Inhibition of Anchorage-Independent Growth of Tumor Cells by IT-62-B, a New Anthracycline

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IT-62-B, a new anthracycline isolated from fermentation broths of *Streptomyces* sp. IT-62, reversed certain tumor cell phenotypes *in vitro* including some of human origin. The observed normal phenotypes were anchorage dependence of cell growth, flattened cell morphology and restoration of actin stress fibers. The extent of the anchorage dependence of cell growth induced by IT-62-B was generally greater than that by doxorubicin or pirarubicin. The cell-flattening effect of IT-62-B on cells of T24 (human bladder), but not on C-33A (human cervix), accompanied inhibition of *fos* gene expression. T24 cells, once flattened by IT-62-B, retained their flat morphology even in drug-free, fresh medium and eventually died in several days. IT-62-B, unlike doxorubicin, only slightly inhibited the topoisomerase II reaction *in vitro* and DNA synthesis in isolated cell nuclei.

Anchorage-independent growth is a reliable characteristic of tumor cells *in vitro* . We previously reported that among antitumor anthracyclines currently in clinical use in Japan, doxorubicin and pirarubicin reduced anchorage independence of growth of K- or H-*ras* transformed cells more strongly than did aclarubicin and MX2[1]. As an extension of these studies, we investigated whether IT-62-B[2], a new anthracycline, could inhibit anchorage-independent growth of tumor cells (including some of human origin), with or without *ras* gene mutations. Here we report that IT-62-B inhibited anchorage-independent growth of various tumor cells and the extent of the inhibition by IT-62-B was generally greater than that by doxorubicin and pirarubicin irrespective of the *ras* gene mutations. Some attempts were made to explain how IT-62-B inhibited anchorage independent growth of cells.

Materials and Methods

Cell Lines

NIH3T3 cell lines transformed with a mutant human H-*ras* gene expressing Q61L[3] (H-*ras* 3T3) or a mutant human K-*ras* gene expressing G12C[4] (K-*ras* 3T3) were provided by Dr. SEKIYA, National Cancer Center Research Institute, Tokyo. An NIH3T3 cell line infected with Rous sarcoma virus SR-D (*src* 3T3) was provided by Dr. KAWAI, the Institute of Medical Science, University of Tokyo, Tokyo. Human bladder carcinoma T24 having a G12V mutation in H-*ras* gene was provided by the Japanese Cancer Research Resources Bank, Tokyo. Human cervical carcinoma HeLa was purchased from Dainipponseiyaku Co., Osaka. Human cervical carcinoma C-33A, free of HPV gene, was purchased from ATCC, U.S.A. Human stomach carcimona SC-6 which showed LOH in p53 gene (Hiratsuka, personal communication) and human lung carcinoma LX-1were provided by the Institute for Chemotherapy, Shizuoka.

Cell Culture in Liquid Medium

The culture media used for the cell lines were as follows. DULBECCO's modified Eagle medium supplemented with 10%fetal calf serum (DMEM·CS) for H-*ras* 3T3, K-*ras* 3T3 and *src* 3T3, EAGLE's minimum essential medium supplemented with 10% fetal calf serum (MEM·FCS) for T24 and HeLa, EAGLE's minimum essential medium supplemented with 1 mM sodium pyruvate and 10%fetal calf serum (MEM·PA·FCS) for C-33A, and, RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI·FCS) for SC-6 and LX-1. Cells were grown in these media in 5% CO_2-containing humidified air at 37°C. To assay the growth inhibition by drugs, the cells were seeded at $1 \sim 4 \times 10^4$ cells/1 ml medium/2 cm^2 well of Coster 24-well tissue culture clusters (day 0). Drugs were added to the wells on day 1, and incubation was

continued until day 3. Cells were photographed during the culture period, if necessary. Cell growth was quantified by cell counting or by a colorimetric MTT assay as reported previously[1].

Cell Culture in Semisolid Medium

The top agar suspensions, 2 ml each, contained the cells (numbers are given in parentheses, below) suspended in the media in which agar had been dissolved at indicated concentrations as follows: H-*ras* 3T3 (10^4) and *src* 3T3 (8×10^3) cells in DMEM·CS with 0.6% agar, T24 (4×10^4) cells in MEM·FCS with 0.4% agar, HeLa (3×10^4) cells in MEM·FCS with 0.6% agar, C-33A (5×10^4) cells in MEM·PA·FCS with 0.4% agar and SC-6 and LX-1 (5×10^4) cells in RPMI·FCS with 0.4% agar. The top agar suspensions were layered on 3 ml of the bottom agar layers, in 21 cm^2 dishes, consisting of the corresponding media in which 0.6% agar had been dissolved. The cells were incubated for 3~4 weeks, and colonies were counted as reported[1].

Staining of Actin Stress Fiber

Cells were seeded at 7.5×10^3 cells/0.15 ml of medium on cover glasses, fixed with 3.7% formaldehyde, made permeable with 0.2% Triton X-100, and stained with rhodamine-phalloidin as reported[1].

Immunohistochemical Staining of Fos Protein

T24 cells were suspended in MEM·FCS at 7×10^4 cells/ml and 0.15 ml portions therefrom were placed on cover glasses preset in 8 cm^2 dishes (Corning 25000 COL 1), and were incubated for 20 hours. The medium was changed for 1 ml of FCS-free MEM and cells were incubated further for 2 days. After the medium was removed the cell layers were covered with 200 μl of a fresh MEM·FCS with or without drugs and incubated for one hour and a half. *In situ* staining of Fos was conducted by the following method to form the avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP Kit, Vector Laboratories, U.S.A.). Cells adhering to each cover glass were freed of the medium by rinsing with 1 ml of cold PBS three times, each cover glass was then immersed in 1 ml of acetone for 5 minutes at -20°C. The fixed cells were washed with 1 ml of PBS three times, blocked with 2% normal goat serum in PBS for 20 minutes at 37°C. After the excess serum was removed, the cells were incubated with 200 μl of 2.5 μg/ml rabbit anti-Fos IgG (c-*fos* (Ab-2), Oncogene Science) for one hour at 37°C, washed with cold PBS for 5 minutes three times, incubated with biotinylated goat anti-rabbit IgG for 30 minutes at 37°C and washed with cold PBS for 5 minutes three times. The cells were incubated with avidin-biotin-alkaline phosphatase complex for one hour at room temperature, rinsed with cold PBS for 5 minutes three times, incubated with the substrate reagent Kit I (Vector Red, diluted with 100 mM Tris·HCl, pH 8.2) for 15 minutes at room temperature and washed with water five times. The stained cells were covered with 50% (v/v) glycerol/PBS and inspected under a fluorescence microscope. C-33A cells (7.5×10^3 cells/0.15 ml of MEM·PA·FCS, placed on a cover glass) were incubated with drugs and stained as above.

DNA Synthesis in Isolated Cell Nuclei

Preparation of T24 cell nuclei and DNA synthesis in the nuclei were performed according to the method of WINNACKER *et al.*[5] with minor modifications. T24 cells were seeded at 6×10^5 cells/10 ml of MEM·FCS/55 cm^2 dish (7 dishes as a unit) and incubated for 2 days. Cell nuclei were isolated on ice as follows. After removing the medium, the cells were washed with 10 ml of Ca^{2+}- and Mg^{2+}- free PBS and scraped in 2 ml of hypotonic HEPES buffer (10 mM HEPES·KOH, pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.05% (v/v) Triton X-100). After 10 minutes at 0°C, the cells were broken open by eight strokes in a Potter homogenizer. Isotonicity was restored by addition of an equal volume of 0.6 M sucrose. The cell lysate was centrifuged at 1500 *g* for 5 minutes and the precipitate was resuspended in 0.8 ml of isotonic HEPES buffer (50 mM HEPES·KOH, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.22 M sucrose), referred to as the cell nuclei fraction. Two microliters of a drug solution was added to 38 μl of the cell nuclei fraction, which was then incubated at 25°C for 10 minutes. To initiate DNA synthesis, 10 μl of reaction mixture consisted of 40 mM NaCl, 5 mM EGTA·KOH, 2 mM ATP, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, 0.02 mM dTTP, 1 μCi [^{3}H] dTTP (20.7 Ci/mmol), 5 mM phosphoenolpyruvate, and 0.75 μg pyruvate kinase was added to the cell nuclei fraction. The mixture was incubated at 25°C for 20 minutes and a 40 μl sample was withdrawn therefrom and transferred to a paper disc (Whatman 3MM, 2.4 cm diameter) to determine the radioactivity incorporated into TCA insoluble materials.

Topoisomerase II Assay

A reaction mixture to assay topoisomerase II activity (20 μl) consisted of 0.5 μg kinetoplast DNA (Chrithidia fasciculata), 1 unit topoisomerase II (human placenta),

Table 1. Effect of anthracyclines on growth and morphology of cancer cells.

Cell	Drug	IC_{50} (ng/ml)		a/b	Morphology (Cell flattening, actin stress fiber)
		Growth in agar medium (a)	Growth in liquid medium (b)		
T24	IT-62-B	3.4	67	0.051	+
	Doxorubicin	15.0	94	0.160	+
	Pirarubicin	4.3	36	0.119	+
	Aclarubicin	5.8	15	0.395	−
HeLa	IT-62-B	2.3	84	0.027	+
	Doxorubicin	5.6	68	0.082	+
	Pirarubicin	4.3	29	0.148	+
	Aclarubicin	10.2	80	0.128	−
C-33A	IT-62-B	4.4	200	0.022	+
	Doxorubicin	9.9	89	0.111	+
	Pirarubicin	5.4	52	0.104	+
	Aclarubicin	9.0	13	0.720	−
SC-6	IT-62-B	4.4	48	0.092	+
	Doxorubicin	1.3	31	0.042	+
	Pirarubicin	1.9	24	0.081	+
	Aclarubicin	6.3	34	0.185	−
LX-1	IT-62-B	4.4	70	0.063	−
	Doxorubicin	1.6	70	0.023	−
	Pirarubicin	9.2	115	0.080	−
	Aclarubicin	1.6	2	0.800	−
H-*ras* NIH3T3	IT-62-B	0.3	130	0.002	+
	Doxorubicin	1.5	46	0.033	+
	Pirarubicin	1.0	23	0.043	+
	Aclarubicin	52.0	72	0.722	−
src NIH3T3	IT-62-B	12.0	160	0.075	+
	Doxorubicin	7.4	54	0.137	−
	Pirarubicin	27.0	70	0.386	−
	Aclarubicin	56.0	80	0.700	−

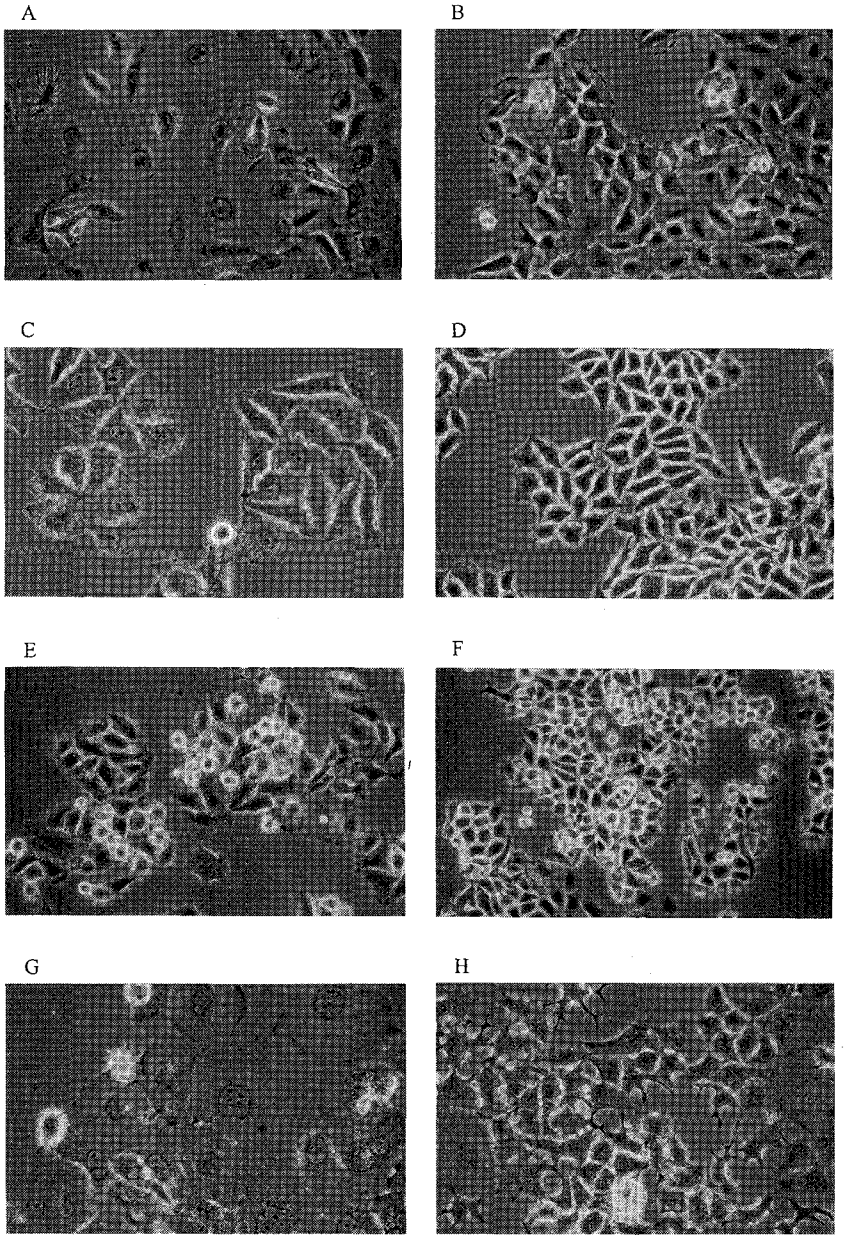
1 mM ATP, 50 mM Tris·HCl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM EDTA, 0.3 μg bovine serum albunim and a drug. After incubation at 37°C for 1 hour, five microliters of stop solution (5% SDS, 0.025% BPB, 50 mM EDTA, 50% (v/v) ficoll 400) was added to the reaction mixture. Eighteen μl portions of the mixture were electrophoresed at 100 v for 1 hour in a 1% agarose gel plate supplemented with ethidium bromide at 0.3 μg/ml.

Results and Discussion

Inhibition of Anchorage-independent Cell Growth

Loss of the anchorage-independent cell growth (cell growth in soft agar) is a reliable and quantitative index *in vitro* of phenotypic alterations from malignant to normal. We therefore determined the extent of cell growth inhibition by IT-62-B in the soft agar medium (for IC_{50}^{agar}) and in the ordinary liquid medium (for IC_{50}^{liq}) with the cell lines described above and the results were compared with those by doxorubicin, pirarubicin and aclarubicin. The smaller the ratio of $IC_{50}^{agar}/IC_{50}^{liq}$ (a/b in Table 1), the more strongly inhibited the anchorage-independent cell growth. IT-62-B inhibited the anchorage-independent growth of T24 (human bladdar), HeLa and C-33A (human cervix), SC-6 (human stomach), LX-1 (human lung), H-*ras* 3T3 and *src* 3T3 more strongly than, or as strong as, did doxorubucin and pirarubicin. Aclarubicin hardly inhibited the anchorage-independent growth of these cell lines. Although the molecular mechanism for the anchorage independence of the growth of cancer cells has not been clarified yet, IT-62-B, as well as doxorubicin and pirarubicin, seems to reverse the characteristics of tumor cells to normal ones.

Fig. 1-1. Phase-contrast micrographs of cells.

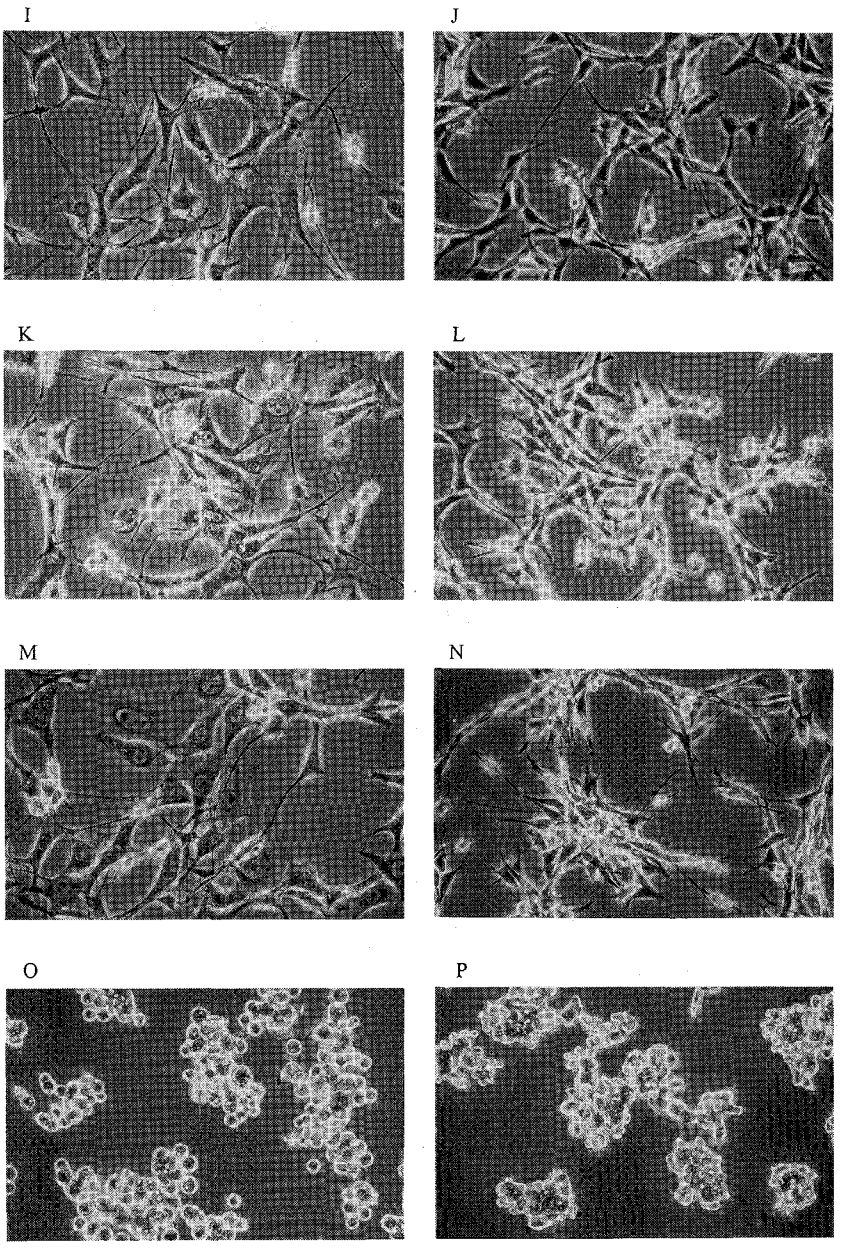


Cells were incubated for 48 hours in the presence (A, C, E, G) or absence (B, D, F, H) of IT-62-B at IC_{30}; (A, B) T24, (C, D) HeLa, (E, F) C-33A and (G, H) SC-6.

Alteration of Cell Morphology and Restoration of Actin Stress Fibers

IT-62-B flattened the morphology of most of the cell lines investigated, T24, HeLa, C-33A, SC-6, K-*ras* 3T3, H-*ras* 3T3 and *src* 3T3, all at concentrations as low as IC_{20} (Fig. 1-1, 1-2). The effect of IT-62-B seemed to have no correlation with *ras* gene mutations since H- and K-*ras* genes of C-33A had no mutations at codons 12, 13 and 61 (data not shown). LX-1, however, did not alter the cell morphology even at high concentrations of the antibiotic (Fig. 1-2). The alteration of cell morphology by IT-62-B always accompanied the restoration of actin stress fibers as exemplified with T24, HeLa and K-*ras* 3T3 (Fig. 2). The alteration of cell morphology by IT-62-B did not require new RNA synthesis nor protein synthesis; T24 cells were flattened by IT-62-B even in the presence of actinomycin D or cycloheximide (data not shown). These results suggested that IT-62-B altered the catalytic activities of some enzymes on the interactions between some proteins participating in controlling the cell morphology, without influencing the amounts of

Fig. 1-2. Phase-contrast micrographs of cells.

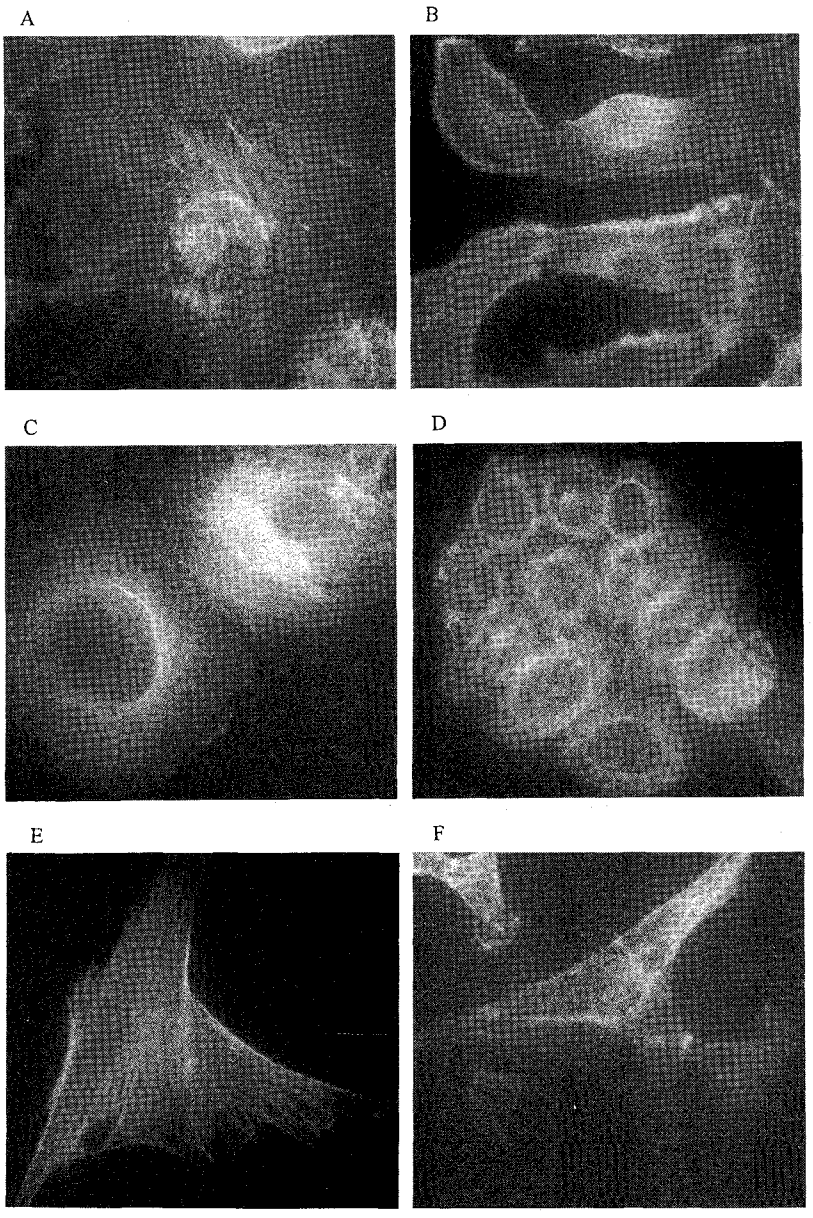


Cells were incubated for 48 hours in the presence (I, K, M) or absence (J, L, N) of IT-62-B at IC_{30}; (I, J) K-*ras* 3T3, (K, L) H-*ras* 3T3 and (M, N) *src* 3T3. LX-1 cells were incubated for 48 hours in the presence (O) or absence (P) of IT-62-B at IC_{70}.

these enzymes or proteins. When T24 cells that had been flattenned by IT-62-B at IC_{70} were incubated further in the drug-free medium, most cells died in a day and the rest of the cells did not return into the original morphology and died eventually in a week. Apoptotic morphological changes were not observed at any stage. At lower concentrations, however, cells were partially flattened and on further culture in drug-free medium, returned to the original morphology and reinitiated proliferation. A parallel experiment with staurosporin, an inhibitor of serine/threonine kinase and tyrosine kinase, showed that the morphological alterations in T24 at IC_{70} of staurosporin was reversible; cells returned to the original morphology in two days in drug-free medium and resumed growth. The comparative results suggested that IT-62-B was not only a simple inhibitor (or activator) of a process(es) involved in controlling cell morphology, but also provoked some irreversible damage, possibly in the cell membrane, especially at high concentrations.

Strong inhibition of anchorage-independent cell growth should be an ideal characteristic for anticancer drugs and hence the cell flattening in T24 or HeLa would

Fig. 2. Restoration of the actin stress fiber in cells.



Cells were incubated for 48 hours in the presence (A, C, E) or absence (B, D, F) of IT-62-B at IC_{50} and were stained with rhodamine-phalloidin as described under Methods; (A, B) T24, (C, D) HeLa and (E, F) K-*ras* 3T3.

be a simple and good index to screen compounds inhibiting, possibly strongly, the anchorage-independent growth. LX-1 was the only exception in that IT-62-B, doxorubicin and pirarubicin, all inhibited the anchorage-independent cell growth but failed to alter the morphology for unknown reasons.

Effect of IT-62-B on the Expression of *fos*

In cell growth signaling pathways, initiated by growth factors and mediated by Ras, the expression of *fos* gene is an event near the terminal step[6]. Overexpression of the *fos* gene is known to be a cause of uncontrolled growth of tumor cells[7]. We investigated whether IT-62-B, and pirarubicin for comparison, would inhibit the expression of *fos* gene induced by serum in T24 and C-33A. Both IT-62-B and pirarubicin inhibited the expression of *fos* gene in T24 cells but not in C-33A cells (data not shown), suggesting that the expression of *fos* gene has no direct correlation with the anchorage-independent cell growth.

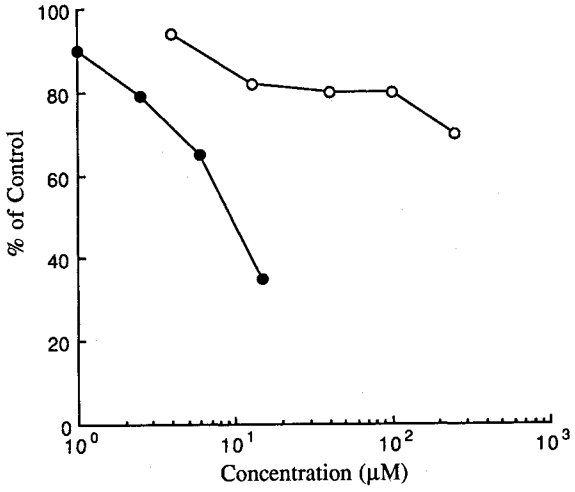
Effect of IT-62-B on DNA Synthesis in Cell Nuclei and the Topoisomerase II Reaction

Antitumor anthracyclines are generally known to

inhibit DNA synthesis, mostly due to inhibition of topoisomerase II[8]. It was an interesting finding that IT-62-B inhibited much less strongly than did doxorubicin both the DNA synthesis in cell nuclei and the topoisomerase II reaction. As shown in Fig. 3, IC_{30} of DNA synthesis for IT-62-B and doxorubicin were 250 μM and 4 μM, respectively, indicating that IT-62-B is only 1/60 as active as doxorubicin in inhibiting DNA synthesis in isolated cell nuclei. IT-62-B was again very weak, compared with doxorubicin, in inhibiting the topoisomerase II reaction, as shown in Fig. 4; IT-62-B little inhibited the reaction at concentrations as high as 40 μM while doxorubicin inhibited the reaction at concentrations less than 5 μM. In conclusion, IT-62-B is a unique anthracycline inhibiting the anchorage-independent growth of many (human) cancer cells with little inhibition of DNA synthesis. This new type anthracycline should be able to inhibit the growth of cancer cells selectively, with little inhibition of normal cell growth. A high chemotherapeutic index is expected.

Fig. 3. Effect of anthracyclines on DNA synthesis in cell nuclei.

○ IT-62-B, ● doxorubicin.



Acknowledgment

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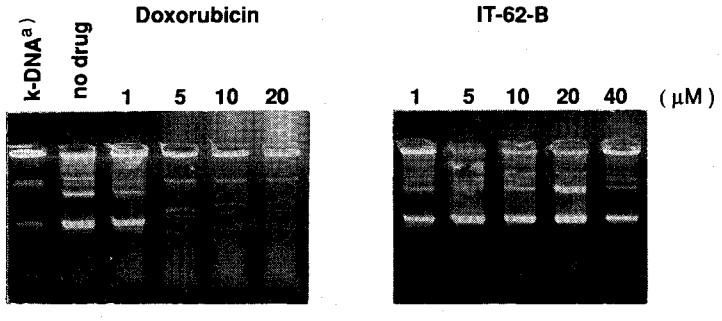
Fig. 4. Effect of anthracyclines on topoisomerase II activity.



a) kinetoplast DNA