New Doxorubicin Analogs Active against Doxorubicin-resistant Colon Tumor Xenografts in the Nude Mouse

Fernando C. Giuliani and Nathan O. Kaplan

ABSTRACT

The antitumor activity of doxorubicin and three new derivatives modified on the position 4' of the amino sugar was tested against five human colon tumors and two human rectal tumors (originating from different patients) and xenografted into nude mice. The drugs tested were: 4'-epidoxorubicin; 4'-deoxydoxorubicin; and 4'-O-methyldoxorubicin. Mice were treated i.v. on a weekly basis for 3 to 4 weeks, starting when the tumors were well established (advanced stage of tumor treatment). No statistically significant effect was observed against the tumors tested with the drugs doxorubicin and 4'-epidoxorubicin. 4'-Deoxydoxorubicin was active against all the colon tumors tested (4 of 5 statistically significant); and 4'-O-methyldoxorubicin was active against 4 of 5 colon tumors tested (statistically significant). Overall, the activity of 4'-O-methyldoxorubicin was less than that of 4'-deoxydoxorubicin against the colon carcinomas tested. Neither analog was active against the two rectal carcinomas tested.

The results of these studies indicate that: (a) the modifications in the chemical structure of doxorubicin can alter the biological properties and thus create new drugs varying in activity against different human tumors; (b) the two antracycline derivatives, 4'-deoxydoxorubicin and 4'-O-methyldoxorubicin, appear to be good candidates for clinical trial against colon carcinoma; and (c) the nude mice system can offer a great potential for identification of new antracycline analogs and, in general, new anticancer agents of clinical interest.

INTRODUCTION

The death rate of patients affected by colorectal cancer, one of the most common types of human tumors, has remained essentially static in the last 20 years despite the effort to develop new therapies. There are only a few clinical antineoplastic drugs effective against colorectal cancer (8). DX3, probably the most "broad-spectrum" single agent in chemotherapy, is not among these (6, 12). A limiting toxic effect in the use of this drug is its dose-dependent cardiotoxicity (20). In order to develop new drugs with higher antitumor activity and without dose-limiting cardiotoxicity, many new antracycline analogs have been synthesized by making structural modifications in either the chromophore or the amino sugar part of DX (1-4, 9, 11, 14). Of the large number of new derivatives and analogs of DX investigated, compounds bearing modifications at position 4' of the amino sugar showed interesting properties (10), particularly epidiX, deoxDX, and O-DX (Chart 1). epidiX displayed noticeable antitumor activity against experimental tumors in mice (4, 9), and toxicity studies in normal and tumor-bearing mice revealed that epidiX was less toxic and less cardiotoxic than was DX (4, 10). In fact, in the Phase I and early Phase II studies performed at the National Cancer Institute of Milan by Bonfante et al. (7), epidiX showed lower acute toxicity than DX and has been found to be active against some human tumors (7). The other 2 derivatives have not yet been used in clinical trial but are presently under experimental studies. deoDX and O-DX showed interesting characteristics of lower cardiotoxicity than the parent compound DX and showed activity in the experimental antitumor system (10). We have examined the activity of the above-described drugs against 5 colon and 2 rectal human tumors, originating from 7 different patients and xenografted in the nude mice. These studies identified 2 DX analogs, deoDX and O-DX, which are active against DX-resistant colon carcinomas.

MATERIALS AND METHODS

Athymic Mice. The animals used in these experiments were 8- to 12-week-old congenitally athymic BALB/c mice of both sexes, homozygous for the nu/nu allele, bred in our laboratory and weighing 18 to 22 g at the start of the experiments. As previously described (22, 23), the mice were maintained under conventional conditions in autoclaved cages with polyester fiber filter covers. Every 3 months, for 3 weeks, piperazine hexahydrate was added to the drinking water to eliminate intestinal nematodes (5).

Human Tumors and Method of Inoculation. For this study, we have been using 7 different colorectal human tumors originating from different patients and transplanted into nude mice. All the tumor lines were established directly by inoculation of fresh tumor tissue from the patient into the nude mice. The rate take for all the tumor lines approached 90 to 100%. At the time of the study, the tumors were between the second and the 23rd passage in the nude mice. At the time of the study, the histological appearance of the serially transplanted tumors was identical to that of the original tumors. The human tumor specimens transplanted into BALB/c nude mice were obtained from the surgeon and transplanted 2 to 3 hr after resection. The specimens were rinsed with sterile medium containing antibiotic and then cut into small pieces for the s.c. implantation. For serial transplantation, the tumor mass was removed under sterile condition from the donor mice, minced in sterile medium containing antibiotics, and then passed through a 18-gauge needle before being inoculated into a s.c. space on the back of the nude mice. CEA was detected in either the blood...
serum or the tumor cells of 6 of the tumors tested (T 157, T 183, T 219, T 348, T 362, and T 380). For each test, 3 mice carrying the same tumor line were used. The sera were assayed with the Roche anti-human CEA antibody fluorescence test. Frozen tumor sections were assayed with an indirect immunofluorescence technique using monoclonal mouse anti-human CEA antibodies (courtesy of Hybritech Inc., La Jolla, San Diego, Calif.). Experiments are in progress to detect the existence of CEA in the blood serum and in the tumor cells of the remaining tumor studied, T 374. The tumor donor patients were never treated before surgery with anthracycline.

Before the serial transplantation, the amount of the mouse lactic dehydrogenase isoenzyme in the xenograft was quantitated as described previously. A limit of 20% was arbitrarily fixed as the maximum allowable level of mouse lactic dehydrogenase isoenzyme in human tissue. The growth curves of the studied tumors are shown in Chart 2, and the main characteristics of these tumors are listed in Table 1. Individual growth of xenografts shows considerable variation; converting tumor volume measurements from absolute to relative values (RV) allows for easier visualization of the antineoplastic activity of the drug and standardizes the tumor size at the start of treatment.

**Measurement of Tumor Size.** After the transplant, the mice were observed weekly and randomized into treated groups of 6 to 10 animals each after the tumors reached palpable size. The tumors were measured weekly in 3 dimensions with a slide caliper. The tumor volume was calculated by the equation $4/3\pi r^3$ modified as follows

$$RV = \frac{V_x}{V_i}$$

where the values of $d$ are the experimental measurements in mm of length, width, and thickness. Each tumor volume was then expressed in relative tumor volume (RV) by the formula

![Chart 1. Chemical structure of 4' DX derivatives.](image1)

- **Chart 1.** Chemical structure of 4' DX derivatives. *Mol. Wt.* of such compounds as hydrochlorides.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>MOL. Wt.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX</td>
<td>OH</td>
<td>H</td>
<td>579</td>
</tr>
<tr>
<td>4'-epiDX</td>
<td>H</td>
<td>OH</td>
<td>579</td>
</tr>
<tr>
<td>4'-deoDX</td>
<td>H</td>
<td>H</td>
<td>563</td>
</tr>
<tr>
<td>4'-ODX</td>
<td>OHCH3</td>
<td>H</td>
<td>593</td>
</tr>
</tbody>
</table>

**Table 1**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tissue of origin</th>
<th>Histopathological characteristics</th>
<th>No. of passages*</th>
<th>Blood serum</th>
<th>Tumor cells</th>
<th>CEA</th>
<th>% Mouse lactic dehydrogenase isoenzyme activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 157</td>
<td>Rectum</td>
<td>Very-well-differentiated invasive adenocarcinoma</td>
<td>17</td>
<td>ND$^c$</td>
<td>+</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>T 348</td>
<td>Rectum</td>
<td>Poorly differentiated adenocarcinoma involving fibrous tissue</td>
<td>4</td>
<td>ND</td>
<td>+</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>T 183</td>
<td>Colon</td>
<td>Well-differentiated mucoid adenocarcinoma</td>
<td>23</td>
<td>+</td>
<td>+</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>T 219</td>
<td>Colon</td>
<td>Well-differentiated adenocarcinoma originally invading the muscularis</td>
<td>23</td>
<td>+</td>
<td>+</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>T 362</td>
<td>Transverse colon</td>
<td>Papillary adenocarcinoma</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>T 374</td>
<td>Colon</td>
<td>Moderately well-differentiated adenocarcinoma</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>T 380</td>
<td>Colon</td>
<td>Moderately well-differentiated adenocarcinoma</td>
<td>2</td>
<td>ND</td>
<td>+</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

* At the time of the experiment.

$^a$ Detected in the tumors at the time of experiment (see “Materials and Methods”).

$^c$ ND, not done.
F. C. Giuliani and N. O. Kaplan

Table 2
Experimental chemotherapy of human colorectal tumors heterotransplanted in nude mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>DX (mg/kg/injection)</th>
<th>epidiX (mg/kg/injection)</th>
<th>deoxyDX (mg/kg/injection)</th>
<th>O-DX (mg/kg/injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.4 6 6.6 10</td>
<td>6 8 12 15</td>
<td>4.5 6</td>
<td>4 6 5</td>
</tr>
<tr>
<td>T 157</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 348</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 219</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 362</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 374</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T 380</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tumor regression:
- $T/C\%$ higher than 50%; ±, $T/C\%$ close to 50%; +, $T/C\%$ from 50 to 35%; ++, $T/C\%$ from 35 to 20%; +++$, T/C\%$ lower than 20%; ---, regression of the tumor to a volume smaller than the volume at the start of treatment.
- Statistically significant evaluated by Student's $t$ test.
- ND, not done.

where $V_x$ is the tumor volume at any given day and where $V_i$ is the initial tumor volume when the treatment was started.

Treatment and Evaluation of Chemotherapeutic Effect.

Drugs were prepared in distilled water at a concentration such that the dose could be given in a volume of 0.1 mg/g body weight. Drugs were supplied by Farmitalia Carlo Erba, Milan, Italy, and those used were DX; epidiX; deoxyDX; and O-DX. All the compounds have been synthesized by Arcamone et al. (1-4), Cassinelli et al. (11), and Di Marco et al. (14) at the Farmitalia Carlo Erba Research Laboratories. The drugs were always administered i.v. on a weekly basis for 3 to 4 weeks. The treatment was started when the tumors reached 1 to 3 ml in volume. The drug doses were selected on the basis of toxicity studies performed in our laboratory on non-tumor-bearing BALB/c nude mice. The treatments were performed with equitoxic doses of the single agents ($\pm$10% lethal dose). In the treated mice, the weight loss average did not generally exceed 10 to 15% of the total body weight. In general, a lower toxicity has been observed with DX and 4'- DX derivatives in non-tumor-bearing BALB/c nude mice than in conventional mice.

Statistical Evaluation. After each RV was obtained, the mean value was calculated to determine $T/C\%$. At any given day, $T/C\%$ was expressed as the average of RV of the treated mice to the average of RV of the control mice. Student’s $t$ test was used to determine if the RV averages of the treated mice differed significantly from those of the controls.

RESULTS

The therapeutic effects of DX, epidiX, deoxyDX, and O-DX upon 7 colorectal tumors originating from different patients are summarized in Table 2.

T 157. The treatment of Tumor T 157 was begun 17 days after tumor implantation (Table 3); the mice received 3 i.v. treatments. None of the drugs showed activity as measured by decrease in tumor growth. Doses of deoxyDX and DX (6 and 10 mg/kg/injection, respectively) slightly delayed the rate of tumor growth.

T 348. The activities of DX and 4'- DX derivatives on T 348 human rectum adenocarcinoma are described in Table 3. The treatment was started 15 days after the transplant. Three i.v. treatments were performed. deoxyDX at the 2 doses tested (4.5 and 6 mg/kg/injection) and O-DX at the higher dose (6.5 mg/kg/injection) slightly delayed the tumor growth but not sufficiently to produce a statistically significant reduction. DX and epidiX did not show any activity.

T 183. The activity on the T 183 human colon adenocarcinoma of the 4 drugs under investigation is shown in Chart 3.

Activity of DX, epidiX, deoxyDX and O-DX against T 157 and T 348 human rectal tumors after being transplanted into nude mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Drug</th>
<th>Dose (mg/kg/injection)</th>
<th>DTS*</th>
<th>Optimal T/C%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T157b</td>
<td>DX</td>
<td>10 28</td>
<td>64 (28)c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>epidiX</td>
<td>15 28</td>
<td>80 (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>deoxyDX</td>
<td>6 28</td>
<td>62 (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-DX</td>
<td>6.5 28</td>
<td>89 (12)</td>
<td></td>
</tr>
<tr>
<td>T 348d</td>
<td>DX</td>
<td>6 15</td>
<td>78 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>epidiX</td>
<td>10 15</td>
<td>58 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>deoxyDX</td>
<td>12 15</td>
<td>104 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-DX</td>
<td>4.5 15</td>
<td>96 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-DX</td>
<td>4.5 15</td>
<td>52 (37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-DX</td>
<td>4.5 15</td>
<td>47 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-DX</td>
<td>4.5 15</td>
<td>73 (22)</td>
<td></td>
</tr>
</tbody>
</table>

* DTS, number of days after tumor transplant that the treatment was started.
* Three i.v. treatments, one each week; 8 mice/group.
* Numbers in parentheses, day of observation (after the start of treatment).
* Three i.v. treatments, one each week; 7 mice/group.

4684 CANCER RESEARCH VOL. 40

Chart 3. Response of T 183 human colon adenocarcinoma xenografts in nude mice to DX, epidiX, deoxyDX, and O-DX. Relative tumor volume is the ratio of the mean tumor volume of a group at any given time to the mean tumor volume of that group at the initiation of treatment. Arrow, treatment; enclosed points, statistically significant points; O--O--control; A, DX (10 mg/kg/injection); A, DX (6 mg/kg/injection); A, epidiX (15 mg/kg/injection); A, epidiX (12 mg/kg/injection); O, deoxyDX (6 mg/kg/injection); O, deoxyDX (4.5 mg/kg/injection); O--O--O-DX (6.5 mg/kg/injection); O, O-DX (4.5 mg/kg/injection).
and Table 4. Four i.v. treatments were performed starting from the 28th day after the implantation of Tumor T 183. DX and epiDX did not show any therapeutic effect. deoDX at the 2 doses tested (4.5 and 6 mg/kg/injection) produced a delay in the tumor growth with statistically significant decrease in tumor volume (T/C% = 49, p < 0.05, and T/C% = 35, p < 0.02, respectively). O-DX produced a slight inhibition of tumor growth when tested at the 6.5-mg/kg/injection dose level (T/C% = 52).

T 219. Table 4 and Chart 4 show the effectiveness of DX and its derivatives on T 219 human colon adenocarcinoma. Four i.v. treatments at weekly schedule were performed on the 32nd day of tumor transplant. The treatment with deoDX at the 2 doses tested (4.5 and 6 mg/kg/injection) produced a temporary regression of the tumor. However, when the treatment was stopped, the tumors began to grow again. There was a statistically significant difference between the tumor volume of the controls and that of the mice treated with deoDX (6 mg/kg/injection; p < 0.02). No significant difference was observed between the controls and mice treated with deoDX (4.5 mg/kg/injection). Tumor T 219 responded markedly to treatment with O-DX at doses of 4 and 6.5 mg/kg/injection, but only with the dose of 6.5 mg/kg/injection was there a statistically significant decrease in tumor size. Only a slight activity against the growth of Tumor T 219 was detected in the groups treated with the higher doses of DX (6.6 mg/kg/injection) and epiDX (8 mg/kg/injection).

T 362. Table 5 and Chart 5 show the activities of DX and DX analogs on T 362 human colon papillary adenocarcinoma xenografted in the nude mice. Four i.v. treatments were performed in the mice bearing T 362 tumors. The treatment began 15 days after the tumor transplant. deoDX produced statistically significant activity at the 2 doses tested, 4.5 mg/kg/injection (T/C% = 31, p < 0.02) and 6 mg/kg/injection (T/C% = 31, p < 0.01). O-DX was active only at the highest dose tested (6.5 mg/kg/injection; T/C% = 44, p < 0.05) but less active than was deoDX. epiDX did not show any activity.

T 374. The response of T 374 human colon adenocarcinoma to DX and 4' DX derivatives is shown in Table 5 and Chart 6. Treatment was started 18 days after the transplant. Four weekly i.v. treatments were performed. Against this tumor, deoDX (6 mg/kg/injection; T/C% = 28, p < 0.02) and O-DX (6.5 mg/kg/injection; T/C% = 40, p < 0.02) were significantly
active. A temporary regression elicited by the drugs was also observed. DX slightly affected the tumor growth; epiDX did not show any effect.

T 380. In Table 5 and Chart 7, the results of the chemotherapy performed against T 380 human colon adenocarcinoma are summarized. The first of 3 treatments began 17 days after the tumor transplant. The treatment with DX and epiDX did not produce any effect on the tumor growth, whereas deoDX at the highest dose tested (6 mg/kg/injection; T/C% = 41) produced some activity which was not statistically significant. In contrast, O-DX was much more active than deoDX on the T 380 colon tumor. The doses tested (4 and 6.5 mg/kg/injection) gave a statistically significant reduction of tumor growth (T/C% = 31, p < 0.025; and T/C% = 29, p < 0.01, respectively).

DISCUSSION

Considerable attention has been devoted recently to investigations of new derivatives of daunorubicin and DX in the hope that modifications of the parent drugs can result in greater antitumor activity and/or decreased toxicity. In this paper, we have described the antineoplastic activity against human colorectal tumors transplanted into athymic mice of 3 new DX derivatives produced by modification at the position 4' of the amino sugar. In the mouse cardiotoxicity tests, these derivatives are less cardiotoxic than the parent compound DX (10).

We have chosen the athymic mouse system to screen the antineoplastic activity of DX derivatives because, although human xenografts in nude mice do not completely represent the real human situation, this mouse model can give relevant information on selected drugs that could be used in clinical trials. The chemotherapeutic treatment was delayed until the tumors became relatively large (advanced stage of tumor treatment) because: (a) the patients usually are not referred for chemotherapeutic treatment until after measurable disease is evident; and (b) treatment too soon after the transplant might cause interference in the tumor take, and the antitumor activity might be due to a different mechanism (e.g., interference in the vascularization) other than to true cytotoxicity of the drug. Clinically, the response rate to DX reported in gastrointestinal carcinoma has been variable, ranging from 0 to 20% (6, 15). Our experimental results are in good agreement with the clinical data. Except for slight activity on T 219 tumor, we never detected any antitumor activity of DX on the 7 colorectal tumors tested. Our results also show that the colorectal tumors tested were also refractory to the epiDX treatment.

The most important result of our experiments is the identification of 2 analogs, deoDX and O-DX, active against DX-resistant colon carcinomas originating from 5 different patients. deoDX was active against 5 of 5 colon tumors tested (4 of 5 statistically significant). The therapeutic effect of deoDX did not seem to be dependent on the rate of tumor growth. In fact, we observed temporary regression in the case of Tumor T 219, which has a growth rate not faster than that of the other tumors tested. A temporary regression was produced by deoDX and O-DX in the T 374 colon tumor. Our data regarding the differing responses to the same drug used on individual human tumors of the same histological type are in agreement with the results obtained by other authors (16, 17, 21). Variations in response could be due to the existence of distinct tumor cell subpopulations with different sensitivity to the chemotherapeutic agents (18, 19) in tumors having the same histological characteristics (13, 24). It is also possible that the sample transplanted into
the nude mouse may not be representative of all the major subpopulations growing in the patient. O-DX was found to be active (biologically and statistically) on 4 of 5 DX-resistant colon tumors. Overall, the activity of O-DX was less than that of deoDX against the colon tumor carcinomas tested. Even in the O-DX-treated groups, the antitumor effectiveness does not seem correlated with the tumor growth rate. Although deoDX and O-DX showed activity on colon tumors, the drugs did not exert any noticeable activity on T 157 and T 348 rectal tumors. Therefore, the 4' DX derivatives seem to be much more active against colon than rectal tumors. We are planning to test the antineoplastic activity of the DX derivatives against other rectal tumors to confirm this observation.

The results reported in this paper confirm that modification in the chemical structure of DX can alter the biological properties and consequently could produce new drugs which are active against certain human tumors. In fact, we have tested the antitumor activity of DX, epiDX, deoDX, and O-DX on several other types of human tumors xenografted in nude mice, and we have found their spectrum of antitumor activities quite different.5

It is of interest to note that deoDX, when tested against mouse colon tumor 38, had the same activity on this tumor growth as did DX.6

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