SYNERGISM BETWEEN 5-FLUOROURACIL AND OXANOSINE IN INHIBITING GROWTH OF *ras*-EXPRESSED CELLS *IN VITRO* AND *IN VIVO*

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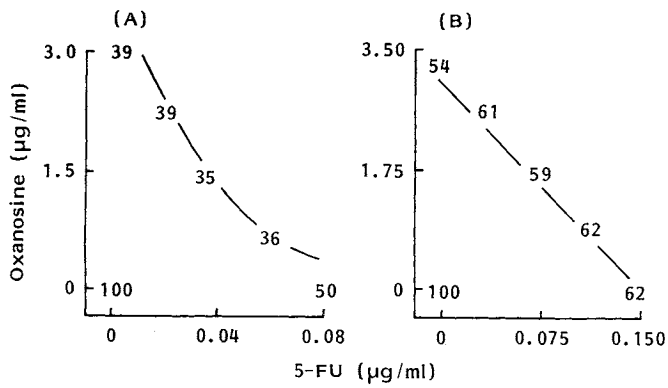
We previously reported that oxanosine as well as 5-FU, among various compounds tested, inhibited growth *in vitro* of v-K-*ras* NRK (*ras*$^+$) cells more strongly than inhibited that of NRK-52E (*ras*$^-$) cells[1]. IC_{50} of oxanosine for *ras*$^+$ and *ras*$^-$ cells was 4.9 μg/ml[a] and >40 μg/ml[b], respectively (b/a for oxanosine = >8), while those of 5-FU were 0.036 μg/ml[a] and 0.17 μg/ml[b], respectively (b/a for 5-FU = 4.6). Another pair of *ras*$^+$ cells and *ras*$^-$ cells, *i.e.*, K-*ras* NIH/3T3 and NIH/3T3, respectively, gave a similar result. The effect of the two drugs in combination[2] on *ras*$^+$ cells was synergistic as shown in Fig. 1. Their effect in combination on *ras*$^-$ cells was rather additive. In all these experiments, cells were cultured with a drug (or drugs) for 2 days without changing the medium and cell growth was then determined (a 48-hour-exposure). Next, we determined the 2-hour-exposure effect of these drugs on cell growth to speculate their possible effect in *in vivo* tests where drug concentrations surrounding target cells should fall rapidly by metabolism and excretion. Cells were incubated in a medium containing a drug (or drugs, simultaneously or successively) for 2 hours, washed free of the drug(s), cultured further for 3 days in the drug-free medium and growth was then determined. For comparison, a 48-hour-exposure experiment was repeated in parallel. As shown in Fig. 2, the differential effect of 5-FU on *ras*$^+$ cells was far greater in the 2-hour-exposure experiment (IC_{50} for *ras*$^+$ and *ras*$^-$ were 0.35 μg/ml[a] and 4.7 μg/ml[b]; b/a = 13) than in the 48-hour-exposure experiment. A prior 2-hour-exposure of *ras*$^+$ cells to 10 μg/ml oxanosine, which on its own had no effect on cell growth, significantly enhanced the effect of subsequent 2-hour-exposure to 5-FU (Table 1, Schedule A). In contrast, there was only weak enhancement with nontumorous control cells (NIH/3T3) and with tumorous cells in which another oncogene, *src*, was expressed (*src* NIH/3T3). Furthermore, the order of exposure to the two drugs was an important factor to make

Fig. 1. Effect of 5-fluorouracil in combination with oxanosine on *in vitro* cell growth.

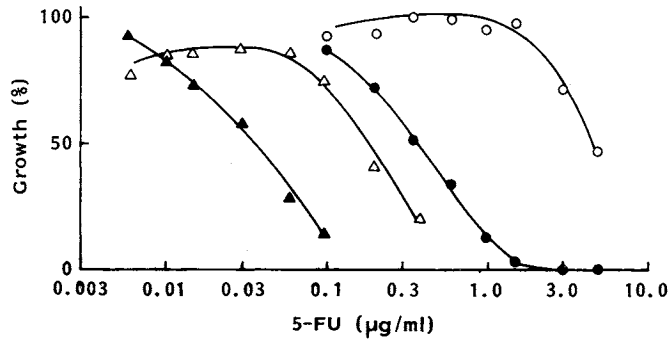
(A) K-*ras* NIH/3T3, (B) NIH/3T3.



An NIH/3T3 cell line transformed with a human activated c-K-*ras* gene carrying a point mutation at codon 12 (K-*ras* NIH/3T3) was provided by Dr. T. SEKIYA, National Cancer Center Research Institute, Tokyo. Culture conditions were as reported[3]. In figures A and B, the isoboles equivalent to 36% and 62% relative growth (vs. each control) are shown, respectively.

Fig. 2. Effect of 5-fluorouracil on *in vitro* growth of K-*ras* NRK and NRK-52E cells.

▲, ● v-K-*ras* NRK, △, ○ NRK-52E.



Cells and culture conditions were as reported[3]. Coster 12-well tissue culture clusters (4 cm^2/well) were used as culture vessels. In each vessel, $1 \sim 2 \times 10^4$ cells were seeded in 2 ml medium (day 0). Effects of a drug on cell growth were determined by two protocols. (1) Cells received a drug on day 1 and were further cultured until day 3 without changing the medium (a 48-hour-exposure: ▲, △). (2) On day 1, cells were exposed to a drug for 2 hours, washed free of the drug with 1 ml of Ca^{2+}- and Mg^{2+}-free PBS, and further cultured in the ordinary medium until day 4 (a 2-hour-exposure: ●, ○).

Table 1. Effect of oxanosine on *in vitro* growth-inhibition by 5-fluorouracil.

Cell	Schedule	IC_{50} of 5-FU (μg/ml)		IC_{50} index without/with oxanosine
		Without oxanosine	With oxanosine (10 μg/ml)	
K-*ras* NIH/3T3	A	2.15	0.45	4.77
	B	1.69	0.48	3.52
	C	2.49	2.54	0.98
H-*ras* NIH/3T3	A	0.93	0.32	2.91
	B	0.95	0.42	2.26
	C	0.76	0.78	0.97
src NIH/3T3	A	0.30	0.18	1.67
	B	0.23	0.18	1.28
	C	0.46	0.42	1.09
NIH/3T3	A	3.05	1.65	1.85

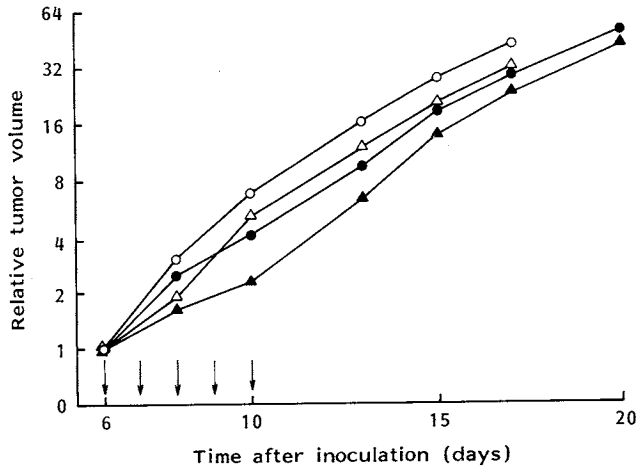
An NIH/3T3 cell line transformed with a human activated c-H-*ras* gene carrying a point mutation at codon 61 (H-*ras* NIH/3T3) was provided by Dr. T. SEKIYA, National Cancer Center Research Institute, Tokyo[4]. Other cell lines and culture conditions were as reported[3]. Cells were seeded as described in the legend to Fig. 2. On day 1, cells were exposed to a drug (or two drugs simultaneously, Schedule B) for 2 hours, washed free of the drug with 1 ml of Ca^{2+}- and Mg^{2+}- free PBS and, in Schedule A and C, the cells were exposed to a second drug for 2 hours, washed and further cultured in the ordinary medium until day 4 (a 2-hour-exposure). Experiments A, B and C were conducted separately. Runs were duplicated.

the *ras*$^+$ specific enhancement effective; no enhancement at all if the order was reversed (Schedule C), while only moderate enhancement if the two drugs were present simultaneously (Schedule B).

We examined if the synergism so far shown *in vitro* between 5-FU and oxanosine could be reflected in inhibiting growth of *ras*$^+$ tumors in mice. As shown in Fig. 3, the combination of oxanosine and 5-FU significantly decreased the progression of tumors as long as the treatment continued (day 6

Fig. 3. Growth curves of K-*ras* NIH/3T3 solid tumors in mice.

● 15 mg/kg 5-FU, △ 328 mg/kg oxanosine, ▲ 15 mg/kg 5-FU + 328 mg/kg oxanosine, ○ no antibiotics, ↓ drug treatment.



K-*ras* NIH/3T3 cells (10^7 cells/0.5 ml medium) were injected sc to a 6-week-old female Balb/c-nuA mouse (Clea Japan Inc.). In two weeks the mouse developed a tumor, 1 cm in diameter. The tumor was excised and cut into about 1 mm^3 fragments and each fragment was inoculated sc to another mouse (day 0). Each determination was made with 5 mice (n=5). On day 6 to 10, oxanosine (sc) and 4 hours later 5-FU (iv) were administered. The effect of each drug alone was also tested. With each tumor, the largest diameter (L) and its perpendicular diameter (W) were measured and the volume was calculated as $1/2 \cdot L \cdot W^2$. The unit (1.0) of "relative tumor volume" was equivalent to 134 mm^3.

to 10), but failed to induce any significant tumor regression. Studies on the molecular mechanism underlying the synergistic effect is in progress. More stable guanosine analogs may give better results.

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