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Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines[1]

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ABSTRACT

We have previously described the application of an automated microculture tetrazolium assay (MTA) involving dimethyl sulfoxide solubilization of cellular-generated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-formazan to the *in vitro* assessment of drug effects on cell growth (M. C. Alley *et al.*, Proc. Am. Assoc. Cancer Res., *27:* 389, 1986; M. C. Alley *et al.*, Cancer Res. *48:* 589–601, 1988). There are several inherent disadvantages of this assay, including the safety hazard of personnel exposure to large quantities of dimethyl sulfoxide, the deleterious effects of this solvent on laboratory equipment, and the inefficient metabolism of MTT by some human cell lines. Recognition of these limitations prompted development of possible alternative MTAs utilizing a different tetrazolium reagent, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT), which is metabolically reduced in viable cells to a water-soluble formazan product. This reagent allows direct absorbance readings, therefore eliminating a solubilization step and shortening the microculture growth assay procedure. Most human tumor cell lines examined metabolized XTT less efficiently than MTT; however, the addition of phenazine methosulfate (PMS) markedly enhanced cellular reduction of XTT. In the presence of PMS, the XTT reagent yielded usable absorbance values for growth and drug sensitivity evaluations with a variety of cell lines. Depending on the metabolic reductive capacity of a given cell line, the optimal conditions for a 4-h XTT incubation assay were 50 μg of XTT and 0.15 to 0.4 μg of PMS per well. Drug profiles obtained with representative human tumor cell lines for several standard compounds utilizing the XTT-PMS methodology were similar to the profiles obtained with MTT. Addition of PMS appeared to have little effect on the metabolism of MTT. The new XTT reagent thus provides for a simplified, *in vitro* cell growth assay with possible applicability to a variety of problems in cellular pharmacology and biology. However, the MTA using the XTT reagent still shares many of the limitations and potential pitfalls of MTT or other tetrazolium-based assays.

INTRODUCTION

The metabolic reduction of soluble tetrazolium salts to insoluble colored formazans has been exploited for many years for histochemical localization of enzyme activities (1, 2). In one of the earliest efforts to develop a practical *in vitro* drug sensitivity test, Black and Speer (3) utilized a tetrazolium/formazan method to assess inhibition of dehydrogenase activity by cancer chemotherapeutic drugs in slices of excised tissue. As an *in situ* vital staining process this phenomenon has also been used for identifying viable colonies of mammalian cells in soft agar culture (4) and for facilitating *in vitro* drug sensitivity assays with human tumor cell populations in primary culture (5). Mosmann (6) described a tetrazolium-based assay which allowed rapid measurement of growth of lymphoid cell populations and their response to lymphokines. Recent reports from our laboratories (7, 8) and others (9, 10) have described modifications of Mosmann's procedure for *in vitro* assay of tumor cell response to chemotherapeutic agents. We have found that this MTA[3] approach allows reproducible estimates of drug sensitivity in a variety of human and other tumor cell lines. Moreover, because of its microscale and potential for automation, the MTA is one of several assays under consideration by the National Cancer Institute for potential application to a large-scale antitumor drug-screening program (7, 8).

The previously described MTA (7, 8) requires DMSO solubilization of MTT-formazan generated by cellular reduction of the MTT tetrazolium reagent. This step is not only laborious, but also may risk exposure of laboratory personnel to large quantities of potentially hazardous solutions in DMSO. Frequent DMSO exposure also produces deleterious effects upon some laboratory equipment. Therefore, to allow the investigation of a simplified MTA and to address potential problems associated with solvent handling, a series of new tetrazolium salts have been developed which, upon metabolic reduction by viable cells, yield aqueous-soluble formazans (11). In this paper we describe the development of one such tetrazolium salt (XTT) and its application to the MTA.

MATERIALS AND METHODS

Cell Lines and Culture. Cell lines (Table 1) were maintained as stocks in RPMI 1640 (Quality Biological, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT) and 2 mM L-glutamine (Central Medium Laboratory, NCI-FCRF). Cell cultures were passaged once or twice weekly using trypsin-EDTA (Central Medium Laboratory, NCI-FCRF) to detach the cells from their culture flasks.

Drugs. All experimental agents were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, DCT, NCI. Crystalline stock materials were stored at −70°C and solubilized in 100% DMSO. Compounds were diluted into complete medium (RPMI 1640 plus fetal bovine serum) plus 0.5% DMSO before addition to cell cultures.

MTT-Microculture Tetrazolium Assay. Cellular growth in the presence or absence of experimental agents was determined using the previously described MTT-microculture tetrazolium assay (7, 8). Briefly, rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations (100-μl volume) into 96-well microtiter plates using a multichannel pipet. After 24 h, drugs were applied (100-μl volume) to triplicate culture wells, and cultures were incubated for 6 days at 37°C. MTT (Sigma, St. Louis, MO) was prepared at 5 mg/ml in PBS (Dulbecco and Vogt formulation, without calcium and magnesium; Quality Biological, Gaithersburg, MD) and stored at 4°C. On Day 7, MTT was diluted 1 to 5 in medium without serum (in the MTA described in Refs. 7 and 8, MTT was diluted in complete medium containing 10% fetal bovine serum), and 50 μl were added to microculture wells. After 4-h incubation at 37°C, 250 μl were removed from

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[3] The abbreviations used are: MTA, microculture tetrazolium assay; DMSO, dimethyl sulfoxide; MEN, menadione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide, inner salt, sodium salt; IC_{50}, 50% inhibitory concentration; NCI, National Cancer Institute; FCRF, Frederick Cancer Research Facility; DCT, Division of Cancer Treatment.

Table 1 *Cell strains used in this study*

Cell line	Origin	Source	MTT/XTT absorbance[a] with the following PMS concentrations[b]				
			MTT, 0.0 mM	XTT			
				0.0 mM	0.001 mM	0.01 mM	0.0250 mM
H23	Lung adenocarcinoma	a[c]	1.176	0.105	0.115	0.492	1.224
H322	Lung bronchioloalveolar carcinoma	a[c]	0.738	0.242	0.256	0.561	1.337
H324	Lung adenocarcinoma	a[c]	0.492	0.031	0.052	0.710	1.110
H358	Lung bronchioloalveolar carcinoma	a[c]	0.545	0.077	0.081	0.266	0.637
H460	Lung large cell carcinoma	a[c]	2.580	0.288	0.342	1.688	2.830
A549	Lung adenocarcinoma	b[d]	2.700	0.233	0.356	2.360	2.804
LOX	Malignant melanoma	c[e]	1.030	0.201	0.266	1.489	2.279
HT-29	Colon adenocarcinoma	b[d]	1.914	0.201	0.196	1.014	1.555
MCF-7	Breast adenocarcinoma	d[f]	1.110	0.086	0.093	0.374	0.828
CCD-19 LU	Lung fibroblast	b[d]	0.356	0.109	0.120	0.369	1.090
MCR-5	Lung fibroblast	b[d]	0.326	0.186	0.211	0.300	0.695
W138	Lung fibroblast	b[d]	0.257	0.045	0.092	0.317	0.747
P388	Murine leukemia	e[g]	0.674	0.101	0.174	0.426	1.262
	Background (n = 13)		0.023 ± 0.006[h]	0.175 ± 0.031	0.186 ± 0.057	0.204 ± 0.034	0.250 ± 0.015

[a] Data represent average absorbance minus background from triplicate wells. Cells were inoculated at 1250 cells/well. Culture duration was for 7 days, and MTT or XTT incubation for 4 h at 37°C.
[b] PMS concentration: 0.001 mM = 0.015 μg/well; 0.01 mM = 0.15 μg/well; 0.025 mM = 0.38 μg/well.
[c] Supplied by Dr. A. Gazdar, Navy Medical Oncology Branch, Division of Cancer Treatment, NCI, Bethesda, MD.
[d] Obtained from the American Type Culture Collection, Rockville, MD.
[e] Supplied by Dr. O. Fodstad, Norwegian Radium Hospital, Oslo, Norway.
[f] Supplied by Dr. K. Cowan, Clinical Pharmacology Branch, Division of Cancer Treatment, NCI, Bethesda, MD.
[g] Supplied by the NCI, DCT Tumor Repository, NCI-FCRF, Frederick, MD.
[h] Mean ± SD of 39 triplicate well background measurements.

each well, and 150 μl of 100% DMSO were added to solubilize the MTT-formazan product. After thorough mixing with a mechanical plate mixer, absorbance at 540 nm was measured with a Dynatech Model MR 600 mciroplate reader.

XTT-Microculture Tetrazolium Assay. The new tetrazolium reagent (XTT) was designed to yield a suitably colored, aqueous-soluble, nontoxic formazan upon metabolic reduction by viable cells. The chemical structures of MTT, XTT, and their respective formazan reduction products are shown in Fig. 1. The presence of two sulfonic acid groups in XTT is the key to its aqueous solubility in both the tetrazolium ion form and the formazan form. XTT has a single net negative charge at physiological pH, and bioreduction of the central positively charged tetrazolium nucleus increases the net charge to two. The corresponding reduction of MTT reduces the net positive charge to zero, and thus the MTT formazan is quite insoluble. For the present investigations, XTT was obtained by a synthetic procedure described elsewhere (11). The XTT reagent is now available from at least one commercial supplier (Polysciences, Warrington, PA).

The XTT-assay methodology was essentially the same as that described using the MTT reagent with the following modifications: XTT was prepared at 1 mg/ml in prewarmed (37°C) medium without serum. PMS (Sigma, St. Louis, MO; Catalogue No. P9625) was prepared at 5 mM (1.53 mg/ml) in PBS. The 5 mM PMS solution was stable at 4°C for at least 3 mo. MEN (Sigma; Catalogue No. M5625) was prepared at 10 mM (1.72 mg/ml) in acetone. MEN was prepared fresh immediately before use. Fresh XTT and PMS were mixed together at the appropriate concentrations. For a 0.025 mM PMS-XTT solution, 25 μl of the stock 5 mM PMS were added per 5 ml of XTT (1 mg/ml). Fifty μl of this mixture (final concentration, 50 μg of XTT and 0.38 μg of PMS per well) were added to each well on Day 7 after cell inoculation. For experiments designed to examine various PMS concentrations, 5 mM PMS was diluted in PBS before addition to the 1-mg/ml solution of XTT. After an appropriate incubation at 37°C (4 h unless otherwise indicated), the plates were mixed on a mechanical plate shaker, and absorbance at 450 nm was measured with the Dynatech Model MR 600. Absorption spectra of tetrazolium reagents, formazan products, and cellular-generated formazans were measured with a Beckman Model MVI scanning spectrophotometer.

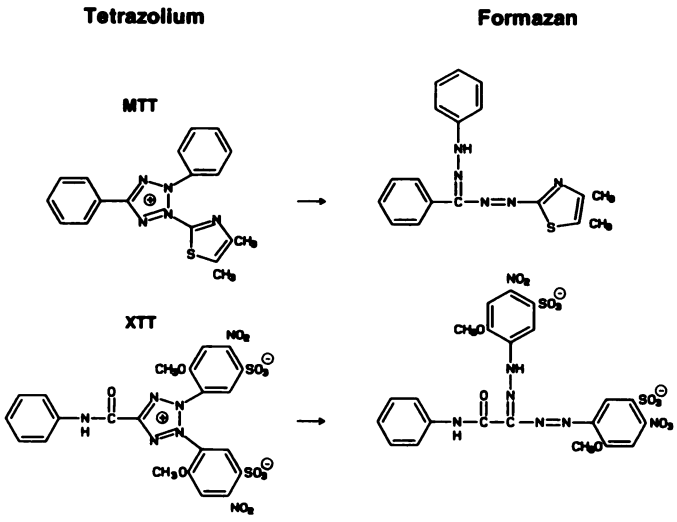

Fig. 1. Structures of MTT and XTT tetrazolium and formazan.

RESULTS

Metabolic Reduction of XTT by Human Cells. To determine the suitability of the new XTT reagent for a microculture growth inhibition methodology, we investigated the ability of several human tumor cell lines to reduce XTT to a measurable aqueous-soluble formazan product. Table 2 shows typical MTT and XTT metabolism data for different cell inoculation densities. Two human lung tumor cell lines were incubated for different times with MTT (only 4-h data shown) or XTT, and the absorbances were measured. Cell line A549 reduced MTT far more efficiently than H322, illustrating two extremes of cellular metabolic ability. Neither cell line was able to efficiently reduce XTT during the 4-h incubation time, thus making obligatory longer incubation times for the generation of a substantial absorbance. These data also indicated that the formazan product resulting from the cellular reduction of XTT was not toxic to human tumor cells over the 96-h period of this experiment. The absorbance continued to increase, and microscopic examination of cultures confirmed that cells remained viable in the presence of the XTT-formazan. Table 1 compares absorbance measurements with MTT and XTT for several cell lines. In all cases the metabolic reduction of the tetrazolium was substantially less for XTT than for MTT (Table 1). Table 1 also

Table 2 *Metabolism of MTT or XTT*

Cell line	Cell density[a]	MTT/XTT absorbance[b] at the following metabolism time[c]					
		MTT, 4 h	XTT				
			4 h	8 h	24 h	48 h	96 h
A549	10	0.15	0.02	0.02	0.07	0.22	0.35
	20	0.28	0.03	0.08	0.18	0.40	0.52
	39	0.55	0.07	0.07	0.32	0.59	0.65
	78	0.95	0.14	0.14	0.44	0.85	0.97
	156	1.39	0.15	0.12	0.53	0.82	1.20
	312	1.61	0.20	0.14	0.55	0.85	1.21
	625	1.68	0.16	0.14	0.54	0.83	1.46
	1,250	1.62	0.17	0.18	0.52	0.82	1.43
	2,500	1.71	0.16	0.19	0.50	0.80	1.92
	5,000	1.62	0.18	0.27	0.52	0.82	1.85
	10,000	1.75	0.18	0.32	0.54	0.86	1.97
H322	10	0.00	0.00	0.00	0.02	0.01	0.03
	20	0.00	0.01	0.00	0.02	0.02	0.04
	39	0.00	0.01	0.01	0.02	0.04	0.06
	78	0.01	0.02	0.02	0.05	0.09	0.13
	156	0.02	0.03	0.04	0.11	0.18	0.30
	312	0.04	0.06	0.09	0.21	0.35	0.45
	625	0.25	0.09	0.15	0.34	0.41	0.63
	1,250	0.48	0.15	0.22	0.48	0.53	0.74
	2,500	0.65	0.17	0.27	0.55	0.63	0.89
	5,000	0.78	0.17	0.26	0.52	0.68	0.93
	10,000	0.86	0.15	0.23	0.48	0.65	0.91

[a] The inoculation cell density: cells inoculated/well.
[b] Data represent average absorbances minus background from triplicate wells. Culture duration was for 7 days.
[c] Plates were incubated at 37°C in 5% CO_2 for the indicated time.

illustrates the marked enhancement of the metabolic reduction of XTT in the presence of the electron-coupling agent, phenazine methosulfate (1). The addition of 0.01 or 0.025 mM PMS (0.15 or 0.38 μg/well) resulted in a marked increase in measured absorbance, and absorbance measurements generally were equal to or greater than those obtained for MTT. One complication of the addition of PMS was an increase of background absorbance (no cells in the well) with increasing concentrations of added PMS (Table 1). With the conventional MTA the liquid medium is aspirated from the assay well prior to solubilization of the formazan product. This step results in lower background absorbance with MTT in comparison to XTT, since with the soluble XTT derivative, the aspiration step is deleted. Background absorbances at PMS concentrations equal or less than 0.38 μg/well are nevertheless acceptable, since control absorbances are at least 3.5-fold greater than background at the optimal PMS concentration.

On occasion, we have observed crystal formation in microculture wells containing PMS and XTT, sometimes resulting in diminished absorbance measurements for some cell lines. The presence of such crystals in a given microplate renders the data difficult to interpret and compromises the reproducibility and validity of an experiment. In addition to high-pressure liquid chromatography and mass spectroscopy analysis of crystals, we initiated studies to examine the potential role of several experimental variables in crystal formation: pH; temperature; cell density; and incubation time. Presently we conclude that PMS is necessary for crystal formation, and that an alkaline pH (which can occur if plates are removed from their CO_2 environment for too long a time) exacerbates the problem. We do not yet have an experimental solution to this occasional interference by crystal formation; thus, until this problem is resolved, careful microscopic examination of individual microculture wells is necessary to ensure the absence of crystal formation in a given experiment. All data presented in this paper result from experiments in which crystal formation was not observed. In addition, we are examining the utility of other electron-coupling agents as a substitute for PMS in an XTT-MTA. In initial experiments, one such agent, menadione (1), has proved promising, resulting in both a manageable background and large enough absorbance values for several cell lines tested (Table 3). After careful microscopic observation, we have yet to observe crystal formation in experiments utilizing XTT in combination with menadione; however, since our total experience with menadione is thus far more limited than with PMS, we cannot yet conclude that crystal formation is totally eliminated with this alternative electron-coupling reagent.

To evaluate the relationship between measured absorbance and viable cell number at the time of tetrazolium addition, cells were plated, allowed to attach for 1 h, and incubated with MTT or XTT plus PMS for 4 h (Fig. 2). At optimal PMS plus XTT conditions (as with MTT), absorbances peak and plateau at different inoculation densities depending on the cell line being studied. From such data (Fig. 2) a range of cell densities which give rise to a detectable and relatively linear range of absorbance values can be determined for each cell line at a given assay duration. An extensive discussion of the effects of inoculation density and culture duration is given in Ref. 8.

XTT-metabolism data for the murine leukemia cell line, P388, are also given in Table 1. The conventional MTA requires the use of a centrifugation step prior to medium aspiration for P388 and other suspension cell lines. Use of the XTT reagent eliminates the need for centrifugation of suspension cell lines. XTT has proved useful for other suspension cultures, including human leukemia cell lines (data not shown).

The absorbance values obtained with two human cell lines as a function of PMS concentration are given in Fig. 3. From data such as these, we have determined that all of the cell lines examined thus far yield adequately quantifiable absorbance measurements when incubated with XTT plus 0.01 to 0.025 mM PMS. Some of the cell lines which metabolized MTT less efficiently also required a larger PMS concentration to yield adequate absorbance values. The addition of PMS had little qualitative or quantitative effect on the MTT response of the cell lines tested (Fig. 3).

Spectral Characteristics of XTT Tetrazolium/Formazan. Spectral analysis of the MTT and XTT/formazan products derived from A549 cells in culture is shown in Fig. 4. Although the absorbance spectrum of XTT is rather different than for MTT, the addition of PMS resulted in little qualitative difference in either spectrum. The absorbance maxima for cellular-generated, DMSO-solubilized MTT-formazan and aqueous-soluble XTT/formazan are 560 and 475 nm, respectively. XTT/formazan can be easily discriminated from the background or from the unreacted XTT tetrazolium reagent.

Application of XTT to Drug Sensitivity Assays. To determine the suitability of the XTT assay for large-scale drug screening, we utilized XTT microculture methodology to generate drug sensitivity profiles for some representative standard compounds and experimental agents. Figs. 5 and 6 show drug profiles generated for Adriamycin-treated H324 cells using both the MTT and XTT reagents. The profiles were very similar for both reagents supplemented with 0.005 to 0.01 mM PMS. Lower PMS concentrations resulted in very low XTT absorbance measurements precluding accurate drug treatment analysis. The drug profiles using MTT were virtually identical for all PMS concentrations studied. Treatment of human tumor cell lines with several other experimental compounds resulted in comparable IC_{50} values (drug concentrations resulting in a 50% inhibition of growth) for either the MTT or XTT methodology (Table 4). Analysis of the data in Table 4 using the Spearman

Table 3 *Comparison of menadione and phenazine methosulfate in the XTT-MTA*

Cell line	MTT/XTT absorbance at the following PMS/MEN concentration[b]						
	MTT, 0	XTT 0.01 mM PMS	XTT 0.025 mM PMS	XTT 0.01 mM MEN	XTT 0.05 mM MEN	XTT 0.10 mM MEN	XTT 0.20 mM MEN
A549	1.947	1.433	2.110	1.065	1.107	3.057	2.973
LOX	1.026	0.403	1.514	1.059	0.789	0.740	1.573
H322	0.535	0.409	0.802	0.651	0.937	1.151	1.312
H460	2.124	1.721	2.427	0.987	1.241	2.763	3.210
MCF-7	1.443	0.553	0.737	0.459	1.434	1.299	1.551
HT-29	1.464	1.084	1.411	0.300	0.953	1.519	2.553
H23	1.042	0.423	1.241	0.217	0.472	1.121	1.324
Background (n = 7)	0.022 ± 0.006[c]	0.210 ± 0.010	0.191 ± 0.011	0.187 ± 0.014	0.120 ± 0.044	0.126 ± 0.038	0.109 ± 0.021

[a] Data represent average absorbance minus background from triplicate wells. Cells were inoculated at 1250 cells/well, culture duration was 7 days, and MTT or XTT metabolism was for 4 h at 37°C.
[b] PMS concentration: 0.01 mM = 0.15 μg/well; 0.025 mM = 0.38 μg/well. MEN concentration: 0.01 mM = 0.086 μg/well; 0.05 mM = 0.43 μg/well; 0.10 mM = 0.86 μg/well; 0.20 mM = 1.72 μg/well.
[c] Mean ± SD of 21 well background measurements.

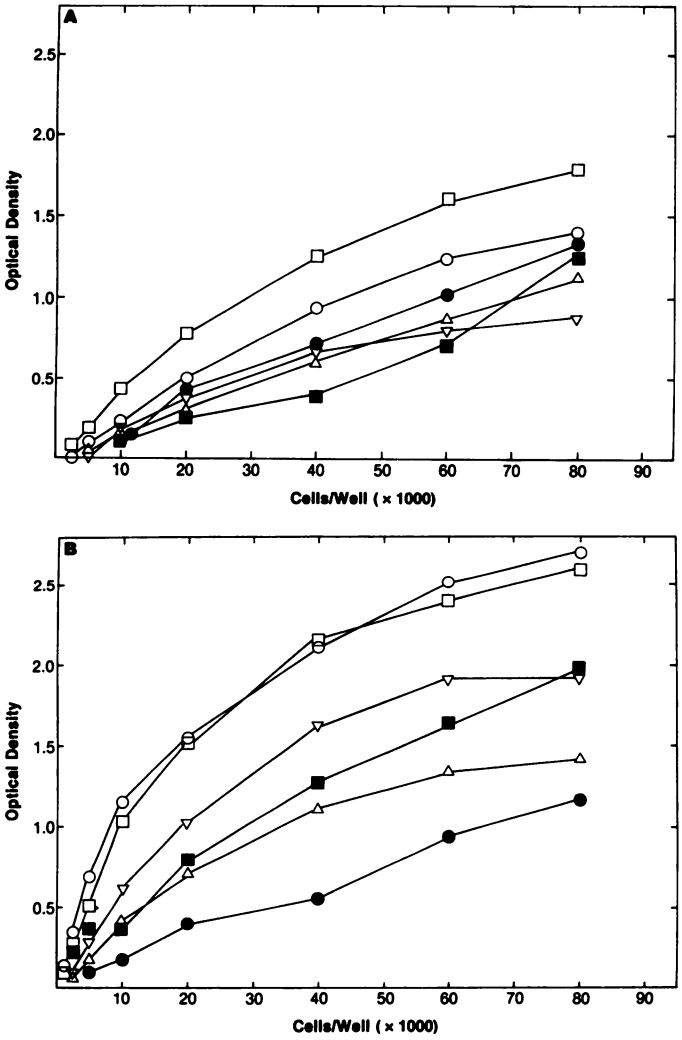

Fig. 2. Absorbance measurement as a function of viable cell density: MTT (*A*) and XTT (*B*). Viable cells were plated at the indicated cell density, allowed to attach for 1 h, and incubated with MTT or XTT plus 0.025 mM PMS (0.38 μg/well) for 4 h. A549 (○), H460 (□), MCF-7 (△), H322 (▽), LOX (●), HT-29 (■).

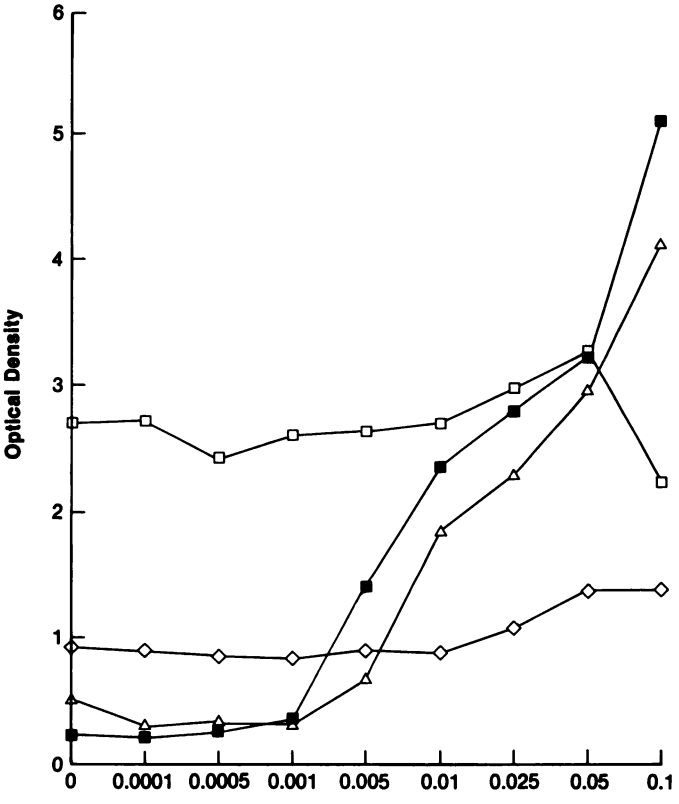

Fig. 3. Absorbance measurement as a function of PMS concentration. The PMS concentration indicated on the *abscissa* is the concentration of PMS in the XTT-PMS solution added to microculture wells. Culture duration, 7 days. A549 MTT (□), A549 XTT (■), LOX MTT (◇), LOX XTT (△).

rank-order correlation method revealed a highly significant association between IC_{50}'s derived from MTT and XTT assays ($r = 0.76$, $P = 0.0001$).

DISCUSSION

In exploring the suitability of a new microculture methodology for large-scale drug screening in the NCI/DCT drug-screening program, we initially developed a useful assay based on the reduction of MTT tetrazolium salt to a formazan product which could be easily and quickly measured in a multiwell scanning spectrophotometer system (7, 8). In this paper, we describe the evaluation of a different tetrazolium reagent which is metabolically reduced by human cells to an aqueous-soluble formazan product. Several design criteria for the new tetrazolium reagent were considered: bioreducibility of the tetrazolium; usable spectrum of the formazan product; low cellular toxicity of both the tetrazolium and formazan; and the aqueous solubility of the tetrazolium and formazan. Certain critical assay modifications were required before the new XTT reagent would approach these requirements. The XTT reagent alone proved unsuitable

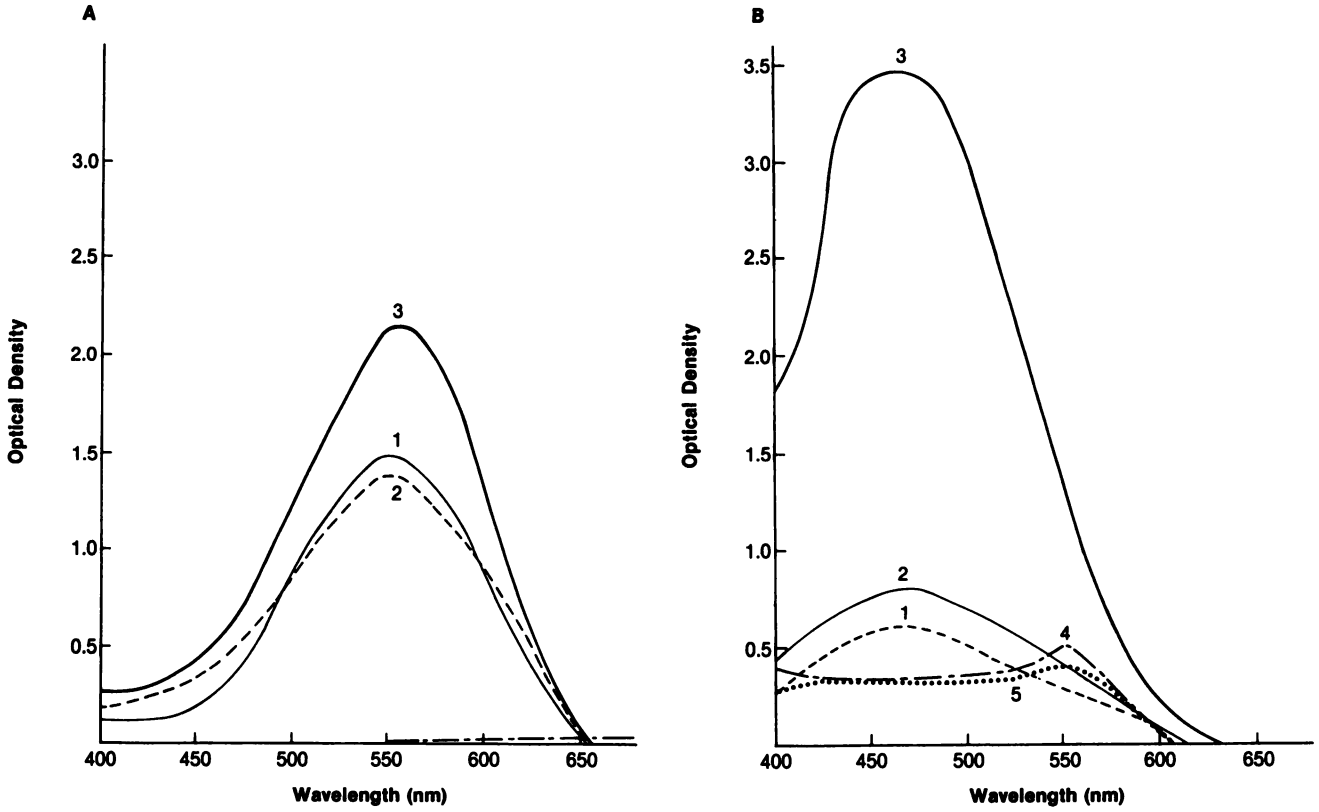

Fig. 4. Absorption spectra of MTT (*A*) and XTT (*B*) formazan products derived from cultured A549 cells (1000-cells/well inoculation, 7-day culture duration, 4-h incubation). Formazan products after incubation with the tetrazolium reagents plus: 0 mM (*1*) 0.001 mM (0.5 μg/well) (*2*), and 0.025 mM (0.38 μg/well) PMS (*3*). Nonreduced XTT tetrazolium (*4*). Background (absence of cells), tetrazolium plus 0.025 mM (0.38 μg/well) PMS (*5*).

for direct incorporation into the MTA; during a 4-h incubation, none of the human cell lines tested was able to sufficiently metabolize XTT to yield a formazan absorbance significantly greater than background. However, supplementation of the XTT incubation mixtures with the electron-coupling agent PMS resulted in adequate absorbance levels. Depending upon the metabolic capacity of a given cell line, the optimal conditions for a 4-h XTT incubation assay are 50 μg of XTT and 0.15 to 0.4 μg of PMS per well.

Fig. 2 illustrates that cellular reduction of XTT resulted in a formazan product which was not itself toxic to the cells under the assay conditions used. Cells retained the capacity to metabolize XTT for at least 96 h without evidence of toxicity. However, since metabolism times of 6 hr or less are required for our high-flux assay applications, and since the addition of XTT terminates the assay, the viability of cells after 24 h or more XTT metabolism times is not immediately relevant to the present usage. The longer incubation times resulted in both elevated absorbance measurements and increased background absorbances. XTT metabolism times of 2 to 6 h proved a suitable compromise between background and cell-generated absorbance.

The new XTT reagent used with PMS allowed application of the MTA to additional cell lines with various growth characteristics previously difficult to accommodate with the MTT-based MTA. For example, the XTT reagent greatly enhanced the usefulness of the MTA for the evaluation of cell growth and inhibition of human fibroblast cell lines (Table 2). Fibroblast cell lines are generally inefficient at metabolism of the MTT tetrazolium reagent; however, usable absorbances were obtained with XTT plus PMS. Also, the use of XTT eliminates a centrifugation step from the MTA methodology for nonadherent cell cultures. The XTT-MTA has proved usable for several suspension cell lines, including P388 and human leukemia cell lines. The XTT reagent may have an advantage for other applications of the MTA for cell growth measurements (*e.g.*, for potential antiviral compounds) where the aspiration step required by the MTT-MTA would be undesirable for either technical or safety reasons. It is beyond the scope of this paper to consider other potential uses of the XTT methodology; *e.g.*, to multicell aggregates and spheroids, however, these potential applications would appear both feasible and straightforward.

The initial experiments designed to assess the utility of the XTT-MTA in drug sensitivity assays indicate that, with certain compromises, XTT can be substituted for MTT to give comparable sensitivity and accuracy. Drug profiles obtained utilizing XTT are similar to the MTT profiles for a variety of human tumor cell lines and several experimental compounds. More extensive XTT-MTA experiments utilizing a panel of 48 human tumor cell lines treated with a wide variety of experimental drugs are under way to further explore the applicability of the XTT reagent to large-scale drug screening.[4] Results of these experiments will be detailed separately.

Even though the XTT-MTA offers several advantages over other *in vitro* assay systems, several inherent shortcomings must be considered. XTT, along with other tetrazolium approaches, depends on cellular reductive capacity, including the activity of mitochondrial dehydrogenases. The assays depend on a correlation between tetrazolium enzymatic reduction (reflected by absorbance measurements) and some associated culture characteristic such as cell number (6, 8, 9) or cell protein (8, 12). This assumption requires that cellular reductive capacity is

[4] A. Monks *et al.*, unpublished results.

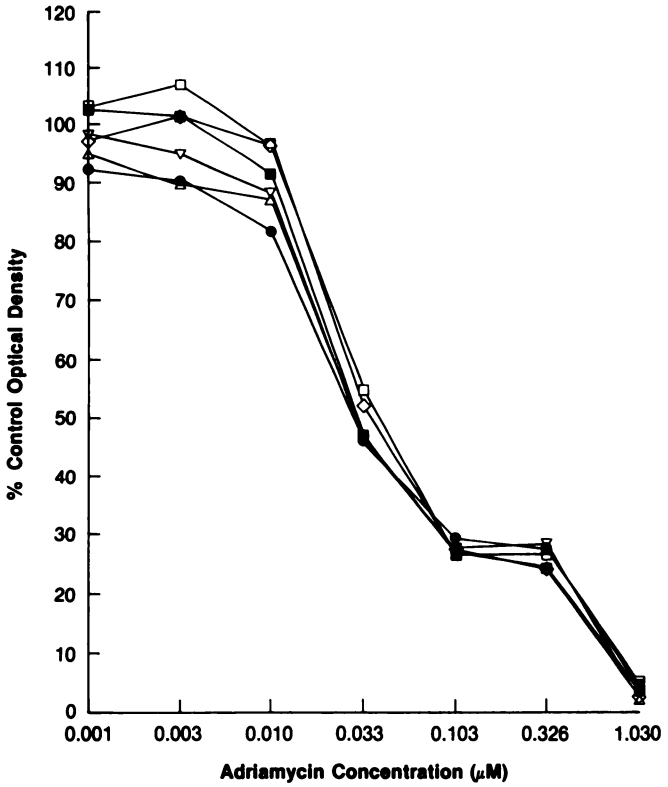

Fig. 5. Dose-response curves for Adriamycin-treated H324 cells (1000-cells/well inoculation, 7-day culture duration, 6-day Adriamycin treatment, 4-h incubation with MTT plus various PMS concentrations). *Points*, mean values from a single experiment calculated from triplicate wells subtracting background. Zero mM (□), 0.001 mM (■), 0.001 mM (◇), 0.01 mM (△), 0.025 mM (●), and 0.10 mM PMS (▽). The PMS concentrations are the concentrations of PMS in the XTT-PMS solution added to microculture wells.

Table 4 *Comparison of IC_{50}s for MTT and XTT*

Compound	Cell line	MTT	XTT
ADRI[b]	A549	1.92×10^{8}	2.28×10^{8}
	H125	2.94×10^{8}	6.39×10^{8}
	H322	5.10×10^{8}	4.84×10^{8}
	LOX	1.03×10^{8}	6.70×10^{9}
	HT-29	2.39×10^{8}	4.38×10^{8}
	MCF-7	1.97×10^{8}	1.77×10^{8}
$HgCl_2$	A549	2.15×10^{5}	2.16×10^{5}
	H125	1.35×10^{5}	1.93×10^{5}
	H322	1.54×10^{5}	1.52×10^{5}
	LOX	5.88×10^{7}	5.22×10^{7}
	HT-29	1.77×10^{5}	1.92×10^{5}
	MCF-7	1.81×10^{5}	1.94×10^{5}
BLEO	A549	5.98×10^{8}	8.74×10^{8}
	H125	2.61×10^{9}	1.30×10^{8}
	H322	1.01×10^{8}	1.80×10^{8}
	LOX	6.35×10^{9}	1.76×10^{9}
	HT-29	5.79×10^{8}	1.88×10^{7}
	MCF-7	5.56×10^{7}	9.31×10^{7}
MIT-C	A549	1.98×10^{8}	7.35×10^{8}
	H125	7.35×10^{8}	1.12×10^{7}
	H322	2.43×10^{8}	1.53×10^{8}
	LOX	3.75×10^{8}	1.53×10^{8}
	HT-29	4.17×10^{8}	8.19×10^{8}
	MCF-7	2.70×10^{8}	2.97×10^{8}
BCNU	A549	4.30×10^{5}	4.46×10^{5}
	H125	2.36×10^{5}	4.20×10^{5}
	H322	3.12×10^{5}	2.45×10^{5}
	LOX	1.30×10^{5}	4.76×10^{6}
	HT-29	4.27×10^{5}	7.94×10^{5}
	MCF-7	3.74×10^{5}	4.49×10^{5}
ACT-D	A549	2.73×10^{8}	2.78×10^{8}
	H125	2.63×10^{9}	3.03×10^{9}
	H322	2.42×10^{8}	1.60×10^{8}
	LOX	2.29×10^{8}	1.75×10^{9}
	HT-29	1.39×10^{8}	7.97×10^{9}
	MCF-7	1.43×10^{8}	1.36×10^{8}
5-FU	A549	1.45×10^{6}	2.02×10^{6}
	H125	5.36×10^{7}	2.73×10^{7}
	H322	6.24×10^{7}	1.67×10^{6}
	LOX	2.29×10^{6}	1.75×10^{6}
	HT-29	1.24×10^{6}	3.36×10^{7}
	MCF-7	7.69×10^{7}	7.15×10^{7}

[a] IC_{50} values calculated from seven concentration-dose responses. Cell inoculation densities: 1000 cells/well for all cell lines except H322 (2000 cells/well). MTA: 7-day culture duration, 6-day drug treatment, 4-h incubation with MTT or XTT plus 0.01 mM (0.15 μg/well) PMS.

[b] ADRI, Adriamycin; BLEO, bleomycin; MIT-C, mitomycin C; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ACT-D, actinomycin D; 5-FU, 5-fluorouracil.

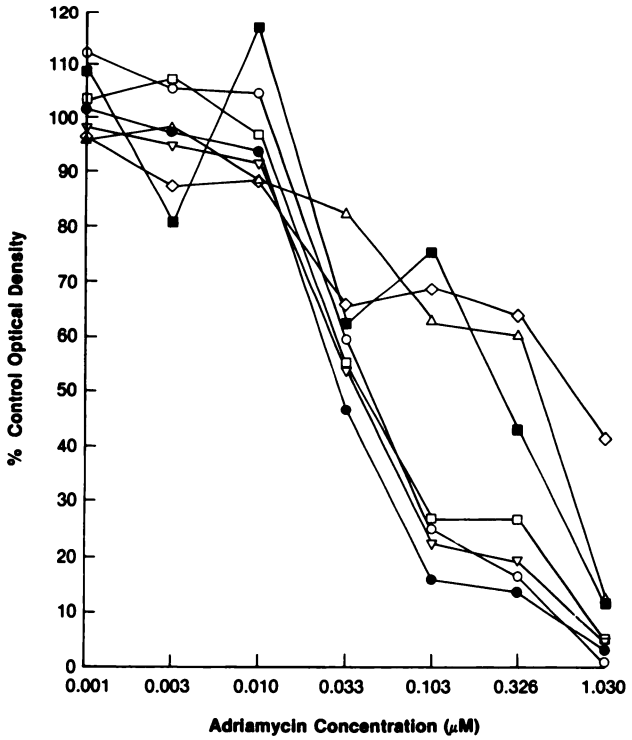

Fig. 6. Dose-response curves for Adriamycin-treated H324 cells (same experimental conditions as in Fig. 5). MTT, 0 mM PMS (□); XTT, 0 mM (■), 0.0001 mM (◇), 0.001 mM (△), 0.01 mM (●), 0.025 mM (▽), 0.10 mM PMS (○).

constitutive and remains relatively constant throughout the time duration of an experiment. However, any regulation of the cellular metabolic machinery resulting in different enzyme activity at any time will render this assumption invalid. Thus, changes of reductive capacity resulting from enzymatic regulation, pH, cellular ion concentration (*e.g.*, sodium, calcium, potassium), cell cycle variation, or other environmental factors may affect the final absorbance reading. For example, Mosman (6) has reported that mitogen-stimulated mouse spleen cells produce more MTT-formazan than do resting cells. Perturbations of these factors by experimental test compounds may further exacerbate this variability. In addition, the XTT-MTA has several unique shortcomings which must be considered before adaption of this assay for generalized drug testing. The present requirements for the addition of an electron-coupling agent increase the complexity of the cellular reduction environment (1, 13) potentially resulting in greater variability and a lack of reproducibility. PMS sometimes can cause nonspecific deposition of formazan (13), and Pearse (1) recommends that intermediate electron acceptors be avoided except to demonstrate activities which cannot otherwise be revealed. The occasional appearance of crystal formation, as yet not completely understood, is a potential interference requiring microscopic surveillance of each individual microwell. Whereas this problem may be less significant for other applications (*e.g.*, antiviral), it

may nevertheless preclude adaption of the XTT methodology to more complex drug-screening paradigms (*e.g.*, involving large cell line panels). Substitution of a different electron transport agent for PMS in the XTT-MTA may eliminate the crystal problem; however, additional work is required to better characterize the menadione-XTT system. In addition, the relatively elevated background levels characteristic of XTT plus PMS result in the inability to utilize this method for some cell lines that exhibit poor metabolic capacity and also decrease the reliability and reproducibility of drug sensitivity measurements at drug concentrations resulting in growth inhibition greater than 80% of control values. At these levels of growth inhibition, the relatively large background absorbances generated by XTT plus PMS (or manadione) in growth medium (Tables 1 and 3) can result in signal/noise ratios of less than one.

While safety and efficiency considerations argue for the possible advantage of the XTT over the MTT-based assay for applications to high-flux drug sensitivity screens, nevertheless, there remain serious problems with the XTT-based MTA, as well as tetrazolium assays in general. Such questions should continue to be of major concern and consideration for adaption of any particular assay protocol for general usage in anticancer, antiviral, or other drug-screening programs. However, the present investigation demonstrates the feasibility of a microculture methodology utilizing a water soluble tetrazolium/formazan reagent, suggesting the inherent advantages in the development of additional reagents which might not require the use of electron-coupling agents. In addition, our present cell line panels of human tumor cell lines (8) would provide a useful resource for studying the biological activity and suitability of such new materials.

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REFERENCES

1. Pearse, A. G. E. Principles of oxidoreductase histochemistry. *In:* Histochemistry, Theoretical and Applied, Ed. 3, Chap. 20. Edinburgh: Churchill Livingston, 1972.
2. Altman, F. P. Tetrazolium salts and formazans. Prog. Histochem. Cytochem., *9:* 1–56, 1977.
3. Black, M. M., and Speer, F. D. Effects of cancer chemotherapeutic agents on dehydrogenase activity of human cancer tissue *in vitro*. Am. J. Clin. Pathol., *23:* 218–227, 1953.
4. Schaeffer, W. I., and Friend, K. Efficient detection of soft-agar grown colonies using a tetrazolium salt. Cancer Lett., *1:* 275–279, 1976.
5. Alley, M. C., and Lieber, M. M. Improved optical detection of colony enlargement and drug cytotoxicity in primary soft agar cultures of human solid tumour cells. Br. J. Cancer, *49:* 225–233, 1984.
6. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, *65:* 55–63, 1983.
7. Alley, M. C., Scudiero, D. A., Monks, A., Czerwinski, M., Shoemaker, R. H., and Boyd, M. R. Validation of an automated microculture tetrazolium assay (MTA) to assess growth and drug sensitivity of human tumor cell lines. Proc. Am. Assoc. Cancer Res., *27:* 389, 1986.
8. Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., and Boyd, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res., *48:* 589–601, 1988.
9. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res., *47:* 936–942, 1987.
10. Cole, S. P. C. Rapid chemosensitivity testing of human lung tumor cell lines. Cancer Chemother. Pharmacol., *17:* 259–263, 1986.
11. Paull, K. D., Shoemaker, R. H., Boyd, M. R., Parsons, J. L., Risbood, P. A., Barbera, W. A., Sharma, M. N., Baker, D., Hand, E., Scudiero, D. A., Monks, A., and Grote, M. The synthesis of XTT: a new tetrazolium reagent bioreducible to a water-soluble formazan. J. Heterocyclic Chem., in press, 1988.
12. Finlay, G. J., Wilson, W. R., and Baguley, B. C. Comparison of *in vitro* activity of cytotoxic drugs towards human carcinoma and leukemia cell lines. Eur. J. Cancer Clin. Oncol., *22:* 655–662, 1986.
13. Kiernan, J. A. Oxidoreductases. *In:* Histological and Histochemical Methods, Theory, and Practice, Chap. 16. New York: Pergamon Press, 1981.

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