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Original Articles

Evaluation of New Anthracycline Analogs with the Human Tumor Stem Cell Assay

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Summary. *Ten anthracyclines, including doxorubicin (DX) and daunorubicin (DNR), and eight analogs with modifications in structure or stereochemistry of the aglycone and/or the aminosugar moiety were simultaneously tested in serial in vitro titration studies against human adenocarcinomas in the human tumor stem cell assay. More than a two-log range in cytotoxicity of the various anthracyclines was observed with the tumors tested. Marked individual differences in sensitivity of specific tumors (breast, lung, peritoneal) were observed for the various analogs. By assessing average effects on survival of tumor colony-forming units (TCFU) in the tumors tested, the three compounds lacking the methoxyl group in position 4 of the aglycone (4-demethoxyDX, 4-demethoxy-4'-epiDX, 4-demethoxyDNR) all proved to be more cytotoxic than their parent compounds. Compounds modified in position 4' of the aminosugar were on average either as toxic (4'epiDX) or more toxic (4'-deoxyDX and 4'-0-methylDX) to TCFU than the parent compound DX. On average, 11-deoxyDX was less toxic than DX or the other eight anthracyclines tested. The results obtained are also in good general agreement with those previously reported for anthracyclines with human tumors in xenografts or cancer patients. These antitumor results viewed in concert with toxicology studies in normal mice (including evidence of a lack of cardiac toxicity) suggest that 4'deoxyDX may prove to be a clinically useful anthracycline analog. We also conclude that use of this clinically predictive in vitro soft agar assay provides a rapid and relatively inexpensive means of simultaneously testing a large number of analogs of a parent compound against a spectrum of human tumors.*

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Introduction

Discovery of new anticancer drugs has been an arduous task requiring extensive preclinical testing prior to entry of a new agent into clinical trials. Identification of biosynthetic or semisynthetic analogs of effective standard agents has been an important strategy which has proven successful in the development of more effective antibiotics for treatment of infectious disease. In that setting, multiple strains of bacteria are utilized to ascertain which of a series of congeners has a differing spectrum of action or potency. This approach has been limited in the development of anticancer drugs because of the logistic difficulties and expense involved in carrying out in vivo screening of multiple analogs against multiple animal tumors or human tumor xenografts. Nonetheless, this approach has been useful for testing new anthracycline analogs [6, 11, 14; F. C. Giuliana et al. 1981, unpublished work]. Recently, an in vitro soft agar clonogenic assay system (the human tumor stem cell assay [HTSCA]) has been developed [15, 16]. It has proven useful for prediction of response of patients to anticancer drugs [1, 16–19, 22]. Of particular importance for new drug testing is the very high true-negative rate (> 95%) and the good true-positive rate (60%–70%) with the assay as applied to a wide variety of forms of cancer [1, 16–19, 22].

HTSCA has potential applicability to new drug screening [16, 17, 19]. In 1980 the National Cancer Institute (NCI) introduced the HTSCA into its new drug screening program to test it as an alternative to the standard in vivo murine tumor sysems [20]. In the program designed by the NCI, initial testing of new compounds is done at a dosage level of 10 μg/ml by continuous contact in the agar. In the present study we have used the HTSCA in a screening mode for simultaneous testing of ten anthracyclines against

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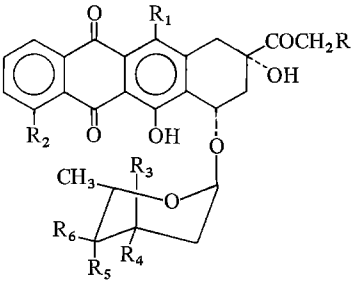
Table 1. Chemical structures of doxorubicin and daunorubicin derivatives



Compound	R	R_1	R_2	R_3	R_4	R_5	R_6	Mol. weight[a]
DX	OH	OH	OCH_3	H	NH_2	OH	H	579
4-DemethoxyDX	OH	OH	H	H	NH_2	OH	H	550
4′-EpiDX	OH	OH	OCH_3	H	NH_2	H	OH	579
4′-DeoxyDX	OH	OH	OCH_3	H	NH_2	H	H	563
4′-O-MethylDX	OH	OH	OCH_3	H	NH_2	OCH_3	H	593
3′,4′-DiepiDX	OH	OH	OCH_3	NH_2	H	H	OH	579
4-Demethoxy-4′-epiDX	OH	OH	H	H	NH_2	H	OH	550
11-DeoxyDX	OH	H	OCH_3	H	NH_2	OH	H	563
DNR	H	OH	OCH_3	H	NH_2	OH	H	563
4-DemethoxyDNR	H	OH	H	H	NH_2	OH	H	533

[a] As hydrochlorides

seven different human adenocarcinomas. The results indicate that in addition to individual overall differences in potency, specific anthracycline analogs may have qualitatively differing activity against specific human tumors.

Materials and Methods

Anthracycline Analogs. Ten anthracycline antibiotics, including two parent compounds (doxorubicin [DX], and daunorubicin [DNR]) and eight analogs (4-demethoxyDX, 4′-epiDX, 4′-deoxyDX, 4′-0-methylDX, 3′,4-diepiDX, 4-demethoxy-4′-epiDX, 11-deoxyDX and 4-demethoxyDNR) were tested. Table 1 summarizes the structural relationships of the various analogs, whose chemistry has been detailed previously by Arcamone and others [e.g., 3, 8, 10]. All the anthracyclines were kindly provided by Dr. F. Arcamone, Farmitalia Carlo Erba, Milano, Italy. All were water-soluble and dissolved in 0.9% sterile NaCl and were maintained at −70° C until the time of testing.

Preparation of Tumor Cell Samples (Malignant Effusions and Solid Tumors). Malignant effusions or solid tumor biopsies from a series of human adenocarcinomas referred to the Human Tumor Stem Cell Assay Laboratory of the University of Arizona Cancer Center were utilized in these studies. Cells from seven patients were used in the final dose-titration studies. Primary tumor sites for this subgroup of patients were as follows: Patients 1 and 2, breast; 3–5, ovarian; 6, lung; and 7, malignant peritoneal effusion in a patient with an unknown primary and extensive hepatic metastasis. Diagnostic studies in this latter patient did not suggest pulmonary, pancreatic, renal, colonic, or ovarian origin of the malignant effusions. Patients 1, 3, and 4 had clinical exposure to DX at some time prior to biopsy. In case 1, DX was given 18–24 months earlier as adjuvant therapy. Case 3 had extensive prior exposure to DX and case 4 a single course 1 month prior to referral for biopsy. Cell suspensions were prepared mechanically according to techniques reported previously [16, 18]. After allocation of cells required for routine cytotoxic drug testing, the remaining cells were transferred into a cryopreservation mixture containing McCoy's 5A medium, 10% heat-inactivated fetal calf serum (FCS) and 10% DMSO, and subjected to slow freezing in an insulated (styrofoam) box which was placed in a freezer at −70° C; and the cells were maintained thereafter at −70° C until the time of testing. Thawing was then carried out rapidly in a 37° C water bath, after which the cells were washed twice and resuspended into CMRL 1066 medium enriched with 10% horse serum and incubated for an additional hour at 37° C in a CO_2 incubator. This additional 37° C incubation improves viability for agar cloning of cryopreserved cells. A comparison of growth of tumor colony-forming units (TCFU) from 68 tumors tested in our laboratory from both fresh and cryopreserved cells is summarized in Table 2. It indicates that 75% of the tumors which gave rise to adequate numbers of colonies for drug evaluation (> 30 colonies/plate) from fresh specimens also provided adequate numbers of colonies after cryopreservation. While the exact cloning efficiencies varied somewhat, quite similar effects of cytotoxic drugs on the percent survival of fresh and cryopreserved TCFU has also been observed in our laboratory [16].

In Vitro Tumor Stem Cell Assay. The clonogenic assay system utilized was a minor modification of the method of Hamburger and Salmon [15, 16]. Conditioned medium was not required in the 0.5% agar feeder layer. Basal growth characteristics were assessed on cryopreserved and reconstituted cell suspensions prior to the large scale testing of the anthracycline analogs. Only specimens with good in vitro growth were utilized. All yielded at least 30 colonies-plate, with a median number of 94 colonies/plate. Colony counting was carried out with an automated image analysis system (Bausch & Lomb Omnicon FAS II) which was optimized for use with the human tumor stem cell assay [16]. Colonies had to have a diameter of > 60 μm to be included in the analysis. As has been detailed previously with standard drugs [16, 18], all plates were first assessed on day 1 to ascertain that a good single cell suspension had

Table 2. Comparison of tumor colony formation by fresh and cryopreserved tumor cells

Tumor type	No. of samples compared	Percentage of specimens with greater than 30 TCFU per plate	
		Fresh cells	Cryopreserved cells
Ovarian	27	63	48
Lung	9	66	55
Colon	3	66	33
Melanoma	14	64	50
Breast	6	50	50
Misc.[a]	9	33	11
Total	68	59%	44%[b]

[a] Stomach, bladder, cervical, neuroblastoma, pancreas, sarcoma
[b] 75% of the tumors which gave rise to colonies from fresh cells also gave rise to adequate numbers of colonies from the frozen aliquot

been plated and counted when adequate growth of tumor colonies was present in control plates (average: day 17) to permit accurate assessment of results in drug-treated plates.

Chemosensitivity Testing of Anthracyclines. For purposes of new drug screening, strategies recently summarized by Salmon [16, 17] were utilized. Testing of the anthracyclines was carried out by the continuous exposure technique [16, 17], which incorporates the agent at twice the final concentration into the 1.0 ml upper layer of 0.3% agar along with the tumor cell suspension (usually 5×10^5 nucleated cells per dish) in enriched CMRL with 10% horse serum. With soluble drugs such as anthracyclines, the drug diffuses quickly into the 1.0-ml bottom layer of 0.5% agar. Preliminary titrations of all ten anthracyclines were carried out at the screening dosage (10 μg/ml) with dose reductions in subsequent assays (generally sequential log 10 reductions) until an end-point titration was achieved wherein no more than three of the ten agents had reduction in survival to less than 1% of the control. For each assay, six control plates and triplicate plates for each drug were employed and the results in drug-treated samples averaged to the nearest whole number for percent survival. Simultaneous multidose testing with plating of higher cell numbers to routinely assess survival at less than 1% of control was not carried out and lower degrees of survival of TCFU were therefore reported as < 1%.

Results

Preliminary Titrations

Initial testing of the ten anthracyclines at the 10 μg/ml level was conducted on TCFU from two breast, two ovarian, two endometrial, and one gastric cancer. At this dosage level, survival was reduced to < 1% of control in all instances with the exception of one of the endometrial cancers. For that specific tumor, survival of TCFU ranged from 11% (4′-0-methylDX) to 22% (DX), with 4′deoxyDX, 4′-epiDX 4-demethoxy-4′-epiDX, and DNR intermediate between those values, in increasing order. The ovarian and gastric adenocarcinomas were also tested at the 1.0 μg level with < 1% of TCFU surviving. Inadequate quantities of cryopreserved cells were available for some of these tumors to permit detailed titrations at lower drug concentrations, and the detailed titrations were therefore limited to seven adenocarcinomas (see below) which were available in sufficient quantities for repeated testing of frozen cells.

Final Titrations

A spread in survival of TCFU over a two-log range was achieved for seven adenocarcinomas tested at the anthracycline dosage of 0.01 or 0.05 μg/ml. The results of these studies are summarized in Table 3. Overall, the greatest degree of inhibition of TCFU was observed with the two breast cancer specimens tested. For the six tumors tested at the 0.01 μg level (cases 2–7, Table 3) a ranking of relative overall in vitro anthracycline sensitivity or resistance was calculated. This was obtained by averaging the percent survival of the ten anthracyclines against each tumor. In order of increasing overall anthracycline resistance these were: Case 2, breast (15% survival); case 5, ovarian (29%); case 4, ovarian (46%); case 6, lung (54%); case 7, unknown primary (57%); and case 3, ovarian (68%). It is of interest that the last-mentioned tumor, which manifested the greatest degree of resistance to all ten anthracyclines, was from an ovarian cancer patient who had received extensive prior therapy with DX. Ovarian case 4 had received one dose of DX 1 month prior to biopsy, while the other four patients in this comparison had no prior anthracycline exposure. The ten anthracycline analogs were compared with each other for relative in vitro potency by averaging the percent survival for each drug against the seven tumors tested (less than 1% survival was assumed to be equal to 1% to derive these averages). 4′DeoxyDX had the greatest in vitro potency (21% survival), followed in decreasing order by 4′-0-methylDX (26%), 4-demethoxy-4′-epiDX (29%), 4-demethoxyDNR (31%), DNR (34%), 4-demethoxyDX (36%), DX (46%), 4′-epiDX (47%), 3′4′-diepiDX (58%), and 11-deoxyDX (61%). Very similar relative potency rankings are obtained if case 1 (five-fold higher dosage testing) and/or cases 3 and 4 (prior clinical anthracycline exposure) are exluded from the computation. If all three cases are excluded, the average in vitro survival of the five most potent compounds (4′-0-methylDX, 4-demethoxy-4′-epiDX, 4-demethoxyDNR, 4′-deoxyDX, and 4-demethoxyDX) in the four remaining

Table 3. Effects of anthracyclines on in vitro percent survival of TCFU from seven human adenocarcinomas

Source of tumor (concentration)[a]	Prior DX exposure	DX	4-Demethoxy-DX	4′-Epi-DX	4′-Deoxy-DX	4′-O-Methyl-DX	3′,4′Diepi-DX	4-Demethoxy-4′-EpiDX	11-Deoxy-DX	DNR	4-Demethoxy-DNR	Average[b] percent survival
1. Breast (0.05 μg/ml)	Yes	6	8	7	< 1	< 1	7	3	3	4	< 1	4
2. Breast (0.01 μg/ml)	No	16	8	9	13	13	46	14	11	15	12	15
3. Ovarian (0.01 μg/ml)	Yes	67	73	80	41	55	75	69	88	63	70	68
4. Ovarian (0.01 μg/ml)	Yes	55	61	50	30	42	43	35	49	48	47	46
5. Ovarian (0.01 μg/ml)	No	31	32	28	18	12	53	14	77	6	17	29
6. Lung (0.01 μg/ml)	No	57	50	60	30	27	89	41	74	51	58	54
7. Peritoneal (0.01 μg/ml)	No	92	23	93	12	34	93	27	128	51	14	57
Average[b] percent survival		46	37	47	21	26	58	29	61	34	31	–

tumors tested was less than 30% of control (range 21%–28% survival). Assessment of the average effects on survival for the human tumors tested (Table 3) revealed that the three compounds lacking the methoxyl group in position 4 of the aglycone (4-demethoxyDX, 4-demethoxy-4′-epiDX, 4-demethoxyDNR) were all more cytotoxic to TCFU than their parent compounds. Compounds modified in position 4′- of the aminosugar (i.e., 4′-epiDX) were on average either as toxic as or more toxic (4′-deoxyDX and 4′-0-methylDX) than the parent compound DX. On average, 11-deoxyDX was less toxic than the parent compound DX or the other eight anthracyclines tested.

Discussion

DX and DNR and various new anthracycline derivatives have been studied in a large variety of experimental systems [6]. In vitro colony inhibition tests on HeLa cells were used as a primary evaluation model by Di Marco et al. [12, 13]: the results related well with the potency of the compounds in mice, but there was not a clear-cut relation between cytotoxicity in vitro and antitumor effectiveness against experimental tumors at the optimal dose. The in vitro HTSCA has previously been reported to be predictive of clinical response in vivo [1, 16, 18, 22], and therefore appeared likely to be a useful system with which to test anthracyclines. The current studies were undertaken to broaden the spectrum of human tumors tested with the new anthracycline analogs, and to evaluate the simpler methodology of in vitro studies with the HTSCA. In the current studies, continuous contact in agar rather than the standard 1-h test was utilized to maximize the chance of observing chemosensitivity to the known analogs. The preliminary titration at the 10-μg level was selected because this initial dose is currrently standard in the NCI's new drug screening effort with the HTSCA [20]. It is clear from the results that all the anthracycline analogs would have been picked up as 'positives' in such a screen, and subjected to further

[a] All compounds tested by continuous exposure of the anthracycline in the agar. Concentrations in parentheses represent the final concentration after equilibration in the agar. With an average culture duration of 17 days the maximum Cxt in vitro at the 0.01 μg/ml dosage would be 4.1 μg h/ml (assuming that the anthracyclines were all stable in the agar)

[b] Average survival figures were computed by assuming that < 1% survival was equal to 1% survival. This assumption was made in view of the limited ability to titrate survival below 1% (see *Methods*)

study. For the anthracyclines, and tumors tested, the 0.01-μg level provided the most useful discrimination in HTSCA. The relevance of the concentration-time product (CXT) achieved with this in vitro dosage level (in the range of 4 μg h [0.01 μg/ml × 408 h or 17 days]) will need to be established with pharmacokinetic studies in vivo. In our experience, for in vitro chemosensitivity with the HTSCA to predict clinical response of known active compounds, reduction in survival of TCFU, to the range of < 30% of control, needs to be observed at less than 10% of the CXT achievable in vivo. For the parent compound, doxorubicin, a clinically tolerable CXT of 1.6–3.8 μg h/ml can be achieved in patients after a bolus dose of 60 mg/m^2 [4]. In recent studies with the HTSCA on ovarian cancer reported by Alberts et al. [2], it was demonstrated that prior clinical exposure to DX reduces the in vitro response rate to this agent as against that observed in vitro with TCFU from previously untreated patients. In that study, in vivo sensitivity to DX was observed on TCFU from 39% of untreated patients and 20% with prior therapy ($P = < 0.01$). A suggestion of the same phenomenon is apparent in the current study, particularly with case 3, who had extensive prior anthracycline exposure and the poorest average reduction in survival with the anthracycline analogs on the six tumors tested at 0.01 μg/ml. When HTSCA is utilized as a primary screen for entirely new agents, tumors from previously untreated patients should probably be used exclusively. However, in an analysis of analogs of known active compounds such as anthracyclines, it is perhaps more informative to include some tumors from patients who have had clinical exposure to the parent compound previously, as long as adequate clinical data are available to classify the specimen properly with regard to prior drug exposure. In that way, an analog lacking cross resistance with the parent compound could potentially be identified. In the present study, in which ten anthracyclines were compared simultaneously in the seven tumors tested, inclusion of both untreated and DX-treated tumors is valid, as the relative ranking of activity is the major end-point, rather than the absolute degree of sensitivity of an individual patient's tumor.

The data summarized in Table 3 show that ten anthracyclines bearing various modifications in the chemical structure and stereochemistry of the aglycone or of the aminosugar have different activity against human tumors in vitro. The compounds studied had been previously tested against HeLa cells in vitro; results reported in Table 4 show the different degree of cytotoxicity detected, and compare it with one of the human tumors. It is of interest to observe that the relative cytotoxicity ratios obtained on HeLa cells correlate in some instances with the cytotoxic effect of a human peritoneal malignant effusion: compounds lacking the methoxyl group in position 4 of the aglycone (4-demethoxyDX, 4-demethoxy-4′-epiDX, 4-demethoxyDNR) were markedly more cytotoxic than the parent compounds in both these systems; compounds modified in position 4′- of the aminosugar were about as cytotoxic as parent drug (i.e., 4′-epiDX) or more cytotoxic (4′-deoxyDX and 4′-0-methylDX); 11-deoxyDX was less toxic than DX. For the other human tumors used in this study there was no clear relation to the HeLa cell-testing results, and individual human tumors tested in vitro had different sensitivity to the anthracyclines. There is therefore no reason to think that HeLa cell testing provides a better model for testing such analogs than a panel of human tumors. In fact, the latter approach is preferable.

Table 4. Comparative in vitro effects of anthracycline analogs on HeLa cells and human adenocarcinoma TCFU from a peritoneal effusion

Compound	HeLa ID_{50} ratio[a] (parent compound/analog)	Adenocarcinoma Percent survival ratio[b] (parent compound/analog)
DX	1.0	1.0
4-DemethoxyDX	9.3	4.0
4′-EpiDX	1.1	1.0
4′-DeoxyDX	1.5	7.6
4′-O-MethylDX	1.9	2.7
3′,4′-DiepiDX	0.1	1.0
4-Demethoxy-4′-epiDX	310	3.4
11-DeoxyDX	0.1	0.7
DNR	1.0	1.0
4-DemethoxyDNR	3.1	3.6

[a] Ratio of ID_{50} s (ng/ml) of parent compound and the analogs. The ID_{50} was determined as the concentration required for 50% inhibition of HeLa cell colony forming units, after cell exposure to the drugs for 24 h in monolayer (ratios calculated from data of DiMarco et al. [12, 13]). The ID_{50} for the parent compound DX was 9.3 ng/ml, and that for DNR, 10 ng/ml

[b] The ratio of percent survival (as compared to control) of the parent compound divided by the percent survival of the analog are shown based on a 0.01 μg/ml exposure dose by continuous contact (see *Methods*)

The high effectiveness of almost all the anthracyclines tested against the two breast carcinomas in vitro is in agreement with experimental [6; F. C. Giuliana et al., unpublished work] and clinical data, which show the high sensitivity of this type of tumor to anthracyclines. Previous studies have shown that some of the new anthracyclines here considered, at their optimal doses, were as effective as DX against mouse mammary carcinoma: 4′-epiDX [7], 4-deme-

thoxyDX and 4-demethoxy-4′-epiDX [13], and 4′-deoxyDX [9]. However, the current in vitro data do not suggest the higher efficacy of DX in comparison with DNR on breast cancer as has previously been suggested based on murine data [11]. Conceivably, in addition to cytotoxicity against the TCFU, other factors, such as modulation of immune response, pharmacokinetics, and toxicity to normal host tissues may also be of importance in determining the high antitumor effectiveness of DX in vivo. However, many of these other factors are similar for DX and DNR and it may not be appropriate to conclude that DX is superior in breast or ovarian cancer, as it must be recognized that extremely scant clinical data are available comparing DX and DNR for their efficacyon human breast or ovarian cancer (or other solid tumors). Alternatively, our relatively small sample size of tumor for HTSCA in this study may have precluded the recognition of a difference in potency between DX and DNR. Comparative testing of analogs in vitro should ideally be carried out at a standard percentage of the CXT or of the concentration achieved by an LD_{10}, or the maximally tolerated dose of the compounds in vivo. 3′-4′-DiepiDX was less potent than DX against L1210 leukemia, but equally effective at its optimal dose [3]; the lower potency in comparison with DX is in agreement with the lower general effectiveness found in this study. Similarly, 11-deoxyDX was about 60 times less potent than DX in mice, but at the optimal dose maintained excellent antitumor activity against P388 leukemia [10]; these data are in agreement with the lower effectiveness of 11-deoxyDX than DX against three of the seven human tumors tested, shown in the present study. The data showing that 4′-deoxyDX and 4′-0-methylDX are more active than DX against three ovarian adenocarcinomas (including two with prior DX exposure) are of great interest.

These two derivatives as well as 4′-epiDX have been tested against a number of human tumors heterotransplanted in nude mice [14; F. C. Giuliana et al., unpublished work]. In the xenograft studies, marked effectiveness of 4′-deoxyDX and 4′-0-methylDX was noted against colon tumors. One out of two different ovarian carcinomas (both rather resistant to DX therapy) was found to be partially sensitive to 4′-epiDX and 4′-0-methylDX, but not to 4′-deoxyDX [14; F. C. Giuliana et al., unpublished work].

The activity observed for 4′-deoxyDX in the current studies as well as in mouse tumor systems along with preliminary pharmacokinetic and toxicology data in mice appears sufficient to warrant the advancement of this anthracycline analog into phase I clinical trials. Important in reaching this decision is evidence for a lack of cardiac toxicity of 4′-deoxyDX in mice [9]. Further studies in HTSCA with this compound in a broader spectrum of tumors could be considered as an 'in vitro phase II clinical trial' [16, 19], which can be carried on simultaneously with the phase I clinical trial in cancer patients. This concept has recently been applied with the HTSCA to a variety of cytotoxic agents and found to be useful for selecting the tumor types (as well as individual patients), with a high likelihood of achieving clinical tumor regression with new phase I–II agents [19].

As regards 4′-epiDX, the data presented here show that this compound has an activity against some human tumors which is of the same order of magnitude as that of DX, and not qualitatively different. These data are in good agreement with the results obtained against mouse experimental tumors, showing that 4′-epiDX has the same antitumor effectiveness as DX at the same dose [7], even if less toxic and slightly less cardiotoxic in experimental animals and humans [8, 21]. Clinical studies suggest that 4′-epiDX can show activity against some human tumors resistant to DX therapy, such as malignant melanoma, colon carcinoma, renal carcinoma [5; G. Robustelli della Cuna et al., unpublished work); these tumors have not been investigated in the present study. All together, these data indicate that modifications in the chemical structure of anthracyclines can result in important differences in their ability to inhibit the growth of human tumor cells of various origin. Further studies are needed with anthracycline analogs to ascertain the predictivity of the in vitro tests as regards selectivity of antitumor effectiveness.

Finally, it is clear from the current study that the strategy of simultaneous screening of a large number of analogs for relative cytotoxicity can be carried out simply, quickly, and at relatively low expense (and requiring only minute amounts of scarce compounds) with the human tumor stem cell assay. Use of cryopreserved cells appears most reasonable, as our preliminary comparisons of effects of standard cytotoxic agents against fresh and cryopreserved cells have yielded quite similar results [16]. In view of the relatively good agreement between the results in our current studies on anthracycline analogs and those obtained independently in xenografts (and in cancer patients), it is likely that HTSCA on cryopreserved as well as fresh cells will gain increasing importance in the preclinical evaluation of new drugs. Development of very large panels of cryopreserved tumors including a wide variety of histopathologies will probably become routine in such efforts. Independent assessment of drug toxicity on normal tissues is of course required.

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