

ACETOXYCYCLOHEXIMIDE AND  
CYCLOHEXIMIDE CONVERT  
TRANSFORMED MORPHOLOGY  
OF *ras*-TRANSFORMED CELLS  
TO NORMAL MORPHOLOGY

Sir:

The discovery of oncogenes gives a great impact on cancer research<sup>1</sup>. In humans, activation of oncogenes by mutagenesis such as point mutation and translocation, rearrangement and amplification of DNA fragments plays a critical role in the initiation of carcinogenesis and/or proliferation of cancer cells. This provides us with a more rational approach to find effective antitumor agents. Thus, it is a hopeful way for overcoming human cancer to find new inhibitors for oncogene products or their functions. In this line of screening program we isolated genistein from fermentation broths of *Pseudomonas* as a specific inhibitor for tyrosine protein kinase<sup>2,3</sup>, a function of the *src* oncogene family. However, *ras* oncogenes are more interesting because they have been detected in a variety of human cancers<sup>4</sup> at very high frequencies<sup>5</sup>. As a result of screening, we found agents in the fermentation broths of some *Streptomyces* species which can convert the morphology of *ras*-transformed mouse NRK cells back to normal and identified them as acetoxycycloheximide and cycloheximide. Here we described the results.

DULBECCO'S modified EAGLE medium (DME) and minimal EAGLE medium (MEM) which did not contain methionine were purchased from Nissui Pharmaceutical Co. and fetal calf serum was from Filton Pty Ltd., Australia. Anti-RAS monoclonal antibody and p21 protein were purchased from Oncogene Science Inc., protein A from Pharmacia and rat IgG from Sigma Co. [<sup>35</sup>S]Methionine was obtained from ICN Radiochemicals, U.S.A. Virginiamycin, capreomycin, lincomycin, puromycin and fusidic acid were supplied from Sigma Co., and chloramphenicol from Sankyo Co., Ltd, josamycin from Yamanouchi Pharmaceutical Co., Ltd. tetracycline and streptomycin from Meiji Seika Kaisha, Ltd., mitomycin from Kyowa Hakko Kogyo Co., Ltd., and blasticidin S from Kaken Pharmaceutical Co., Ltd. Thiostrepton was a kind gift of S. J. LUCANIA of The Squibb Institute. YMC-A312 column for HPLC, was obtained from Yamamura Chemical Co. and silicic acid from Merck.

NRK cells, Harvey sarcoma virus-transformed NRK (Hav-NRK) cells and Swiss3T3 cells transformed with *abl* gene (AbtX-33), Rous sarcoma

virus-transformed 3Y1 (SRA) cells, Ki-*ras* transformed NIH3T3 cells and adenovirus-transformed 3Y1 (WY3) cells were generous gifts from HIROSHI YOSHIKURA, SADA AKI KAWAI, NOBUO TSUCHIDA and KAZUKO SHIROKI of the University of Tokyo, respectively.

NRK and Hav-NRK cells were cultured in DME supplemented with 10% fetal calf serum in 5% CO<sub>2</sub>, water-saturated incubator. For screening of microbial products, the Hav-NRK cells were seeded at  $2 \times 10^3$  cells in 200  $\mu$ l in a 96 well plate and grown overnight at 37°C. Test samples (usually 10  $\mu$ l) were added to a well, incubation was continued for 15 to 17 hours at 37°C, and then the cell morphology was observed under an optical microscope. Effects on the other cells were determined in a similar manner.

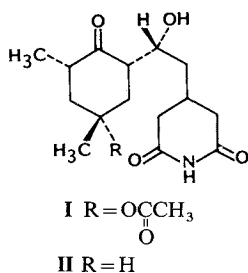
Inhibition of protein synthesis was determined by the method of KERRIDGE<sup>6</sup> with a slight modification as follows: Hav-NRK cells grown in a 3.5-cm diameter dish were collected by centrifugation, washed twice with 1 ml each of MEM and then suspended in MEM. Ten  $\mu$ l of solution containing acetoxycycloheximide or cycloheximide at an appropriate concentration was added and the mixture was incubated for 1 hour at 37°C. Then, 10  $\mu$ l of [<sup>35</sup>S]methionine (1  $\mu$ Ci/ml) was added and the incubation was continued for an additional 30 minutes at 37°C. After removing the medium and washing twice with 1 ml of phosphate-buffered saline, 1 ml of 5% TCA was added, and the mixture was kept for 30 minutes at 0°C. Radioactivity of the precipitate dissolved in 200  $\mu$ l of 0.2N NaOH was determined by a liquid-scintillation counter.

Hav-NRK cells grown in a 6.0-cm diameter dish were collected by centrifugation, washed twice with 1 ml of MEM and then suspended in MEM. Ten  $\mu$ l of solution containing acetoxycycloheximide (48.9 ng/ml) or cycloheximide (390 ng/ml) and 10  $\mu$ l of [<sup>35</sup>S]methionine (100  $\mu$ Ci) were added and the mixture was incubated overnight at 37°C. After removing the medium and washing four times with 1 ml each of phosphate-buffered saline at 4°C, 500  $\mu$ l of PBS-TDS (Na<sub>2</sub>PO<sub>4</sub> 10 mM, NaCl 0.155 M, Triton X-100 1%, sodium deoxycholate 0.5%, SDS 0.1%, sodium azide 0.2% and NaF 0.004%, pH 7.2) was added and the mixture was incubated for 10 minutes at 4°C. The supernatant was collected by centrifugation at 15,000  $\times g$  for 30 minutes. p21 protein in the supernatant was analyzed by using anti-RAS monoclonal antibody according to the Manual of the producer. Electrophoresis was performed by the method of LAEMMLI<sup>7</sup>.

Active substances were isolated from cultured broth of *Streptomyces* species such as *Streptomyces hygroscopicus* by extraction with ethyl acetate, column chromatography on silicic acid, preparative TLC and HPLC on YMC-A312 with 40% methanol. These substances were identified as acetoxycycloheximide (I) and cycloheximide (II) by high resolution (HR)-MS, IR spectrometry and  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectrometry (Fig. 1).

The morphology of the transformed cells (Hav-NRK cells) characterized by small-sized,

Fig. 1. Chemical structures of acetoxycycloheximide (I) and cycloheximide (II).

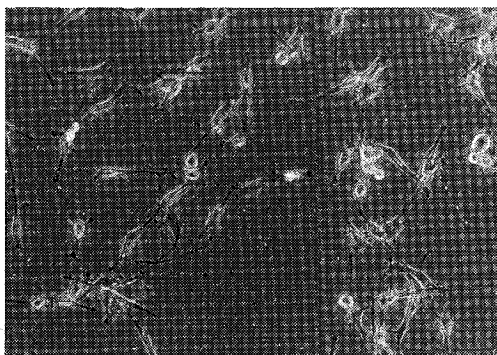


densely stuffed and spindle-shaped cells is shown in Fig. 2A. These cells can reach high cell densities, while the normal cells are flattened out and sensitive to contact inhibition (Fig. 2B). When a fermentation product from *Streptomyces* species was added to a Hav-NRK cell culture, the cells changed their morphology from the transformed shape to the normal one. With pure acetoxycycloheximide the morphological changes were observed at a concentration of 24.5 ng/ml (72 nM, Fig. 2C) or more, while with cycloheximide the morphology reverted to normal at a concentration of 390 ng/ml (1.4  $\mu\text{M}$ , Fig. 2D) or more. When the cells were washed free of these agents and allowed to grow in the fresh medium, the normal morphology gradually changed back to the transformed one.

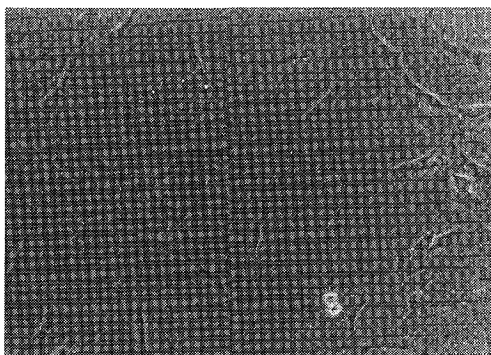
On the other hand, when acetoxycycloheximide or cycloheximide was added at 20  $\mu\text{g}/\text{ml}$  to the cultures of Rous sarcoma virus-transformed 3Y1 cells, Ki-ras transformed NIH3T3 cells, adenovirus-transformed 3Y1 cells or Swiss3T3 cells transformed with *abl* gene, no morphological change was observed. Thus, it is suggested that acetoxycyclohex-

Fig. 2. Microscopic pictures of Hav-NRK cells (A), NRK cells (B), Hav-NRK cells treated with 24.5 ng/ml of acetoxycycloheximide (C) and Hav-NRK cells treated with 390 ng/ml of cycloheximide (D).

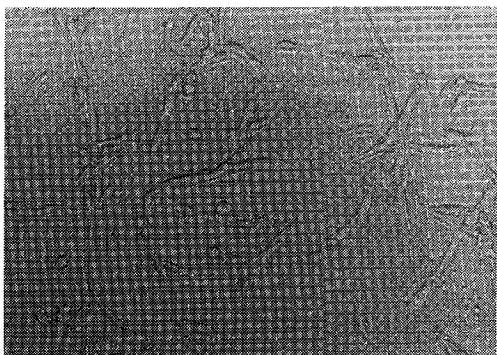
(A)



(B)



(C)



(D)

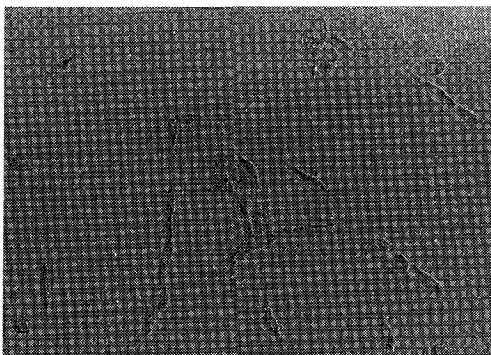
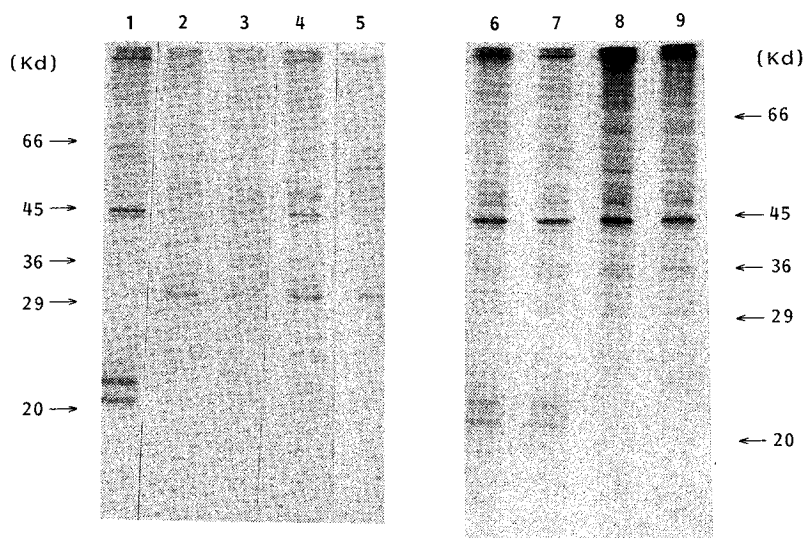


Fig. 3. Analysis of the proteins precipitated with anti-RAS monoclonal antibody by SDS-polyacrylamide gel electrophoresis and autoradiography.



Lane 1: No inhibitor; lane 2: the precipitate with protein A-rat IgG (non-specific precipitation); lane 3: cycloheximide, 390 ng/ml; lane 4: acetoxy-cycloheximide, 48.9 ng/ml; lane 5: the same as lane 2 but containing acetoxy-cycloheximide, 48.9 ng/ml; lanes 6~9: competition experiments, concentrations of rat p21 protein were 1 ng, 10 ng, 100 ng and 1  $\mu$ g for lanes 6, 7, 8 and 9, respectively. Kd: Kilodalton.

imide and cycloheximide inhibited specifically the process of transformation of the cells by Harvey-type *ras*-oncogene.

Cycloheximide is known to be a specific inhibitor of protein synthesis of eukaryotic cells<sup>8)</sup> and is used widely as such. The inhibition of protein synthesis was therefore determined in Hav-NRK cells. Acetoxy-cycloheximide and cycloheximide inhibited the protein synthesis by 50% at concentrations of 9.8 and 160 ng/ml, respectively. These results suggested that inhibition of protein synthesis might be involved in the recovery of normal morphology of Hav-NRK cells, because both agents caused such morphological changes at concentrations about 2.5 times those required for inhibition of protein synthesis. However, antibiotics known to inhibit protein synthesis of eukaryotic organisms such as puromycin and blasticidin S did not change the morphology of Hav-NRK cells at or less than 50  $\mu$ g/ml, although puromycin at 50  $\mu$ g/ml and blasticidin S at 200  $\mu$ g/ml almost killed the cells. Inhibitors of prokaryotic protein synthesis such as chloramphenicol, josamycin (a macrolide antibiotic), tetracycline, thiostrepton, streptomycin, virginiamycin, capreomycin, lincomycin, fusidic acid and mitomycin did not also cause the morphological

recovery at 50  $\mu$ g/ml. It is suggested therefore that the morphological change of the *ras*-transformed Hav-NRK cells is specifically caused by acetoxy-cycloheximide and cycloheximide. Analysis of the proteins precipitated by anti-RAS monoclonal antibody revealed that the synthesis of p21 proteins was preferentially inhibited by cycloheximide or acetoxy-cycloheximide, compared to other proteins (Fig. 3, lanes 3 and 4). In these experiments, the biosynthesis of total protein was inhibited by 73.3 and 59.9% by cycloheximide and acetoxy-cycloheximide, respectively, while that of p21 protein was decreased to almost zero and 7.9%, respectively. On the other hand, puromycin at 30  $\mu$ g/ml and blasticidin S at 10  $\mu$ g/ml inhibited the biosynthesis of total protein by 61 and 61% respectively, while that of p21 was decreased to 64 and 69%, respectively. Thus, these results indicate that acetoxy-cycloheximide and cycloheximide change the morphology of the transformed cells by inhibiting the biosynthesis of p21 protein preferentially. Although two bands were detectable near 21,000 daltons in the autoradiogram, competition experiment clearly showed that they were derived from p21 protein (Fig. 3, lanes 6, 7, 8 and 9). One may be a degradation product.

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