RAPID COMMUNICATION Jpn. J. Cancer Res. 83, 324–328, April 1992

A Novel Tetracyclic Peptide, Trapoxin, Induces Phenotypic Change from Transformed to Normal in *sis*-Oncogene-transformed NIH3T3 Cells

Hiroshi Yoshida and Kenji Sugita[1]

Division of Oncology, Department of Microbiology, Shionogi Research Laboratories, Shionogi & Co., Ltd., 4-12, Sagisu 5-chome, Fukushima-ku, Osaka 553

A novel tetracyclic peptide, trapoxin [cyclo(L-phenylalanyl-L-phenylalanyl-D-pipecolinyl-L-2-amino-8-oxo-9,10-epoxy-decanoyl)], was found to induce the flat phenotype in v-*sis*-transformed NIH3T3 cells at a quite low concentration of 1 ng/ml. Actin stress fiber could be detected after trapoxin treatment. Almost complete reversion into the flat phenotype was observed at 6 h after the administration of the compound. The effect of trapoxin was reversible, when the cell culture was incubated for more than 24 h after its removal. The intracellular level of *sis*-mRNA did not decrease with trapoxin treatment at a concentration (50 ng/ml), sufficient to reverse the transformed morphology. Substitution of pipecolinic acid with proline in trapoxin did not change the activity. WF3161, in which leucine was substituted for a phenylalanine of trapoxin, showed only one-sixteenth of the activity of trapoxin. Reduction of the epoxide residue of trapoxin destroyed the activity.

Key words: Trapoxin — Morphological reversion — *sis* oncogene — Epoxide

Recent extensive studies have revealed a close correlation of oncogenes with carcinogenesis.[1] *sis* is an oncogene possessing a sequence homologous to a gene encoding platelet-derived growth factor (PDGF)[2] and is expressed in some tumor cell lines derived from osteosarcoma and gliomas.[3,4] The *sis*-oncogene product is assumed to confer the characteristic cellular morphology, as well as the ability of autocrine growth.[5] The transformed phenotype induced by overexpression of c-*sis* is similar to that induced by v-*sis*,[6] indicating that they might induce the transformation of the cells through the same mechanism, that is, binding to the receptor of PDGF. During screening for an agent inducing flat reversion of the morphology of v-*sis*-transformed NIH3T3 cells (a kind gift from Dr. K. C. Robbins, National Institute of Health, USA), we found that a novel tetracyclic peptide, trapoxin (Fig. 1a), produced by a fungus, showed an extremely strong activity with low toxicity against v-*sis*-transformed cells.[7] In this paper, we describe the biological activity and the structure-activity correlation of trapoxin.

We found that trapoxin induced phenotypic change from transformed to normal in v-*sis*-transformed NIH-3T3 cells over a wide range of concentration (1–100 ng/ml), while it did not significantly change the morphology of normal NIH3T3 cells at a concentration of 100 ng/ml. Conversely, trapoxin inhibited the growth of the normal and v-*sis*-transformed NIH3T3 cells at a higher concentration (the concentration for 50% inhibition of cell growth is 200 ng/ml). The effect of trapoxin was reversible when the cells were treated for 24 h with trapoxin (8 ng/ml) to make them flat and further incubated for more than 24 h after the removal of trapoxin (Fig. 2). To find out how long the cells have to be exposed to trapoxin to make their shape flat, the morphological change was followed after addition of trapoxin to v-*sis*-transformed cells. The morphological change into a flat phenotype was observed in more than 75% of the cells after exposure for more than 6 h, whereas it was observed in less than 50% at 4 h (Fig. 3). The flat cells induced by trapoxin exhibited contact inhibition, which is one of the characteristics of normal cells (data not shown). In general, one of the most characteristic phenotypes of transformed cells is the loss of bundles of actin stress fiber.[8] Therefore, we examined the appearance of the actin stress fiber in v-*sis*-transformed cells after treatment with trapoxin. Under fluorescence microscopy, the clear bands of actin stress fiber were observed in v-*sis*-transformed cells after trapoxin treatment at the concentration of 8 ng/ml for 24 h, but not in the control culture without the treatment (Fig. 4). To determine whether the morphological reversion was caused by the decrease in *sis*-mRNA level, we measured the amount of *sis*-mRNA by the Northern blotting method using a v-*sis* oncogene probe (1.23 kbp, Takara Shuzo, Kyoto) in v-*sis*-transformed cells with and without trapoxin treatment. Trapoxin did not decrease the level of *sis*-mRNA at less than 50 ng/ml, at which concentration the transformed phenotype was clearly reversed (Fig. 5).

[1] To whom correspondence should be addressed.

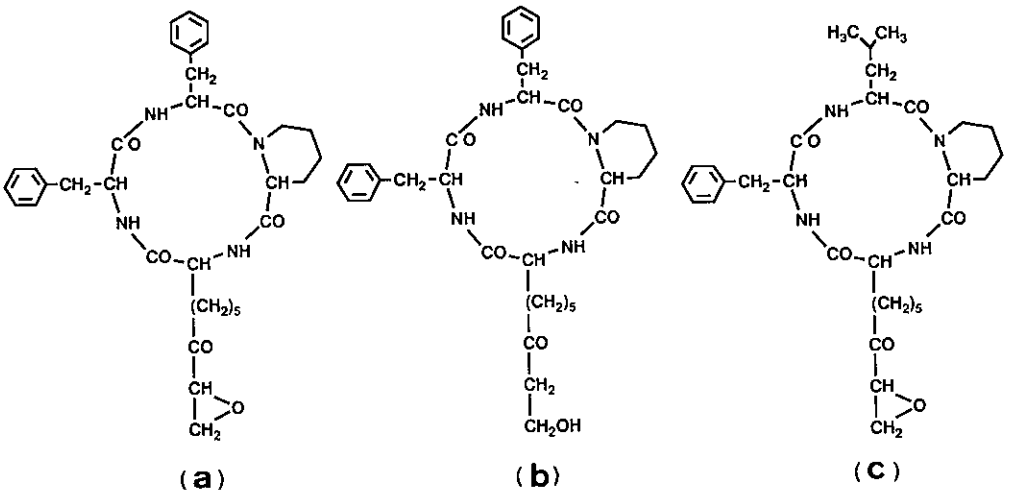
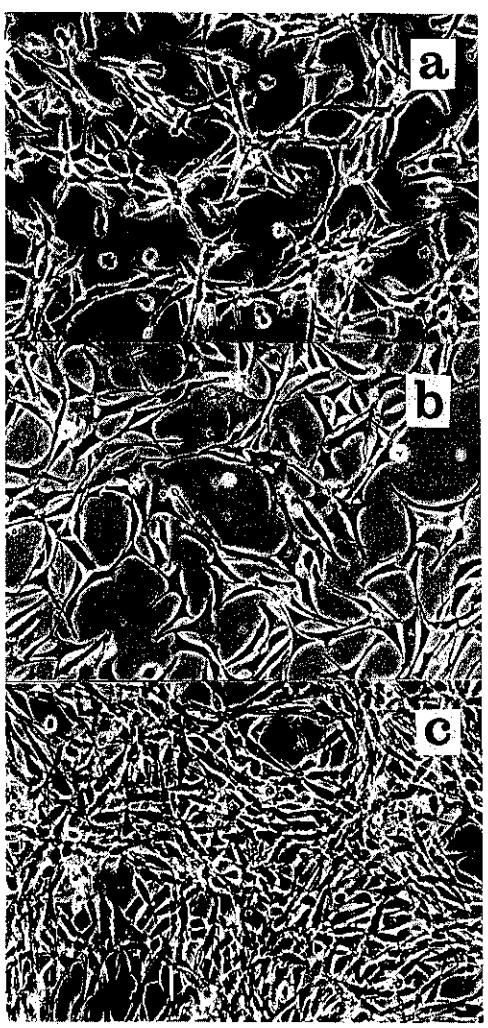


Fig. 1. Structures of trapoxin and related compounds. (a) trapoxin, (b) reduced form of trapoxin, and (c) WF3161.



To determine what structural features of trapoxin are important for activity in inducing the flat phenotype, we prepared related compounds and compared their activities with that of trapoxin. Reduction of the epoxide residue (Fig. 1b) destroyed the activity; the product was inactive even at the concentration of 250 ng/ml. WF-3161[9] (Fig. 1c, a kind gift from Dr. Y. Hori, Fujisawa Pharmaceutical Co., Ltd., Tokyo), in which leucine was substituted for a phenylalanine of trapoxin, exhibited only one-sixteenth of the activity of trapoxin. However, replacement of pipecolinic acid of trapoxin with proline did not decrease the activity.

There are several reports on agents which induce phenotypic change from the morphology of various oncogene-transformed cells to the normal flat morphology,[10-15] such as herbimycin for *src* tyrosine kinase,[10] erbstatin for tyrosine kinase of epidermal growth factor,[11] and azatyrosine for *ras*-transformed cells.[12] Concerning inhibitors of *sis*-transformed cells, only the heterocyclic polyanionic compound suramin[16] has been reported. This compound blocks the binding of PDGF to the receptor on the cell membrane,[17] although it requires a high concentration (more than 200 μg/ml) to show this activity and its inhibition is not specific to PDGF, as it inhibits not only the binding of PDGF, but also that of bFGF.[18]

Fig. 2. Induction of the flat morphology of v-*sis*-transformed NIH3T3 cells by trapoxin and reversion to the transformed phenotype after its removal. The v-*sis*-transformed NIH3T3 cells were inoculated into petri dishes (ϕ 6 cm, Falcon) at 2.5×10^4 cells/ml in 5 ml of D-MEM (10% FBS) and cultured overnight in a 5% CO_2 incubator and then for 24 h in the absence (a) or the presence (b) of trapoxin (8 ng/ml) and further incubated for 48 h after its removal (c).

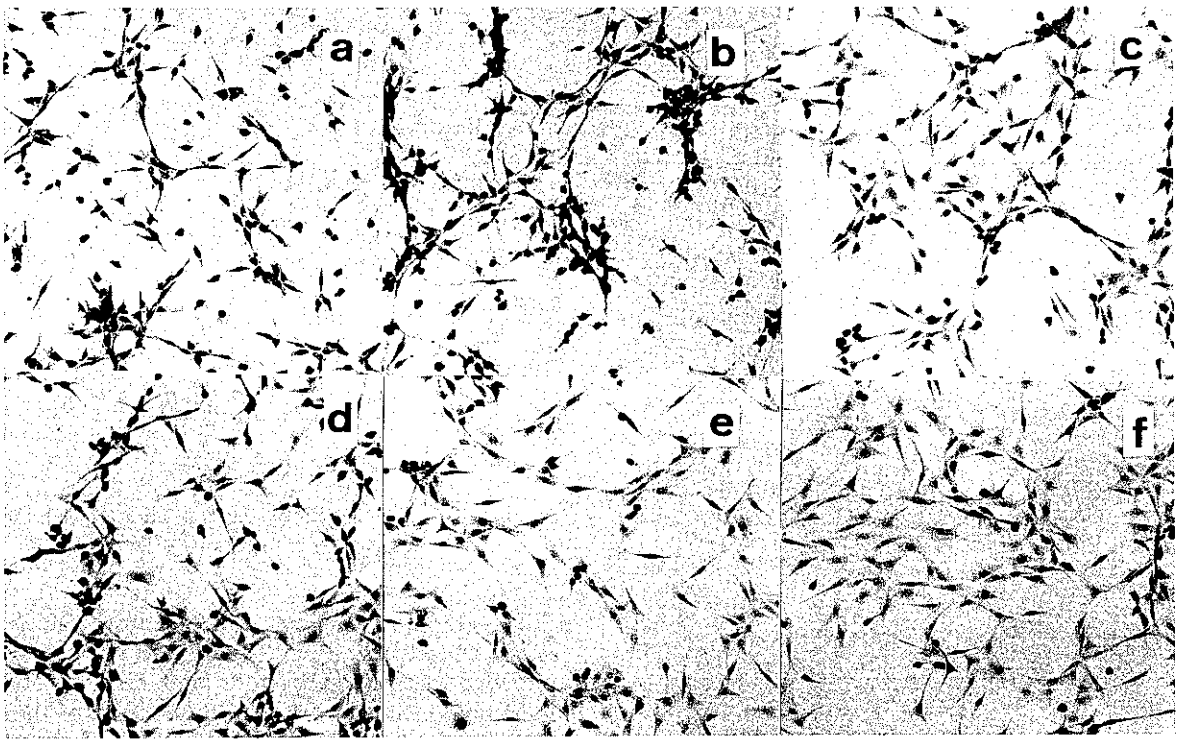
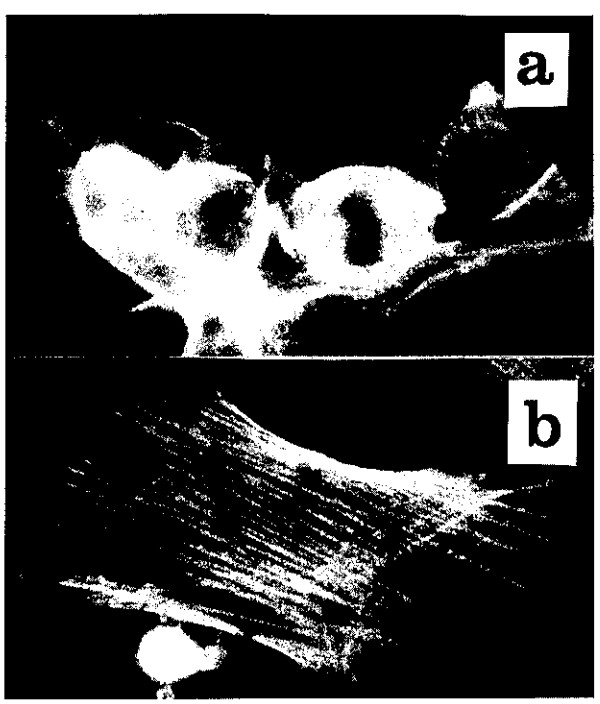


Fig. 3. Kinetics of trapoxin activity to reverse the phenotype of v-*sis*-transformed NIH3T3 cells. The morphology of the cells was observed at 0 h (a), 2 h (b), 4 h (c), 6 h (d), 8 h (e), and 10 h (f) after the addition of trapoxin (8 ng/ml).



We found that trapoxin, a novel tetracyclic peptide which was isolated from the fungus *Helicoma ambiens*,[7] induced the flat phenotype of v-*sis*-transformed cells at a much lower concentration (1 ng/ml) than that of suramin. Trapoxin did not induce the normal flat morphology in any other oncogene-transformed cells tested (v-Ha-*ras*-, v-*src*-, v-*fos*-, v-*abl*-, and v-*fgr*-transformed cells) (data not shown). Thus, trapoxin seemed to be a specific inducer of the normal flat phenotype of v-*sis*-transformed cells. A little earlier, we had found that trichostatin A (TSA), an inhibitor of histone deacetylase,[19] changed the abnormal piled-up morphology of v-*sis*-transformed NIH3T3 cells to normal flat morphology after exposure for more than 6 h, and the morphology induced by TSA was similar to that induced by *n*-butyrate.[20] Therefore, we compared the biological activity of trapoxin with that of TSA, and found that they

Fig. 4. Restoration of actin stress fiber in v-*sis*-transformed cells with trapoxin treatment. For fluorescence microscopy, actin stress fiber in the cells was observed after staining with rhodamine-phalloidin.[8] (a) Untreated cells. (b) Cells treated with trapoxin (8 ng/ml) for 24 h.

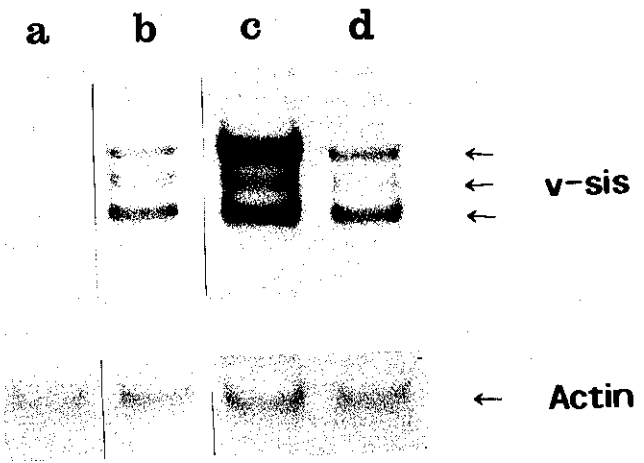


Fig. 5. No decrease of *sis*-mRNA level by trapoxin. Normal (a) and v-*sis*-transformed (b–d) NIH3T3 cells were inoculated into a 75 cm^2 bottle (Falcon) at 5×10^4 cells/ml in 15 ml of D-MEM, and incubated for 48 h with 10 ng/ml (c), and 50 ng/ml (d) of trapoxin. Preparation of total RNA from v-*sis*-transformed and normal NIH3T3 cells with and without trapoxin treatment, electrophoretic separation of RNA, and Northern blotting followed the methods reported previously.[21,22]

were similar in several properties: the induction of the flat morphology, the short time required to show the activity, the reversibility of action, and the absence of a decrease in *sis*-mRNA level. Furthermore, a preliminary experiment showed that the TSA-resistant mutant cells (a kind gift of Prof. T. Beppu, University of Tokyo) which possess a mutation in histone deacetylase,[19] were also resistant to trapoxin. All these results suggest that the target molecule of trapoxin might also be involved in the histone acetylation-deacetylation equilibrium. The epoxide residue is critical for the activity, since reduction of this portion apparently destroyed the activity. Furthermore, substitution of a phenylalanine of trapoxin with leucine caused significant reduction of the activity (1/16). Since trapoxin probably binds to protein(s) through its epoxide residue, the next step will be to determine the immediate target of trapoxin.

The authors wish to thank Dr. T. Beppu, Dr. M. Yoshida and Dr. H. Igarashi for their discussion, Dr. H. Itazaki, Mr. K. Nagashima, and Mr. K. Koizumi for their help, and also Dr. T. Yoshida for his support during this work.

(Received November 7, 1991/Accepted January 11, 1992)

REFERENCES

1) Bishop, J. M. Molecular themes in oncogenesis. *Cell,* **64,** 235–248 (1991).
2) Robbins, K. C., Antoniades, H. N., Devare, S. G., Hunkapiller, W. and Aaronson, S. A. Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. *Nature,* **305,** 605–608 (1983).
3) Heldin, C. H., Johnsson, A., Wennergren, S., Wernsted, C., Betsholtz, C. and Westermark, B. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chain. *Nature,* **319,** 511–514 (1986).
4) Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. C., Eddy, R., Show, T. B., Philpott, K., Mellor, A. L., Knot, T. J. and Scott, J. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumor cell lines. *Nature,* **320,** 695–699 (1986).
5) Bishop, J. M. Viral oncogene. *Cell,* **42,** 23–38 (1985).
6) Gazit, A., Igarashi, H., Chiu, I.-M., Srinnivasan, A., Yaniv, A., Tronick, R. S., Robbins, K. C. and Aaronson, S. A. Expression of the normal human *sis*/PDGF-2 coding sequence induces cellular transformation. *Cell,* **39,** 89–97 (1984).
7) Itazaki, H., Nagashima, K., Sugita, K., Yoshida, H., Kawamura, Y., Yasuda, Y., Matsumoto, K., Ishii, K., Uotani, N., Nakai, H., Terui, A., Yoshimatsu, S., Ikenishi, Y. and Nakagawa, Y. Isolation and structural elucidation of new cyclotetrapeptides, trapoxins A and B, having detransformation activities as antitumor agents. *J. Antibiot.,* **12,** 1524–1532 (1990).
8) Pollack, R., Osborn, M. and Weber, K. Pattern of organization of actin and myosin in normal and transformed cultured cells. *Proc. Natl. Acad. Sci. USA,* **72,** 994–998 (1975).
9) Umehara, K., Nakahara, K., Kiyoto, S., Iwami, M., Okamoto, M., Tanaka, H., Kohsaka, M., Aoki, H. and Imanaka, H. Studies on WF-3161, a new antitumor antibiotic. *J. Antibiot.,* **36,** 478–483 (1983).
10) Uehara, Y., Hori, M., Takeuchi, T. and Umezawa, H. Screening of agents which convert 'transformed morphology' of Rous sarcoma virus-infected rat kidney cells to 'normal morphology': identification of an active agent as herbimycin and its inhibition of intracellular *src* kinase. *Jpn. J. Cancer Res.,* **76,** 672–675 (1985).
11) Umezawa, H., Hori, T., Tajima, H., Imoto, M., Ishiki, K. and Takeuchi, T. Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS Lett.,* **260,** 198–200 (1990).
12) Shindo-Okada, N., Makabe, O., Nagahara, H. and Nishimura, S. Permanent conversion of NIH3T3 cells transformed by activated c-Ha-*ras,* c-Ki-*ras,* N-*ras,* or c-*raf,* and of human pancreatic adenocarcinoma containing activated c-Ki-*ras* to apparently normal cells by treatment with the antibiotic azatyrosine. *Basic Life Sci.,* **52,** 309–312 (1990).

13) Itoh, O., Kuroiwa, S., Atsumi, S., Umezawa, K., Takeuchi, T. and Hori, M. Induction by the guanosine analogue oxanosine of reversion toward the normal phenotype of Ki-*ras*-transformed rat kidney cells. *Cancer Res.*, **49**, 996–1000 (1989).
14) Samid, D., Flessate, D. M. and Friedman, R. M. Interferon-induced revertants of *ras*-transformed cells; resistance to transformation by specific oncogenes and retransformation by 5-azacytidine. *Mol. Cell Biol.*, **7**, 2196–2200 (1987).
15) Sakai, R., Ikeda, I., Kitani, H., Fujiki, H., Takaku, F., Rapp, U., Sugimura, T. and Nagao, M. Flat reversion by okadaic acid of *raf* and *ret*-II transformants. *Proc. Natl. Acad. Sci. USA*, **86**, 9946–9950 (1989).
16) Hawking, F. Suramin: with special reference to onchocerciasis. *Adv. Pharmacol. Chemother.*, **15**, 289–322 (1978).
17) Williams, L. T., Tremble, P. M., Lavin, M. F. and Sunday, M. E. Platelet-derived growth factor receptors form a high affinity state in membrane preparations. *J. Biol. Chem.*, **259**, 5287–5294 (1984).
18) Moscatelli, D. and Quarto, N. Transformation of NIH-3T3 cells with basic fibroblast growth factor or the *hst/K-fgf* oncogene causes downregulation of the fibroblast growth factor receptor: reversal of morphological transformation and restoration of receptor number by suramin. *J. Cell Biol.*, **109**, 2519–2527 (1989).
19) Yoshida, M., Kijima, M., Akita, M. and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J. Biol. Chem.*, **265**, 17174–17179 (1990).
20) Sugita, K., Koizumi, K. and Yoshida, H. Morphological reversion of *sis*-transformed NIH3T3 cells by trichostatin A. *Cancer Res.*, **52**, 168–172 (1992).
21) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
22) Davis, L. G., Dibner, M. D. and Battey, J. F. "Basic Methods in Molecular Biology," pp. 143–146 (1986). Elsevier Science Publishing, New York.