IN VITRO AND *IN VIVO* EFFECTS OF DIFFERANISOLE A ON SOME TUMOR CELLS[1]

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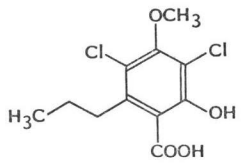
Some types of tumor cells can be induced to differentiate to normal mature cells by treatment with certain substances, the differentiated cells lose their tumorigenicity. This suggests that induction of cell differentiation might be a powerful approach to cancer therapy[2].

Recently, as a result of screening for substances inducing the differentiation of animal cells, we have identified a novel substance with this property in the broth filtrate of a *Chaetomium* sp. strain. It has been named differanisole A. The methods of isolation and structure determination have been reported in the preceding papers[3,4]. The chemical structure is shown in Fig. 1.

This communication describes the *in vivo* effect of differanisole A against tumors cells implanted into mice, as well as the effect on *in vitro* differentiation of tumor cells and on their tumorigenicity.

First we examined the induction of differentiation of erythroleukemia cells (B8) by determining hemoglobin content of the cells; cells plated at $3\sim5\times10^5$ cells/ml were exposed to various concentrations of differanisole A at 37°C for 5 days. After incubation, 0.3 ml of the resulting cell suspension was added to 1.5 ml of following mixture; 7.5 volume of 0.2% dimethylaniline, 7.5 volume of 20 mM 4-aminoantipyrine, and 85 volume of 0.1 M phosphate buffer. An aliquot of 0.15 ml of 3% hydrogen peroxide was added to the suspension. After 15-minute incubation at 37°C, the absorbance was measured at 550 nm[5]. Treatment with $5\sim20$ μg/ml of differanisole A increased hemoglobin content in B8 cells by $37\sim47$%.

Fig. 1. Structure of differanisole A.



Having confirmed that differanisole A was effective against B8 cells, we examined the effects on the other tumor cell lines; mouse myeloid leukemia Ml cells, human promyeloid leukemia HL60 cells, and mouse melanoma B16 cells. In order to evaluate differentiation induction of these cells, we measured lysozyme activity for Ml and HL60 cells, and tyrosinase activity for B16 cells. Lysozyme activity was determined by the modified lyso-plate method of OSSERMAN and LAWLOR[6]. HL60 cells (2×10^5 cells/ml) were cultured in the presence of differanisole A at 37°C for 3 days. After centrifugation, the cell pellets were lysed by mixing vigorously with 6.7 mM phosphate buffer (pH 6.6). To this lysate, 0.75 mg/ml heat-killed *Micrococcus lysodeikticus* was added and after incubating for 3 hours, turbidity was measured with a spectrophotometer at 540 nm. Tyrosinase activity in B16 cells was determined spectrophotometrically by following the oxidation of L-DOPA to dopachrome[7]: B16 cells were incubated with differanisole A at a density of 1×10^7 cells/95 mm plastic dish at 37°C for 4 days. Cells harvested from dishes were homogenized and sonicated in 0.25 M sucrose. After centrifugation, tyrosinase activity in the supernatant fraction was determined.

Though there was marked deviation in each experiment, $5\sim20$ μg/ml of differanisole A seemed to induce the lysozyme activity in Ml cells. It was ineffective in inducing lysozyme activity in HL60 cells. Treatment with 10 μg/ml of differanisole A increased the tyrosinase activity in B16 cells by 45%. These findings show that the differentiation inducing activity of differanisole A depends on the cell lines used and the differentiation markers employed.

On the other hand, for *in vivo* studies, we used mouse neuroblastoma C-1300 cells, which can be transformed to neuroblasts by various substances. C-1300 cells were cultured in RPMI 1640 medium supplemented with 10% heat-

Fig. 2. Effect of differanisole A on the tumorigenicity of C-1300 cells.

○ Control, ● differanisole A 3 μg/ml, ▲ differanisole A 10 μg/ml.

* $P<0.01$.

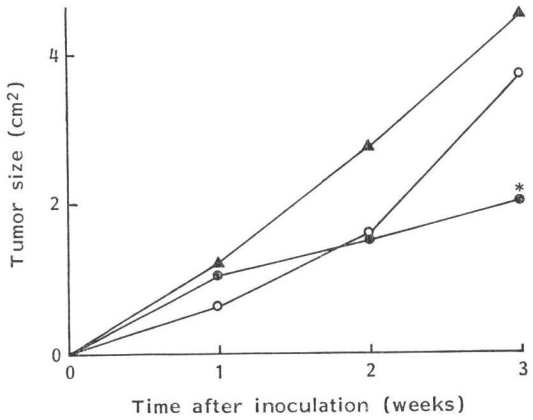
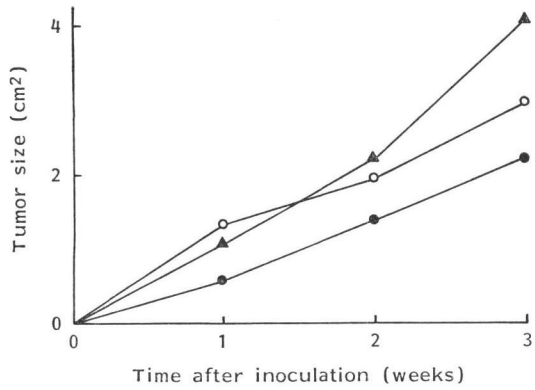


Fig. 3. Antitumor effect of differanisole A on C-1300 cells.

○ Control, ● differanisole A 10 mg/kg/day, ▲ differanisole A 30 mg/kg/day.



inactivated fetal bovine serum. For testing the effect on their tumorigenicity, 5×10^5 cells were inoculated into each 90-mm-diameter tissue culture dish with 13.5 ml of the medium, and 1.5 ml of sample solution was added using various concentrations of differanisole A. After incubating for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 in air, the cells were washed with phosphate-buffered saline and the number of viable cells were counted with a haemocytometer. Cell viability was determined by the trypan blue exclusion method, and 8×10^5 viable cells were subcutaneously injected into the inguinal area of A/J mice (9~10 weeks old female, $n=6$, obtained from Shizuoka Laboratory Animals Center).

For testing the *in vivo* antitumor effect, 8×10^5 C-1300 cells grown in the culture medium in the absence of differanisole A were inoculated as described above and the sample solution was given intraperitoneally once a day for 14 days after the tumor implantation. In these two *in vivo* experimental systems, each tumor area was measured once a week for 3 weeks.

The effect on tumorigenicity of C-1300 cells is shown in Fig. 2. Treatment with 10 μg/ml of differanisole A was not effective. Treatment with 3 μg/ml of differanisole A was effective, and the growth of C-1300 cells remained 55% of untreated control 3 weeks after tumor implantation ($P<0.01$ by Student's t-test). As Fig. 3 shows, the tumor growth was partly inhibited by the intraperitoneal injection of 10 mg/kg/day of differanisole A (not significant by Student's t-test). However, 30 mg/kg/day of differanisole A was ineffective. High doses such as 100 mg/kg/day were lethal. Mice died within 17 days of the day of tumor implantation. Against subcutaneous implanted B16 melanoma, differanisole A did not show the inhibition of the growth by intraperitoneal 14 consecutive daily injection at a dose range of 10~100 mg/kg/day (data not shown).

It is known that differentiation-inducing activity reaches a maximum in Ml cells treated with dibutyryl cyclic AMP[8] or poly(I)·poly(C)[9] at a certain concentration, but they are less effective at higher concentrations. The present results of *in vivo* experiments against C-1300 cells suggest that, as in the reported cases, there is an optimum dose or concentration for the differentiation-inducing activity of differanisole A.

Although many substances are known to induce differentiation of tumor cells *in vitro*, few have been reported to give a positive *in vivo* effect. Consequently, it is interesting to note that differanisole A is effective *in vivo* against C-1300 cells. Further investigations on the mechanism of action of differanisole A are in progress.

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