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In vitro cytotoxicity against fresh human tumors and P388 leukemia predicts the differential *in vivo* activity of a series of anthracene anticancer drugs

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To date, random anticancer drug screening has proven to be relatively inefficient and non-specific with respect to selecting active compounds for most tumor types (except for leukemia/lymphoma). Although large numbers of compounds from diverse sources were evaluated for many years in the P388 mouse leukemia model, only a few clinically useful drugs have been identified by this *in vivo* screening method. Thus, there is intense interest in the development of more effective *in vitro* screening models for new anticancer drugs. In the present paper we have compared the discriminating power for fresh human tumors from patients, human tumor cell lines developed from 11 patients and murine P388 leukemia in tumor colony forming assays as indicators of cytotoxicity for a series of anthracene antitumor agents. Two of a series of 21 novel bisantrene analogs, R6 (*N,N*^{1}-bis[2-(dimethylamino)ethyl]-9,10-anthracenebis(methylamine)) and R26 (*N,N*^{1}-bis(1-ethyl-3-piperidinyl)-9,10-anthracenebis(methylamine)) produced significant cytotoxicity against the 11 human tumor cell lines and were therefore selected for additional *in vitro* and *in vivo* studies. R26 was specifically selected for further testing since it had similar *in vitro* potency as mitoxantrone, but showed no cross-resistance against mitoxantrone-resistant WiDr colon or doxorubicin-resistant 8226 myeloma cell lines. In contrast to the cell line data, only one of the 22 fresh human tumors showed significant *in vitro* sensitivity (i.e. <50% survival of tumor colony forming units) to either R6 or R26 tested at high concentrations. Both of these bisantrene analogs also proved inactive at 1.2–1.6 μM concentrations against P388 leukemia *in vitro*, whereas mitoxantrone and bisantrene were highly active in this model at a concentration of 0.2 μM. In order to compare the *in vitro* data with antitumor activity *in vivo*, R26, the most active bisantrene analog, and mitoxantrone, the most active of the two anthracene parent compounds, were tested against P388 leukemia and M5076 ovarian sarcoma in mice. In both models mitoxantrone showed significant activity whereas R26 produced minimal or no antitumor effects. We conclude that fresh human tumors, but not defined human tumor cell lines, predict the *in vivo* cytotoxicity of a series of anthracene anticancer agents. Although this conclusion may not apply to the screening of other classes of antitumor agents, we propose an *in vitro* screening process which first utilizes numerous human tumor cell lines of many different biologies (to screen a large number of new compounds each year), followed by confirmatory tests in fresh human tumors using colony forming assays to screen up to a smaller number of 1000 compounds. Finally, appropriate *in vivo* tumor models based on histologic specificity would be used to screen a few consistently active new compounds for advancement to clinical trials. Thus, the first screening stage would be highly sensitive and non-specific, the second *in vitro* stage more specific and the third *in vivo* stage relevant by histologic tumor type.

Key words: Anthracene, P388 leukemia, cytotoxicity.

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Introduction

The screening program for cytotoxic anticancer agents continues to undergo re-evaluation and change at the National Cancer Institute (NCI) and elsewhere. Emphasis has been placed on improving the specificity, rapidity and cost-effectiveness of drug screening. Under NCI sponsorship from the mid-1950s to the mid-1980s random antitumor screening of as many as 15,000 compounds per year from diverse sources was completed in mice bearing P388 leukemia intraperitoneally.[1] Unfortunately, only a few clinically useful anticancer drugs have been identified by this screening method.

During the 1980s, new *in vitro* systems, including human tumor colony forming assays in soft agar, were developed to try to improve the predictive power of initial activity screens to differentiate active and inactive compounds. The human tumor colony forming assay (HTCA)[2,3] was subsequently validated in a series of clinical trials and was associated with a 91% true negative and a 61% true positive incidence of predicting anticancer drug resistance and sensitivity, respectively.[4–11]

The HTCA has been used to identify specific tumor types for phase II testing of both new cytotoxic agents[12–15] and for selecting active analogs of existing compounds.[16,17] Additionally, the NCI sponsored studies to determine whether HTCA results from fresh human tumors compliment data obtained in *in vivo* screening models concerning cytotoxic drug activity.[18] A major interest focused on the use of the HTCA to select drugs for further preclinical development which were specifically inactive *in vivo* against P388 leukemia.

Encouraging early results were obtained in the initial HTCA drug screening tests with fresh tumors.[18,19] However, the NCI drug screening program is now primarily using human tumor cell line cytotoxicity data to select new compounds for further preclinical testing[20,21] and an emphasis on fresh human tumor testing has diminished. A series of studies has compared various strengths and weaknesses of *in vitro* drug screening using human tumor cell lines vs fresh human tumors[22–25] and the current findings suggest continued utility for assays using fresh human tumors. The present paper compares cloned fresh human tumors, human tumor cell lines and murine P388 leukemia assays as predictors of *in vitro* cytotoxicity for a series of anthracene antitumor compounds.

Materials and methods

Drugs

Bisantrene and mitoxantrone were supplied by Lederle Laboratories (Pearl River, NY). A series of 21 novel analogs based on anthracene and bisantrene were synthesized by TP Wunz and WA Remers (Tucson, AZ).[26] Purity >98% was confirmed by NMR spectroscopy. Two of these analogs which showed remarkable activity *in vitro*,[26] R6 and R26, were used in this study. Drugs were reconstituted in normal saline (mitoxantrone, R6), 5% dextrose in water (bisantrene) or dimethyl sulfoxide (R26). Drug aliquots were frozen and stored at −80°C until use.

Human tumor clonogenic assay

The HTCA was carried out with minor modifications as described by Salmon and Hamburger for epithelial cancers of the ovary.[4,27–30] Cells were exposed for 1 h to varying concentrations of drugs, ranging from 0.01 to 200 μM, so that at least a 1 log dose–response curve could be constructed. Definition of *in vitro* chemosensitivity was based on calculation of the percentage of tumor colony forming units (TCFUs) in drug-exposed plates relative to those in control plates at drug concentrations which represented 1/10 of the cumulative plasma exposure *in vivo* (i.e. ~0.1 × the *in vivo* plasma concentration · time product).[31] New agents were tested at a range of 1 h concentrations which reduced survival to ≤10% of controls. Sensitivity to a specific drug was defined as ≤50% survival of TCFUs.

Human tumor cell lines

Colon cancer cell lines (Colo 205, HCT 15, LoVo, DLD-1, COLO 320 DM and SW 480) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). An ovarian cancer cell line (UACC 66) was obtained from A Leibovitz (Arizona Cancer Center, Tucson, AZ).[32] The WiDr (sensitive) and WiDr (mitoxantrone-resistant, passage 232) cell lines were obtained from R Wallace (Medical Research Division, American Cyanamid, Pearl River, NY). Myeloma cell lines, 8226 (sensitive) and 8226 (40-fold doxorubicin-resistant) were obtained from WS Dalton (Arizona Cancer Center, Tucson, AZ).[33]

Cell lines were maintained in RPMI 1640 (Irvine, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM), (Sigma, St Louis, MO), and incubated at 37°C in 5% CO_2. All cell lines with the exception of Colo 205, COLO 320 DM and myeloma 8226, grew as monolayers and were harvested with trypsin–EDTA (0.025%, Gibco, Grand Island, NY). The Colo 205 and COLO 320 DM grew attached and in suspension and were mechanically removed from flasks. The 8226 cell lines grew as suspensions. Cell lines were used no more than four passages from the frozen stock.

Murine cell line

P388, mouse lymphocytic leukemia (ATCC 501490), was obtained from the NCI-Fredrick Cancer Facility, DCT Tumor Repository (Fredrick, MD). Cells were maintained in supplemented serum RPMI 1640 at 37°C in 5% CO_2. Cells grew as a suspension.

Human tumor cell line clonogenic assays

All cell lines were harvested in an exponential growth phase and processed to a single cell suspension. The number of cells used in the Hamburger–Salmon clonogenic assay ranged from 5000 to 20,000 cells per 35 mm tissue culture dish, depending upon the plating efficiency of the individual cell line. Table 1 shows the doubling time and plating efficiencies for the 11 cell lines used in pre-clinical studies of the anthracene compounds.

Table 1. Doubling time and plating efficiencies for 11 human tumor cell lines used as a pre-screen for cytotoxic activity of a series of anthracene anticancer drugs

	Doubling time (h)	Plating efficiency (%)
COLO 320 DM	37	13
DLD-1	40	30
Colo 205	25	14
HCT 15	41	8
SW 480	45	26
LoVo	48	8
WiDr (sen.)	31	22
WiDr (mitoxantrone-resist.)	33	7
UACC 66	48	5
8226 (sen.)	27	24
8226 (doxorubicin-resist. ×40)	28	12

Frozen drug aliquots were thawed and immediately diluted to appropriate concentrations with normal saline. Final DMSO concentrations were never greater than 0.1% for a 1 h drug exposure (vehicle controls were included in all assays).

Drug incubations were done at 37°C for 1 h in serum-supplemented RPMI 1640. Concentrations ranged from 10 μM to 0.012 or 0.0012 μM incubated with sufficient numbers of cells so that the clonogenic assay could be plated in triplicate for each of the drug concentrations and controls. After drug incubation, cells were washed twice with 5 ml of McCoy's 5A (Irvine, Santa Ana, CA) supplemented with 2% FBS, penicillin (100 units/ml) and streptomycin (100 units/ml) and plated as previously described.[2,4,27] Plates were incubated for 7–10 days at 37°C in 5% CO_2, after which 1 ml of 1 mg/ml INT (2-*p*-iodophenyl-3-(*p*-nitro-phenyl)-5-phenyltetrazolium chloride) (Pfaltz and Bauer, Stamford, CT) stain[27] was added to plates for 24 h. Colonies >60 μ were then enumerated by an automated image analyzer (FAS II Omnicon®, Bausch and Lomb, Rochester, NY).[28] Bisantrene and mitoxantrone were included in each experiment as positive cytotoxic controls.

Fresh human tumor clonogenic assays

Tumors were received from local cancer surgeons in transport media (McCoys 5A, Irvine, Santa Ana, CA) supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml) and transferrin (0.01 mg/ml) (Sigma, St Louis, MO).

Samples were minced into 2–3 mm pieces and then further digested with 8 ml of 0.15% type I collagenase, 0.015% DNase mixture (Sigma, St Louis, MO) and 8 ml of hypo-osmotic media[29] in a stirring flask at 100 rpm for 1.5 h at 37°C. The digest was then filtered through an 80 μm mesh Nitex® fiber filter (Tetko, Monterey Park, CA) and washed with McCoys 5A supplemented with 10% FBS. The resulting cell suspension was examined and if cell clumps were present, the suspension was passed through a 30 μ Nitex mesh. If necessary, a 25 or 30 μ mesh filtration was then used to obtain a single cell suspension. One modification in the original Hamburger–Salmon clonogenic assay[2,4,22,27] involved the use of sea plaque agarose (Sigma, St Louis, MO) in place of agar.

In contrast to the 1 h drug exposures used with the human tumor cell lines, the anthracene compounds were mixed with the fresh tumor cell suspensions in supplemented RPMI 1640 and added to the upper agarose layer to facilitate continuous drug exposure.[30] Large cell types (>20 μ in diameter) were plated at approximately 2×10^5 cells per plate, while cells <20 μ were plated at $2.5–3 \times 10^5$ cells per plate. Plates were incubated for 7–21 days at 37°C in 5% CO_2, stained with INT stain[18] and colonies counted on the FAS II Image Analyzer. The plant lectin, Abrin (Sigma, St Louis, MO) was used as a positive control in each experiment.

Mice

Eight- to 10-week-old mice were obtained from Jackson Laboratories, Bar Harbor, ME (male DBA/2J) and from Charles' River, Portage, MI (female CD2F1 and female B6C3F1).

Mouse tumor models

In vivo mouse studies were performed at the Arizona Cancer Center and at the Southern Research Institute, Birmingham, AL, under a contract with Dr Daniel Griswold.

P388 leukemia. Approximately 4.4×10^6 cells were implanted into the intraperitoneal space on day 0 in male DBA/2J or female CD2F1 mice. Mitoxantrone (1.8 and 2.7 mg/kg) and the R26 anthracene analog (44, 67, 100 and 125 mg/kg) were injected ip on days 1, 5 and 9. The anthracene analog R6 (30, 60, 70, 80, 90 and 100 mg/kg) was injected ip once on day 1 or daily for 5 consecutive days on days 1–5. Control groups of 10 mice received ip saline on days 1, 5 and 9 or daily on days 1–5. Doses for compounds R6 and R26 were determined by initial toxicology studies in non-tumor-bearing DBA/2J or CD2F1 mice.[26]

M5076 ovarian sarcoma. Cells were obtained from a tumor repository at the Southern Research Institute and were administered subcutaneously into the flank of B6C3F1 female mice by Dr Daniel Griswold and investigators to evaluate the relative antitumor activities of mitoxantrone and compound R26. Both mitoxantrone (1.8 and 2.7 mg/kg) and compound R26 (30, 44, 67 and 100 mg/kg) were injected ip on an every 4-day (×3 injections) schedule.

Results

Human tumor cell line clonogenic assays

Two of the series of 21 novel bisantrene analogs, R6 (*N,N'*-bis[2-(dimethylamino)ethyl]-9,10-anthracenebis(methylamine)) and R26 (*N,N'*-bis(1-ethyl-3-piperidinyl)-9,10-anthracenebis(methylamine))[26] proved significantly cytotoxic *in vitro* against the 11 human tumor cell lines and were therefore selected for additional *in vitro* and *in vivo* studies. The chemical structures of bisantrene, mitoxantrone and these two bisantrene analogs are shown in Figure 1. R6 and R26 represent two anthracenes from a series of bis[[2-(dialkylamino)-ethyl]amino]methyl derivatives of bisantrene which were produced by modifying bisantrene's alkyl-hydrazone side chain. Alterations involved changes in the heteroatom, the degree of unsaturation and the length of the alkyl chain separating the anthracene nucleus from the basic centers in the side chains.[26]

The *in vitro* IC_{50} μM concentrations (i.e. concentrations of drug associated with inhibition of 50% of TCFUs of bisantrene, mitoxantrone, R6 and R26 against each of 11 human tumor cell lines are shown in Table 2. R26 had similar potency to mitoxantrone, but showed no cross-resistance against the mitoxantrone-resistant WiDr colon or doxorubicin-resistant 8226 myeloma cell lines. Although R6 was less potent than R26, it was considerably more cytotoxic than bisantrene against six of the 11 lines.

P388 leukemia cell line clonogenic assays

Both mitoxantrone and bisantrene proved highly potent *in vitro* against P388 leukemia colony forming units at all concentrations tested (Table 3). In contrast, R6 and R26 were inactive at ≤ 0.16 μM and only minimally active at ≥ 1.2 μM (continuous exposure) against P388 murine leukemia *in vitro*.

Fresh human tumor clonogenic assays

Twenty-two fresh human tumors were tested *in vitro* with R6 (0.16 and 1.6 μM) and R26 (0.12 and

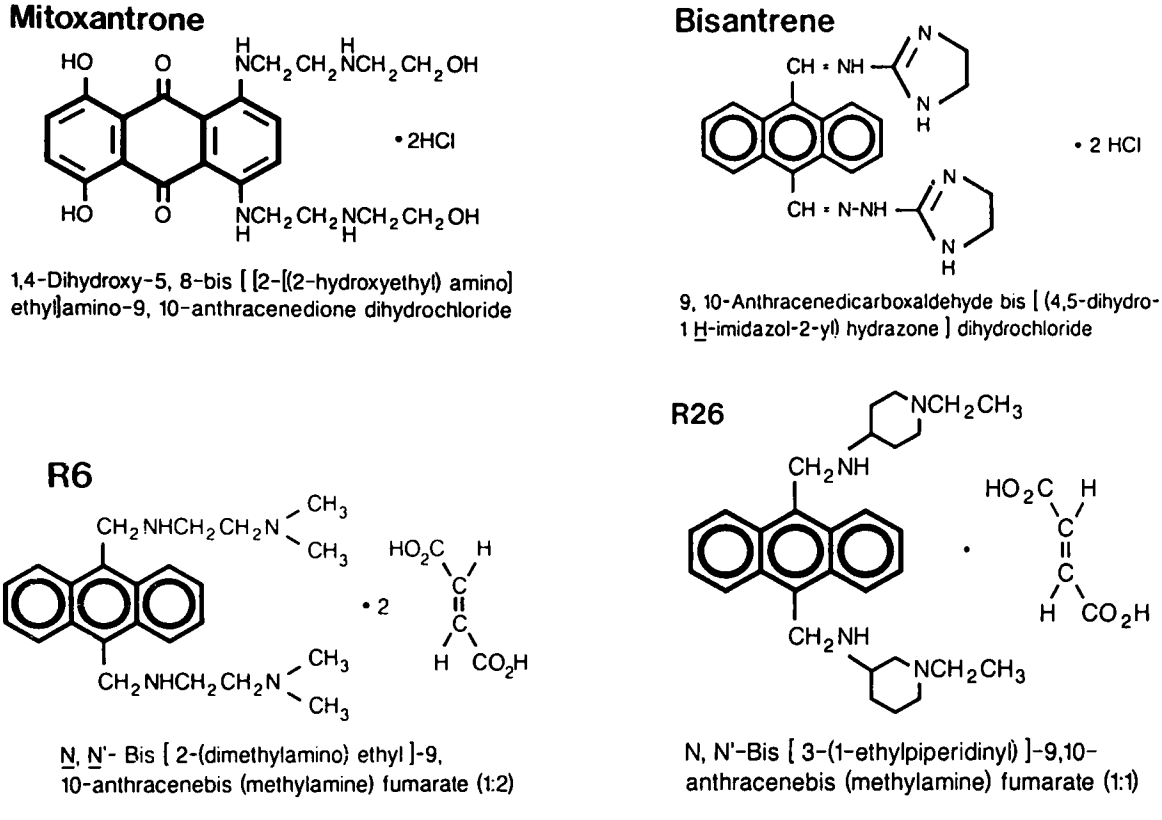

Figure 1. Chemical structures of the anthracene compounds mitoxantrone, bisantrene and the bisantrene analogs R6 and R26.

1.2 μM) by continuous exposure (Table 4). Only one of these tumors showed sensitivity (i.e. <50% survival of TCFUs) to either of the bisantrene analogs (i.e. R26 against one ovarian cancer). The 10 melanomas, seven breast cancers and five lung cancer specimens were insensitive to both R6 and R26.

Mouse tumor model studies

The majority of the *in vivo* studies were designed to evaluate the antitumor activity of anthracene analog R26, because this compound proved 5- to 100-fold more potent than compound R6 *in vitro* against 10 of the 11 different human tumor cell lines (Table

Table 2. Inhibitory concentrations in 50% of cells (IC_{50}) associated with the *in vitro* cytotoxicity of four anthracene anticancer agents against 11 different human tumor cell lines[a]

Tumor cell line	Bisantrene (μM)	Mitoxantrone (μM)	Analog R6 (μM)	Analog R26 (μM)
DLD-1 (Colon)	2.73	0.06	0.23	0.03
HCT 15 (Colon)	UA[b]	0.12	0.34	0.04
LoVo (Colon)	2.41	NT	0.14	0.02
Colo 205 (Colon)	0.21	0.01	<0.01	<0.01
COLO 320 (Colon)	UA	0.01	0.05	0.01
SW 480 (Colon)	1.57	0.05	0.14	0.02
WiDr-S (Colon)	0.42	0.04	0.23	0.01
WiDr-R (Colon)	0.53	0.77	0.23	0.02
UACC 66 (Ovarian)	0.74	0.09	1.82	0.02
8226-S (Myeloma)	0.16	0.02	0.19	0.01
8226-R (Myeloma)	UA	0.06	NT	0.02

[a] Mean continuous exposure in colony forming assays in soft agar ($n = 3$).
[b] UA, unachievable IC_{50} at concentrations tested; NT, not tested.

Table 3. Activity of anthracene compounds against P388 leukemia colony forming units (CFU) in soft agar ($n = 3$)

Anthracene (conc. tested by cont. exposure)	% Survival of CFUs		
	0.1 μg/ml	1.0 μg/ml	10 μg/ml
Mitoxantrone (0.19–19 μM)	<1	<1	<1
Bisantrene (0.21–21 μM)	0	0	0
R6 (0.16–16 μM)	100	88	0
R26 (0.12–12 μM)	100	47	NA[a]

[a] NA, data not available.

Table 4. Number of fresh human cancers sensitive[a] to anthracene compounds in the human tumor clonogenic assay

Cancer	R6	R26
Ovarian	0/8	1/7
Melanoma	0/5	0/5
Breast	0/3	0/4
Lung	NT[b]	0/5

[a] <50% survival of tumor colony forming units at drug concentrations of 0.1 or 1.0 μg/ml by continuous exposure.
[b] NT, not tested.

2). Compound R26 was compared with the anthracene compound mitoxantrone because of identical *in vitro* potency characteristics[26] and because R26 was active against the mitoxantrone-resistant WiDr cell line (Table 1).

P388 leukemia. R6 doses of 30–100 mg/kg (ip) were injected once, or daily for 5 days. The 90 and 100 mg/kg single doses were associated with early deaths. There was a 25% increase in life spans (%ILS) at 60 mg/kg (daily × 5 schedule) and 35% ILS with 80 mg/kg (daily × 1 schedule). These %ILS data suggest marginal antileukemic activity for compound R6. No leukemic mice were cured by R6 in experiments performed at the Arizona Cancer Center.

%ILS data, survival durations and approximate $\log_{10}$ changes in tumor burden for mitoxantrone compared to R26 in P388 leukemic CD2F1 mice (Southern Research Institute) are shown in Table 5. A limited R26 survival study in non-tumored CD2F1 mice suggested an optimal non-toxic dose of 67 mg/kg administered ip three times at 4-day intervals. In leukemic mice this produced a 33% ILS over that of control mice, whereas mitoxantrone produced 152% ILS. It is estimated that there were about 4×10^6 P388 leukemia cells when therapy was initiated 24 h post-implant. These data indicate that mitoxantrone reduced the tumor burden by about five orders of magnitude, while R26 blocked the leukemia cell increase by nearly two orders of magnitude.

Table 5. Comparative activity of mitoxantrone and anthracene analog R26 against P388 leukemia in CD2F1 mice

Treatment ip; days 1, 5, 9		Therapeutic response[a]			
		Median		Approx. no. of cells alive at end of experiment	Approx. log change in tumor burden at end of experiment[b]
Agent	Dosage (mg/kg/dose)	Day of death	% ILS		
Control	(untreated)	10.5	—	—	—
R26	125.0	2.0	−81	Toxic	
	100.0	7.0	−34	Toxic	
	67.0	14.0	+33	2.1×10^8	+1.7
	44.0	13.5	+28	2.5×10^8	+1.7
Mitoxantrone	2.7	26.5	+152	33	−5.1
	1.8	20.0	+90	4.8×10^5	−1.0

[a] Based on median day of death. Historical doubling time of 0.47 day was used in estimating cell kill and the number of cells alive at end of experiment.
[b] Log_{10} change = net log change in viable tumor cell population at the end of treatment as compared to the start of treatment; e.g. a −6 log change means that there was a 99.9999% reduction and a +3 log change means there was a 1000-fold increase in tumor burden at the end of treatment.

M5076 ovarian sarcoma. Compound R26 and mitoxantrone were tested ip against M5076 ovarian sarcoma implanted subcutaneously in the mouse flank. An every 4-day × 3 ip dosing schedule was used. The first dose was begun 24 h after tumor implantation and R26 doses ranged between 30 and 100 mg/kg. R26 treatment was not associated with a significant %ILS or tumor growth delay as determined by median times to attain 500 and 1000 mg flank tumor weights (Table 6). Although mitoxantrone did not increase murine life span, it was associated with a slightly greater tumor growth delay when compared to compound R26 (i.e. average of 2.7 days growth delay for mitoxantrone vs 0.525 days for R26).

These data show that despite equivalent activity to mitoxantrone and bisantrene in 11 established human tumor cell lines, neither R6 nor R26 produced significant antitumor effects in freshly harvested human tumors *in vitro* or in two mouse tumors tested *in vivo*. They also had poor cytotoxic potency against P388 leukemia cells *in vitro*.

Discussion

Newer *in vitro* screening models using fresh human tumor cells, such as the HTCA[2-4] have gained increasing use in drug development. Interest in the HTCA has continued because the assay appears to accurately predict both clinical response and resistance to a wide variety of anticancer drugs in individual patients.[5-11] Perhaps of equal significance, the HTCA has proven useful when applied to secondary drug screening trials, such as for obtaining individual tumor-type chemosensitivity data on novel compounds identified by random screening methods.[12-15] For these reasons the NCI contracted with several institutions to use the HTCA to identify entirely new anticancer agents which might be overlooked by the conventional murine leukemia model.[18,19] The results of these studies established that the HTCA accurately identifies non-toxic, clinically ineffective agents as true negatives with 97% accuracy and identifies other agents not detected with P388 leukemia *in vivo*. However, HTCA suffers from the limitations of any *in vitro* screening system. These problems include: (1) underpredicting activity for compounds that require metabolic activation (false negative); (2) overlooking certain antimetabolites whose activity is blocked by culture medium ingredients (false negative); and (3) overprediction of therapeutic activity for non-selective toxins such as plant lectins (false positive).

Although the HTCA has proven useful in the selection of at least two previously overlooked P388 leukemia negative compounds,[19] for logistics and cost its use was limited to only a few hundred compounds per year. As a result, *in vitro* drug screening programs have been moving toward testing drugs against banks of established human tumor cell lines studied by automated, microtiter-type growth inhibition assays.

Screening assays involving a vital tetrazolium dye have been used to evaluate the cytotoxicity of standard and experimental agents against panels of 10–20 different human tumor cell lines of breast,

Table 6. Comparative activity of mitoxantrone and anthracene analog R26 against subcutaneously implanted M5076 ovarian sarcoma in female B6C3F1 mice (n = 20/group)

Treatment		Lifespan (days post-implant)		% ILS	Time (days) to reach tumor weight		Days delay[b] (T − C)
Agent	Dose (mg/kg)[a]	Median	Range		500 mg	1000 mg	
Control	(untreated)	28.0	24–40		12.9	14.8	
R26	100	30.5	29–40	8	12.9	15.4	0.3
R26	67	30.0	28–34	7	13.2	16.0	0.7
R26	44	33.5	30–40	19	13.1	17.0	1.2
R26	30	28.0	28–32	0	11.8	15.8	−0.1
Mitoxantrone	2.7	31.5	26–38	12	14.7	18.0	2.5
Mitoxantrone	1.8	30.5	28–>40	8	14.2	19.3	2.9

[a] Route of drug administration was intraperitoneal every 4 days ×3, treatment begun on day 1 after tumor implantation.
[b] T − C, the unweighted average of the differences of the median times post-implant for the treated and control groups to attain each of the two evaluation sizes.

colon, lung, and ovarian cancer types.[20,21] However, the tetrazolium assay may have several shortcomings in broad screening programs.[25] These include: (1) inability to produce multi-log dose–response curves for standard anticancer drugs compared to clonogenic assays; (2) lack of dose–response curves for highly schedule-dependent drugs, such as fluorodeoxyuridine, when using short-term (i.e. <7 days) dye assays;[25] and (3) the unsuitability of using slowly growing human tumor cell lines which have prolonged doubling times.

In the present study, *in vitro* clonogenic assays with P388 leukemia accurately predicted *in vivo* antitumor activity for mitoxantrone and a lack of activity for two novel anthracene compounds (e.g. R6 and R26 tested against murine leukemia and M5076 ovarian tumor models). Similar results were described by Marsh *et al.*[34] in a retrospective comparison of 13 different agents tested in the HTCA assay and P388 leukemic mice. The correlation was best ($r = 0.92$) for drug concentrations producing 70% inhibition of colony formation *in vitro* with 5-day drug administration schedules in leukemic mice. This is consistent with our finding that P388 leukemia tested in colony-forming assays *in vitro* proved more reliable than did a panel of 13 human tumor cell lines for the selection of the clinically active anthracene. Because of the narrow range of compounds evaluated, the utility of the *in vitro* P388 leukemia model cannot be generalized to a broad range of cytotoxic compounds. And it should be recalled that drugs selected as 'active' by *in vivo* leukemia screening models such as P388 and L1210 leukemias are primarily those which cause myelotoxicity or have antileukemia/lymphoma effects.[12,13,19]

The fresh human tumor data in the HTCA also accurately predicted the relative lack of *in vivo* activity of the R6 and R26 anthracenes. This sharply contrasts with the studies in freshly cloned human tumors which documented a high degree of cytotoxicity for the parent anthracene compounds, mitoxantrone and bisantrene.[14,15,30] Thus, the use of the HTCA as a drug screening model has a number of real and potential advantages. These include: (1) the selection of active drugs which may not produce dose-limiting myelotoxicity (not shown in the current study); (2) the possibility that each fresh human tumor may be equivalent to one *in vivo* murine tumor model in terms of predictive potential; (3) the ability to store fresh human cancers at $-80°C$ for rapid screening tests and as reproducible controls for new agents; and (4) the presence of lymphocytes and macrophages in fresh tumors aids in the evaluation of biologic modifiers as well as cytotoxic drugs.

Although the HTCA is costly and labor-intensive when applied to fresh human tumors, by testing combinations of three to four new agents simultaneously, its screening capacity is significantly enhanced. This could facilitate the evaluation of more than 1000 compounds per year. Because different drug combinations can be tested repetitively against the same group of tumors, it would be relatively straightforward to identify the 'active ingredient'.

The current studies focused on only one class of cytotoxic compounds, the anthracenes. Thus, the results and conclusions may not extend to anthracyclines, alkylating agents or antimetabolites. With this caveat, we propose a revised *in vitro* screening process which retains the initial use of human tumor cell lines of multiple histologies to evaluate new compounds. 'Active' agents would then be tested in fresh HTCAs. Finally, appropriate *in vivo* tumor models based on histologic specificity observed in the prior two assays would be utilized to more thoroughly screen the few remaining active compounds. Thus, the first screening stage would be hypersensitive and non-specific, the second *in vitro* stage more specific, and the third *in vivo* stage relevant by histologic tumor type.

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