-stained blot as confirmation of the quantities of RNA loaded.

rather increased to 194%. Thus it was confirmed that hypothemycin selectively reduced the expression of some Ras-inducible genes in tumor cells having an activated c-k-*ras* gene.

Reduction of metalloproteinase production in culture by hypothemycin We next studied the reduction of the production of Ras-inducible MMPs of Colon 26 in culture cells by zymography. Gelatin zymography (Fig. 5a) showed that the amount of MMP-9 in culture supernatants was reduced with an increasing concentration of hypothemycin, while the amount of MMP-2 was decreased only at high concentration. In casein zymography (Fig. 5b), the amount of MMP-3, another Ras-inducible MMP,[16] was also reduced with as little as 0.01 μg/ml of hypothemycin. The decrease in expression of Ras-inducible genes due to hypothemycin was thus confirmed at the translational level, as well as at the transcriptional level.

Inhibition of anchorage-independent growth by hypothemycin Since anchorage-independent growth is one of the malignant features of tumor cells, including Ras-transformed cells, we examined the growth-inhibitory activity of hypothemycin against Ras-transformed cells under both anchorage-dependent and anchorage-independent culture conditions. Treatment of DT cells with hypothemycin preferentially inhibited the growth in soft agar medium (Fig. 6). Reduction of colony-forming efficiency at 0.5 μg/ml hypothemycin was 97% in soft agar medium, but 17% in liquid culture. These results demonstrated that hypothemycin selectively inhibited anchorage-independent growth of tumor cells.

Antitumor effect of hypothemycin *in vivo* The antitumor efficacy of hypothemycin was evaluated *in vivo*. As shown in Fig. 7a, daily treatment with 12.5 mg/kg hypothemycin significantly inhibited the growth of Colon 26 tumor with an IR of 54% at day 16 ($P<0.05$). A dose-dependent growth-inhibitory activity of hypothemycin was also observed in two human tumor models. Both human cell lines used here (Ma44 cells and HCT116 cells) have activated *c*-K-*ras* genes (Dr. T. Komiya, personal communication).[2] Treatment with 12.5 mg/kg and 25 mg/kg hypothemycin significantly reduced the Ma44 tumor growth with IR values of 48% and 63%, respectively, at day 15 ($P<0.01$) (Fig. 7b). In the HCT116 tumor model, 25 mg/kg hypothemycin significantly inhibited the tumor

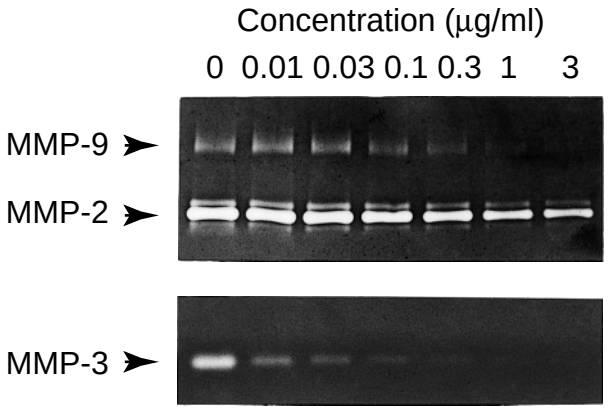

Fig. 5. Reduction of matrix metalloproteinase production by hypothemycin in culture medium. Murine colon adenocarcinoma cells, Colon 26, were treated with 0, 0.01, 0.03, 0.1, 0.3, 1, or 3 μg/ml of hypothemycin for 24 h. Conditioned medium (5 μl/well or 19 μl/well) was loaded onto the gel and analyzed by means of gelatin zymography (a) or α-casein zymography (b), respectively. The detected signals were identified on the basis of their relative molecular weights (arrowheads).

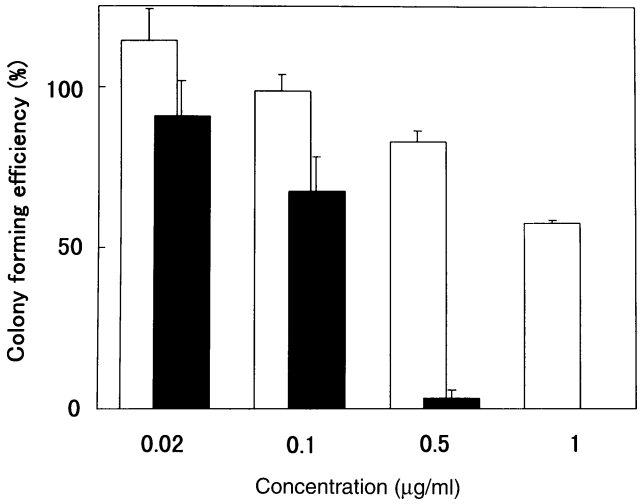

Fig. 6. Inhibition of anchorage-independent growth of transformed cells by hypothemycin. DT cells were treated with 0, 0.02, 0.1, 0.5, or 1 μg/ml of hypothemycin in both liquid (open bar) and soft agar (solid bar) media. Mean colony-forming efficiency of triplicate experiments and the SD are shown.

growth with an IR of 57% at day 19 ($P<0.01$) (Fig. 7c). During these treatments, no mouse died due to toxicity and body weight loss was within a tolerable range (less than 20% of initial body weight). These results demonstrate that hypothemycin, an inhibitor of Ras-inducible

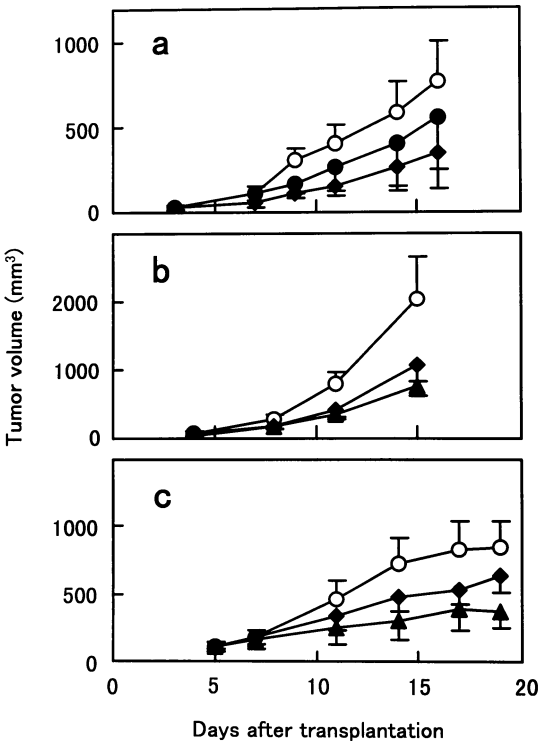

Fig. 7. Inhibition of tumor growth *in vivo* by hypothemycin. Mice were treated with vehicle only (○) or with 6.25 mg/kg (●), 12.5 mg/kg (◆), or 25 mg/kg (▲) of hypothemycin as described in "Materials and Methods." Mean tumor volume and SD are shown. The following tumor cells were used: a, Colon 26; b, Ma44; c, HCT116.

gene expression, exerted a potent antitumor efficacy *in vivo*.

DISCUSSION

We have devised a new reporter gene-based assay for Ras inhibitors. As it is cell-based, compounds with general cytotoxicity are excluded by the comparison of inhibition rates of luciferase activity in DT-C cells and DT-R cells. To validate the assay, we tested known commercial antitumor drugs and found that compounds targeted at DNA (adriamycin, mitomycin C, CDDP) or DNA-processing enzymes (CPT, etoposide) gave negative results. Among the mitosis inhibitors, vinca alkaloids were slightly active, while taxanes were not. Thus, with stringent criteria, this assay was proved to be useful for screening a new class of antitumor drugs.

Kumar *et al*. previously reported a reporter-gene based assay for Ras inhibitors,[28] which would restore the Ras-suppressed transcription of α-actin promoter, and identified a pyrazoloquinoline, SCH51344.[29] However, neither

reduction of the expression of Ras-inducible genes nor antitumor activity in an animal model was reported for SCH51344.

Hypothemycin is an antibacterial agent[22] with growth-inhibitory activity against several tumor cell lines *in vitro*.[30] In this study, we have shown that hypothemycin selectively inhibits anchorage-independent growth of Ras-transformed cells in comparison with anchorage-dependent growth (Fig. 6). These results suggest that the growth-inhibitory effect of hypothemycin is not due to general cytotoxicity but to blockade of the transforming signals derived from Ras.

Hypothemycin reduced the expression of some Ras-inducible genes. It was observed that the degree of reduction of each gene is variable (Fig. 4). We considered that this was due to differences of Ras-signaling dependency of transcription of the genes. Among the Ras-inducible genes whose expression was inhibited by hypothemycin (Fig. 4), *VEGF* is the only gene in whose promoter region no RRE element has been reported.[31] However it was demonstrated that the Ras-mediated signal increased the expression of VEGF[32] not only by enhancing transcription, but by increasing the stability of the transcripts.[33] Our results suggest that hypothemycin can inhibit Ras-inducible genes other than these with RRE.

In contrast to the Ras-inducible genes, transcription of MMP-2 was not inhibited, or was even induced by hypothemycin treatment (Fig. 4). *MMP-2* gene is constitutively transcribed and no RRE element has been reported in its promoter region.[27] Thus, hypothemycin selectively suppressed the transcription of Ras-inducible genes. The mechanism of induction of *MMP-2* transcript is unknown. But gelatin zymography revealed that this increase of *MMP-2* transcript was not reflected at protein level (Fig. 5a), indicating the existence of a mechanism to maintain the regular expression level of MMP-2.

MMP-1, MMP-3 and MMP-9 are known to be involved in tumor invasion into surrounding tissue and metastasis.[16] TGF-β and VEGF play important roles in tumor angiogenesis.[17] It was suggested that reduced expression of these genes in tumors leads to inhibition of invasion and angiogenesis *in vivo*. Thus, we speculate that hypothemycin may exert its antitumor efficacy by reducing the expression of Ras-inducible genes, in addition to its growth-inhibitory effect. We are examining whether this effect of hypothemycin, reducing the expression of Ras-inducible genes, contributes to its growth-inhibitory effect *in vivo*.

ACKNOWLEDGMENTS

We thank T. Tanimoto, H. Tani, H. Hinoo, and T. Kamigauchi for providing hypothemycin and Dr. Robert M. Hoffman for a critical review of this paper.

(Received May 14, 1999/Revised June 30, 1999/Accepted July 7, 1999)

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