Phenotypic Reversion Induced by Anthracyclines in ras Oncogene-Expressed Cells; Structure–Activity Relationships

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Several antitumor anthracyclines, including those in preclinical stages, were examined for their action in reversing tumorous phenotypes of H- or K-ras 3T3 cells (NIH3T3 cells transformed by human H- or K-ras oncogene) into normal phenotypes, such as flattened cell morphology, anchorage dependent cell growth, *etc.* (referred to as anti-ras activity). The study elucidated relationships between the chemical structure of anthracyclines and the anti-ras activity. The human tumor cell line T24, which has a mutated H-ras gene, responded to the anthracyclines, as did K- or H-ras 3T3 cells, in respect to the phenotypic alterations. Pirarubicin was more than 4 times as active as aclarubicin in inhibiting the growth of solid tumors of K-ras 3T3 cells in nude mice, possibly reflecting a difference in anti-ras activity between the two antibiotics.

Keywords anthracycline; ras; structure–activity relationship; solid tumor; morphology; actin fiber

We previously reported that among antitumor anthracyclines currently in clinical use in Japan, doxorubicin, pirarubicin and FAD-104, but not aclarubicin or MX2, induced some normal cell phenotypes in NIH3T3 cells that had been transformed by K- or H-ras oncogenes (referred to as K- or H-ras 3T3 cells); the phenotypic alterations included flattened cell morphology, restoration of actin stress fibers, anchorage dependence of cell growth, and an increase in nucleoside diphosphate (NDP) kinase activity (collectively referred to as anti-ras activity).¹⁾ Studies were extended as follows: (1) to elucidate relationships between the chemical structure of anthracyclines and the anti-ras activity by examining additional analogues of preclinical stages, (2) to confirm a similar response to anthracyclines of a human tumor cell line, T24, which has an activated ras gene, and (3) to examine a possible correlation between the anti-ras activity and the effectiveness in inhibiting the growth of solid tumors of K-ras 3T3 cells in nude mice. The present paper describes these results.

MATERIALS AND METHODS

Cell Lines NIH3T3 cell lines transformed with a human c-H-ras gene (H-ras 3T3)¹⁾ or a human c-K-ras gene (K-ras 3T3)¹⁾ were provided by Dr. Sekiya, National Cancer Center Research Institute, Tokyo. Human bladder carcinoma T24 was provided by the Japanese Cancer Research Resources Bank, Tokyo.

Cell Culture in Liquid Medium Cells were grown under the conditions described below in Dulbecco's modified Eagle medium supplemented with 10% calf serum (DMEM·CS for H-ras 3T3 and K-ras 3T3) or in Eagle's minimum essential medium supplemented with 10% fetal calf serum (MEM·FCS for T24), in 5% CO₂-containing humidified air at 37 °C. To assay growth inhibition by drugs, the cells were seeded at $1\text{--}1.5 \times 10^4$ cells/1 ml medium/2 cm² 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 H-ras 3T3 cells were seeded at 4×10^3 cells/5 ml DMEM·CS·0.6% agar/21 cm² dish and incubated for 14 d, and colonies were counted as reported.¹⁾

Staining Actin Stress Fiber Cells were seeded at 7.5×10^3 cells/0.15 ml 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⁾

Solid Tumors of K-ras 3T3 Cells in Nude Mice K-ras NIH3T3 cells (10^7 cells/0.5 ml medium) were injected s.c. into a 6-week-old female Balb/c-nuA mouse (Clea Japan, Inc.). In two weeks the mouse developed a tumor with a size of approximately 1 cm in diameter. The tumor was excised and cut into about 1 mm³ fragments, and single fragments were inoculated s.c. into several mice (day 0). Tumor sizes in a group of 5 mice were averaged for each determination ($n=5$). 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 79 mm³.

RESULTS AND DISCUSSION

Structure Activity Relationships of Anthracyclines with Respect to Anti-ras Activity To complement our previous observations on the structure–activity relationships of anthracyclines with respect to reversing tumorous phenotypes in K- and H-ras 3T3 cells, various antitumor anthracyclines at preclinical stages were also examined as to whether they flatten the morphology of K-ras 3T3 cells, restore actin stress fibers in the cells, and inhibit the growth of H-ras 3T3 cells more strongly in the agar medium (anchorage independent growth) than in a liquid medium (anchorage dependent growth). Alterations in the cell

Fig. 1. Structures of Anthracyclines

TABLE I. Effect of Anthracyclines on Morphology, Actin Structure and Growth of ras-Expressed Cells

Anthracycline	Morphology ^{a)}	Restoration of actin fibers ^{b)}	Ratio of IC ₅₀ in agar/liquid ^{c)}
Doxorubicin	+	+	0.033
Pirarubicin	+	+	0.043
FAD-104 ¹⁾	+	+	0.049
Daunorubicin	+	—	0.096
Epirubicin ^{d)}	+	+	0.217
4-Demethoxy-11-deoxydaunorubicin ³⁾	+	—	0.259
Aclarubicin	—	—	0.722
MX2 ¹⁾	—	—	0.382
3'-Deamino-3'-morpholinodaunorubicin ⁴⁾	—	—	0.421
Steffimycin B ⁵⁾	—	—	0.685
Steffimycin D ⁵⁾	—	—	0.730
2-Demethyl-steffimycin D ⁵⁾	++	+	0.111

a) Flat morphology of both K- and H-ras 3T3 cells at concentrations higher than IC₅₀ (+) or IC₃₀ (++) while (—) designates no alteration of the morphology at any drug concentration. b) Actin stress fibers were restored (+) or not restored (—) in K-ras 3T3 cells. c) Values inversely correlate with the activity inducing anchorage dependent growth of H-ras 3T3 cells, as discussed previously.¹⁾ d) 4'-Epi-adriamycin.

morphology and the actin structure were inspected preferably with K-ras 3T3 cells, since they looked thinner and adhered more firmly to culture wells than the H-ras 3T3 cells. In contrast, the experiment determining anchorage-dependent vs. -independent growth was conducted with H-ras 3T3 cells, since they grew faster in the agar medium than the K-ras 3T3 cells. Results are

summarized in Table I. The numbering system for the anthracycline structure, exemplified by doxorubicin, is shown in Fig. 1. Actin stress fibers were always detectable (+ under index *b*, Table I) in flattened cells (+ under index *a*, Table I). Compounds which were + under both *a* and *b*, contrary to those with both —'s, inhibited cell growth more strongly in the agar medium than in a liquid medium, giving smaller values under index *c* (Table I). To discuss the structure activity relationships, the compounds were classified into three groups based on their anti-ras activities; "strongly" anti-ras (+ under both *a* and *b*, and $0.049 \geq c$), "moderately" anti-ras (+ under both *a* and *b*, and $0.259 \geq c \geq 0.096$), and "not" anti-ras (— under both *a* and *b*, and $c \geq 0.382$). Based on this classification, the results were interpreted as follows. (1) 3'-NH₂ or 3'-OH is necessary; aclarubicin, MX2, and 3'-deamino-3'-morpholinodaunorubicin, all having 3'-NR₂, were "not" anti-ras. (2) A carbonyl groups at 13 may be essential; the compounds with this carbonyl were "strongly" to "moderately" anti-ras, while those without this carbonyl were "not" anti-ras, though they had other structural modifications. (3) A C-14 hydroxyl is desired; daunorubicin and 4-demethoxy-11-deoxydaunorubicin, both having hydrogen instead of a hydroxyl at 14, were still "moderately" anti-ras. (4) 4'-OH or 4'-OR, in an axial configuration, is desired; 4'-epirubicin, having 4'-OH in an equatorial configuration, was "moderately" anti-ras. (5) 4-OCH₃ rather than 4-OH is desired; doxorubicin, pirarubicin and FAD-104, all having 4-OCH₃, were strongly anti-ras, while aclarubicin and MX2, both having

Fig. 2.⁶⁾ Alteration of Cell Morphology and Actin Structure in T24 Induced by Doxorubicin

T24 cells were incubated for 48 h in the presence (A and C) or absence (B and D) of 0.1 µg/ml doxorubicin which inhibited the growth of T24 cells by 30%. Cells were photographed ($\times 100$) (A and B), or stained with rhodamine-phalloidin and photographed ($\times 400$) (C and D) as described under Materials and Methods.

Fig. 3. Growth Curves of K-ras 3T3 Solid Tumors in Mice

Standard deviations of the aclarubicin curves were similar to those of the control curve, thus omitted. □, 5 mg/kg pirarubicin; ■, 10 mg/kg pirarubicin; △, 5 mg/kg aclarubicin; ▲, 10 mg/kg aclarubicin; ○, no antibiotics; ↓, drug treatment.

4-OH, were not anti-ras. Comparison between daunorubicin (having 4-OCH₃) and 4-demethoxy-11-deoxydaunorubicin (having 4-H) would be more direct. The index *c* value of daunorubicin (0.096) was significantly

smaller than that of 4-demethoxy-11-deoxydaunorubicin (0.259).

The cell flattening activity of 2-demethylsteffimycin D (++) under *a*) did not parallel its activity inducing anchorage dependent growth (0.111, or "moderately," under *c*).

Anti-ras Activity to T24, a Human Bladder Carcinoma Cell Line Carrying a Mutated H-ras Gene The results described above, as well as those reported previously, were derived from experiments using rodent cell lines which had been artificially transformed by any of ras oncogenes. We therefore tried to confirm anti-ras activity in human tumor cells of natural origin, carrying a mutated ras gene. Doxorubicin flattened T24 cells and reconstructed actin fibers in the cells at a concentration of 0.1 µg/ml, which inhibited cell growth by 30% (Fig. 2). Other "strongly" anti-ras compounds, as opposed to "not" anti-ras ones, gave similar results. An attempt to determine the ratio of anchorage-dependent vs. -independent cell growth using T24 cells was not successful, however, because of their slow growth in the agar medium.

Correlation between Anti-ras Activity and Inhibitory Effect on Growth of K-ras 3T3 Solid Tumors in Nude Mice Now that the bedside diagnosis of oncogenes and

tumor suppressor genes is becoming practical,²⁾ better chemotherapy is expected using drugs that have selectivity to the genetic alteration of target tumors. Is a "strongly" anti-ras drug more effective than a "not" anti-ras one in inhibiting the *in vivo* growth of ras-mutated tumors? We examined pirarubicin ("strongly" anti-ras) and aclarubicin ("not" anti-ras), using an *in vivo* model of ras-mutated solid tumors; K-ras 3T3 cells inoculated s.c. were allowed to grow as solid tumors in nude mice while drugs were administered i.v. thereafter. As shown in Fig. 3, at doses of 5 and 10 mg/kg, pirarubicin was effective in a dose-dependent manner while aclarubicin was hardly effective. Aclarubicin at 20 mg/kg (data not shown) appeared almost as effective as pirarubicin at 5 mg/kg up to the 10th day, but 3 mice out of 5 (60%) died of toxicity after the 12th day, indicating that the dose was intolerable. The obvious difference in effectiveness between pirarubicin and aclarubicin could be a reflection of the difference in anti-ras activities and a clue to ras-specific chemotherapy. The two antibiotics, sharing the advantage of lower cardiac toxicity than that of doxorubicin, somewhat differ from each other in the antitumor spectrum. Since there is some

consistency between types of tumors and causative oncogenes,²⁾ we propose to review the antitumor spectra of the two antibiotics, as well as those of other clinically used anthracyclines, with regard to the presence or absence of ras oncogene mutations in different tumors.

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REFERENCES AND NOTES

- 1) K. S. Tsuchiya, T. Kanbe, M. Hori, Y. Uehara, Y. Takahashi, T. Takeuchi, *Biol. Pharm. Bull.*, **16**, 908 (1993).
- 2) J. M. Bishop, *Cell*, **64**, 235 (1991).
- 3) H. Umezawa, Y. Takahashi, M. Kinoshita, H. Naganawa, K. Tatsuta, T. Takeuchi, *J. Antibiotics*, **33**, 1581 (1980).
- 4) Y. Takahashi, M. Kinoshita, T. Masuda, K. Tatsuta, T. Takeuchi, H. Umezawa, *J. Antibiotics*, **35**, 117 (1982).
- 5) K. S. Tsuchiya, Y. Moriya, K. Yamazaki, M. Hori, N. Hosokawa, T. Sawa, H. Iinuma, H. Naganawa, C. Imada, M. Hamada, *J. Antibiotics*, **43**, 1489 (1990).
- 6) Color prints on request.