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Metabolic stability of experimental chemotherapeutic agents in hepatocyte: tumor cell co-cultures

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Summary. A U. S. National Cancer Institute screening program for new anticancer drugs, based on the growth of primary human tumor cells in an in vitro soft agar colony formation assay, has resulted in the identification of a number of compounds that have cytotoxic activity against primary human tumor cells in vitro but are inactive in the conventional in vivo murine P388 leukemia animal model pre-screen. To investigate whether metabolic inactivation ov the compounds might be a factor in the lack of in vivo cytotoxicity we have co-cultured rat hepatocytes with A204 rhabdomyosarcoma and murine P388 leukemia cell lines in the soft agarose colony formation assay for 24 h during exposure to the compounds. Twenty compounds with a range of in vitro activities were studied. Thirteen compounds exhibited cytotoxicity against A204 cells in culture; nine of them were less active when co-cultured with hepatocytes, two were activated by hepatocyte co-culture, and two showed no effect of hepatocyte co-culture. P388 cells were more sensitive to the antiproliferative effects of the compounds than A204 cells. Two compounds that were not active against A204 cells exhibited cytotoxicity against P388 cells. One compound was inactivated by hepatocyte co-culture and one showed no effect. Five compounds showed no cytotoxicity toward either A204 cells or P388 cells. Two of the compounds showing hepatocyte inactivation in vitro possess activity in one or more in vivo tumor models. Thus, evidence for metabolic inactivation in hepatocyte co-culture is not always an indication for lack of in vivo antitumor activity. Hepatocyte co-culture methodology provides a simple and objective means, amenable to large-scale screening, of distinguishing metabolic activation or inactivation of a given compound from other pharmacokinetic and pharmacodynamic factors with a minimum of material.

Introduction

The search for new anticancer drugs from among natural products and newly synthesized chemicals has relied primarily on screening in murine tumor systems in vivo, such as the present "pre-screen", the P388 transplantable leukemia, and the L1210 lymphocytic leukemia and B16 melanoma tumor models. In 1980 the U. S. National Cancer Institute (NCI) began study of a new in vitro screening system based on the soft agar colony formation assay popularized by Salmon et al. [7]. In this ongoing multi-institutional research program, freshly isolated primary human tumor cells of different histologic origins are cultured in the presence of new putative anticancer drugs; inhibition of in vitro tumor cell colony formation in soft agar cultures is being studied as a potentially useful screen to supplement the P388 in vivo animal model system [8, 9].

As previously reported [9], this new NCI in vitro screening program has already resulted in the identification of a number of compounds which show strong cytotoxic activity against freshly isolated human solid tumor cells in soft agar culture. In some instances, these compounds had shown no significant activity when tested previously in vivo against the P388 murine pre-screen. To investigate whether metabolic inactivation might be responsible for the lack of in vivo cytotoxicity of some of these compounds, we have examined a number of active compounds identified in the NCI human tumor colony forming assay in vitro screening program using a previously described hepatocyte co-culture methodology [2]. A total of 20 experimental compounds, which had exhibited a range of cytotoxic activity in the primary human tumor cell culture system, were assessed in hepatocyte:tumor cell co-cultures using a continuous human tumor cell line or murine P388 cells as targets.

Materials and methods

Human rhabdomyosarcoma (A204) and murine leukemia (P388) continuous cell lines were obtained from Frederick Cancer Research Facility (Frederick, Md). A204 cells were maintained as bulk cell culture monolayers in multiple 75-cm^2 flasks containing a standard growth medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units penicillin/ml, 100 μg streptomycin/ml, 2 m*M* *L*-glutamine and 25 m*M* HEPES (Gibco, Grand Island, NY). P388 cells were grown in suspension culture at low density in the same medium. Medium was changed three times per week for A204 cells and once a week for P388 cells. A204 cells in exponential growth were passaged each week for a maximum of 15 weeks using DMEM containing 0.25% trypsin and 0.01% EDTA. P388 cells were subcultured by dilution. One day before harvesting of the P388 cells fresh medium was

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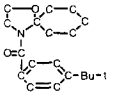
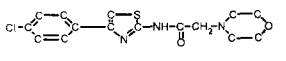
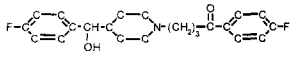
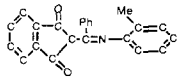
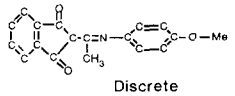
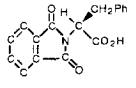
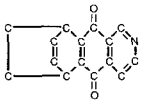
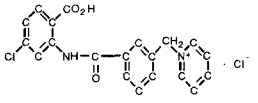
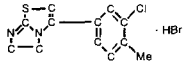
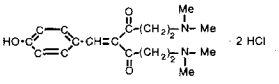
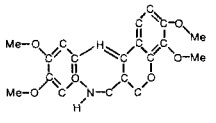
added to the suspension culture to stimulate cell growth. Subsequent bulk cultures were re-established from cell aliquots stored in liquid nitrogen. Cell lines were verified to be mycoplasma-free by culture (Virology Laboratory, Mayo Clinic) and by fluorescence microscopy [4].

Soft agarose cultures were performed in quadruplicate in 35-mm culture dishes containing a base layer consisting of 0.5 ml standard culture medium with 0.5% low melting point agarose (Seaplaque, FMC Corporation) for A204 cells and 0.6% agarose for P388 cells. On *day 0* A204 cells in bulk culture were dissociated with trypsin and EDTA, washed once in growth medium, and subcultured by layering 1×10^4 viable cells in 0.5 ml growth medium with 0.3% agarose over each base layer. P388 cells in bulk culture were layered in 0.5 ml growth medium with 0.4% agarose over a base layer which had been previously chilled. Culture dishes were then refrigerated for 15 min. Cultures were examined with the aid of an inverted microscope, and only cultures containing uniformly distributed single-cell suspensions (< ten 30-μm diameter cell clusters and no 60-μm clusters) were accepted for subsequent evaluation. Cultures were maintained in cell culture incubators at 37 °C, 5% CO_2, 95% air and 100% relative humidity. On *day 1* (24 h later) an upper layer of 1 ml growth medium containing 1×10^5 hepatocytes, drug and/or drug vehicle was applied to each culture. Cyclophosphamide was included as a positive control in all studies. On *day 2* (24 h later) hepatocytes were removed by aspiration, and agarose culture surfaces were washed twice with 0.5 ml prewarmed growth medium and then overlaid with 1 ml fresh growth medium. Colony formation was examined at daily intervals by conventional light microscopy. Cell lines formed sufficient numbers of detectable colonies (> 60-μm diameter) for analysis following 7–12 days' incubation. Viable colonies were stained using a metabolizable tetrazolium salt, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT, Aldrich Chemical Co., Milwaukee, Wis) and analyzed as previously described [2]. Colony formation was expressed relative to control cultures without drug or hepatocytes. Each study was repeated on at least two, but usually three separate occasions.

Rat hepatocytes were isolated by a modification of the method of Berry and Friend [3] developed by Stewart and Inaba [10]. The liver of an anesthetized male Sprague-Dawley rat weighing 150–200 g was flushed with Ca^{2+}-free oxygenated Krebs bicarbonate-buffered saline at 37 °C through a cannula in the portal vein. The liver was excised and placed in a perfusion apparatus and Ca^{2+}-free oxygenated bicarbonate-buffered saline containing 0.5% collagenase recirculated through the liver for 30 min. The liver capsule was opened and the hepatocytes liberated by gentle shaking at 37 °C in oxygenated bicarbonate-buffered saline containing 0.25% dialyzed bovine serum albumin. The cells were filtered through nylon mesh

Table 1. Compounds studied

Compound[a]	Structure	In Vitro Human Tumor Activity[b]	In Vivo P388 Activity[c]
A	Discrete	34/34 (100%)	-
B	Discrete	20/25 (80%)	-
C		21/46 (46%)	-
D	Discrete	22/64 (34%)	+[d]
E		2/34 (6%)	-
F	Discrete	4/44 (9%)	-
G	Discrete	2/32 (6%)	-
H		9/66 (14%)	-
I		31/44 (70%)	-
J		5/44 (11%)	-
K	Discrete	4/43 (9%)	-
L		5/31 (16%)	-
M		34/35 (97%)	-
N	Discrete	14/25 (56%)	+
O		3/36 (8%)	-
P		6/33 (18%)	-
Q		12/31 (39%)	-
R	Discrete	27/34 (79%)	+[d]
S		10/34 (29%)	-
T	Discrete	10/32 (31%)	-

[a] NCI accession numbers for compounds not obtained under commercial discrete agreements are: C, NSC 343549; e, NSC 339675; H, NSC 343513; I, NSC 343378; J, NSC 343385; L, NSC 338600; M, NSC 338698; O, NSC 341964; P, NSC 343529; Q, NSC 340307 and S, NSC 353646. Structures and accession numbers are not available for discrete commercial compounds

[b] Response rate in a variety of human primary tumors at 10 μg/ml continuous exposure (NCI screening data)

[c] Activity against murine leukemia P388 in vivo (NCI screening data)

[d] Active in dimethylsulfoxide, inactive in water

(150-μm pore size) and centrifuged at 800 rpm for 10 min. Viability and density of isolated hepatocytes were determined by trypan blue exclusion within 2 min of combining 0.9 ml sterile 0.08% trypan blue (Gibco, Grand Island, NY) with 0.1 ml hepatocyte suspension containing approximately 10^6 cells/ml at room temperature. Hepatocyte cultures densities refer to total hepatocyte number and not to viable cells (generally more than 90%). Hepatocyte functionality was verified in terms of the *O*-dealkylation rate as reported by Fry [5].

Cyclophosphamide was purchased from Sigma Chemical Co., St. Louis, Mo. All other drugs (listed in Table 1) were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Drug solutions were freshly prepared within 30 min of culture application. In the initial phase of evaluation, agents were tested at four concentrations (0.1, 1, 10, and 100 μg/ml) to determine the effective concentration range of each agent for a given tumor cell line in the presence and absence of hepatocytes. In subsequent phases of evaluation each agent was evaluated at four concentrations differing by a serial dilution factor of 0.7, 2, or 4, so as to cover as much of the effective concentration range as possible (i.e., 10%–90% inhibition of colony formation). All agents and concentrations (assigned letters and numbers, respectively) were tested in single blind fashion.

Colony formation data were fitted to a monoexponential survival curve using the NONLIN nonlinear least-squares regression analysis program [6]. Variance of the drug or prodrug concentration required to produce 50% inhibition of colony formation (IC_{50}) was obtained from the variance of the intercept and slope using a Taylor series expansion [2]. Groups of data with and without hepatocytes were compared using Student's *t*-test.

Results

A204 Cell co-culture

Preliminary experiments were conducted to determine for each drug the range of concentrations with antiproliferative activity against A204 human rhabodomyosarcoma continuous tumor cells in the soft agarose colony formation assay. Not all drugs exhibited activity against A204 at the highest concentration tested (100 μg/ml). Some typical responses are shown in Fig. 1. Compound D is an agent whose activity was not affected by hepatocyte co-culture. Compound R is an agent whose activity was significantly decreased by hepatocyte co-culture. Compound H is another agent whose activity was decreased by hepatocyte co-culture and where a shoulder occurred in the dose-response curve. This type of response curve indicates limitations in obtaining an estimate of the IC_{50} by monoexponential regression analysis of all the data points. In cases where a shoulder was observed, data points on the shoulder were omitted from the linear regression analysis of IC_{50}. Finally, for a few agents, such as compound I (Fig. 1), a significant increase in antiproliferative activity was obtained with hepatocyte co-culture. A summary of the effect of hepatocyte co-culture on drug activity for typical responses is shown in Table 2. All response curves were repeated twice, and usually three times, to confirm the results. For some active agents hepatocyte co-culture had no significant effect upon antiproliferative activity,

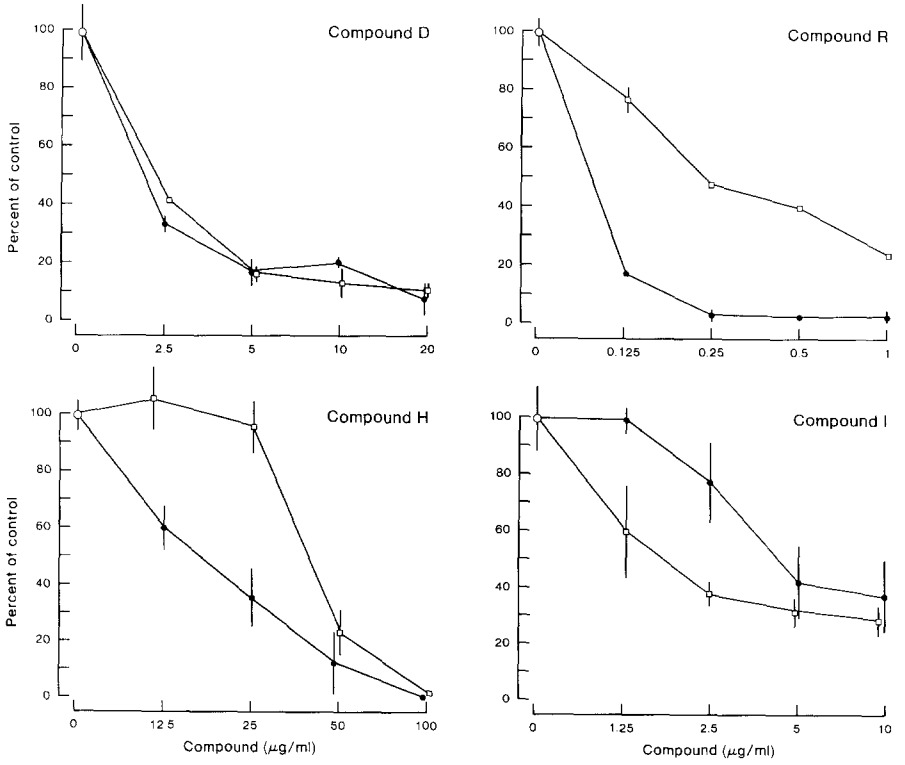


Fig. 1. Typical dose-response curves for four selected agents on hepatocyte: A204 cell co-culture; (●) absence of hepatocytes, and (□) presence of hepatocytes. Each *point* is the mean of quadruplicate determinations; *bars* are SE of mean

Table 2. Effect of hepatocytes on cytotoxicity against human A204 cells in the soft agarose colony formation assay

Compound	50% Growth inhibitory concentrations[a]		Effect
	Without hepatocytes (μg/ml)	With hepatocytes (μg/ml)	
D	1.71 ± 0.37	2.07 ± 0.24	No significant effect
M	2.06 ± 0.62	2.15 ± 0.43	
A	0.75 ± 0.14	1.82 ± 0.20[b]	Hepatocyte inactivation
B	1.05 ± 0.20	2.47 ± 0.25[b]	
E	6.19 ± 0.97	10.8 ± 2.2[c]	
H	16.9 ± 2.1	37.3 ± 3.0[a,b]	
N	7.07 ± 0.65	13.6 ± 0.5[b]	
P	8.45 ± 0.57	15.8 ± 2.2[b]	
Q	9.12 ± 1.22	23.4 ± 2.7[a,b]	
R	0.05 ± 0.01	0.62 ± 0.15[b]	
S	30.1 ± 5.1	76.5 ± 1.2[a,b]	
I	4.60 ± 1.34	1.95 ± 0.42[c]	Hepatocyte activation
T	18.2 ± 6.2	8.9 ± 3.08[c]	
CTX	144.0 ± 4.0	15.8 ± 1.1[b]	
C	> 100	> 100	Inactive
F	> 100	> 100	
G	> 100	> 100	
J	> 100	> 100	
K	> 100	> 100	
L	> 100	> 100	
O	> 100	> 100	

A204 cells (10^4) were grown in soft agarose for 24 h prior to application of medium containing 10^5 rat hepatocytes with and without drug. Hepatocytes and drug were removed 24 h later. Colony formations was measured following 10 days of incubation. Quadruplicate determinations of at least four drug concentrations were used. Values are mean ± SD. Table entries represent concentrations of drug resulting in a 50% decrease in colony count (≥ 60 μm diameter)

CTX, cyclophosphamide

[a] A dose response curve with a shoulder fitted to the log-linear portion of the curve as described in the text

[b] $p < 0.01$ compared with the appropriate control without hepatocytes

[c] $p < 0.05$

namely compounds M and D. Other agents showed a marked decrease in antiproliferative activity when co-cultured with hepatocytes. Compounds B, Q, H, and S showed more than a 100% increase in the IC_{50} value, while compounds A, E, R, N, and P showed smaller but significant decreases in activity upon co-culture with hepatocytes.

P388 Cell culture

Hepatocytes were co-cultured with murine P388 leukemia cells in soft agarose culture to study a more limited number of drugs, including drugs with little or no activity against A204 cells. P388 cells proved difficult to grow under standard conditions employed for human tumor cells in soft agar culture. Instead of forming discrete colonies the P388 cells appeared to form sheets of cells, which could not be evaluated. These problems were overcome by (a) growing the P388 cells in medium containing 0.4% agarose over a base layer containing 0.6% agarose; (b) chilling the base layer prior to application of the layer containing the P388 cells; and then (c) refrigerating the dishes for 15 min to ensure uniform gelling and subsequent growth of discrete colonies.

P388 cells were in general more sensitive than A204 cells to the antiproliferative effects of the drugs tested (Table 3). The IC_{50} for compounds H and R were about an order of magnitude lower for P388 than for A204 cells, while compounds J and C, which were inactive against A204 cells, were active against P388 cells. Some compounds, G, L, K, F, and O, were inactive against both A204 cells and P388 cells. Of the two agents inactive against A204 cells but active against P388 cells, compound J showed no significant effect of co-culture with hepatocytes, while compound C was inactivated by hepatocyte co-culture.

Discussion

Eight of fifteen compounds with activity in the in vitro human tumor colony formation assay (that is, with activity against about 10% or more of tumors tested) but inactive in the in vivo P388 pre-screen model underwent significant inactivation in the in vitro hepatocyte:tumor co-culture system. Two compounds exhibited significant in vitro hepatocyte activation although they had no in vivo activity. Five compounds were inactive against both A204 human tumor cells and P388 cells in vitro. Two compounds exhibiting significant in vitro hepatocyte inactivation did have activity in the P388 in vivo model. Indeed, even for the most significantly hepatocyte-inactivated agent in this series, compound R, it was possible to elicit in vivo activity.

Table 3. Effect of hepatocytes on cytotoxicity against murine P388 cells in the soft agarose colony formation assay

Compound	50% Growth inhibitory concentrations		Effect
	Without hepatocytes (μg/ml)	With hepatocytes (μg/ml)	
H	2.97 ± 0.71	4.00 ± 0.82	No significant effect
I	2.13 ± 0.32	1.92 ± 0.82	
J	15.0 ± 4.0	30.0 ± 14	
C	1.69 ± 0.46	7.14 ± 1.80[a]	Hepatocyte inactivation
R	0.007 ± 0.002	0.05 ± 0.21[a]	
CTX	> 100	5.8 ± 0.9[a]	Hepatocyte activation
F	> 100	> 100	Inactive
G	> 100	> 100	
K	> 100	> 100	
L	> 100	> 100	
O	> 100	> 100	

P388 cells (10^4) were grown in soft agarose for 24 h prior to application of medium containing 10^5 rat hepatocytes with and without drug. Hepatocytes and drug were removed 24 h later. Colony formations was measured following 10 days of incubation. Quadruplicate determinations of at least four drug concentrations were used. Values are mean ± SD. Table entries represent concentrations of drug resulting in a 50% decrease in colony count (≥ 60 μm diameter)

[a] $p < 0.05$ compared with appropriate control without hepatocytes

Thus, evidence for metabolic inactivation in hepatocyte co-culture is not necessarily an indication for lack of in vivo antitumor activity. No doubt, other pharmacokinetic and pharmacodynamic factors (e.g., tissue distribution, excretion, protein binding, and metabolism by nonhepatic tissue) contribute to the limited in vivo activity of these compounds.

Results from these assays coupled with other pharmacologic information can provide important indications regarding optimum therapeutic employment of new agents. For example, compound R, which shows a 12.4-fold decrease in activity in the presence of hepatocytes, is under consideration for development as a parenteral agent in the treatment of malignant ascites. Pharmacokinetic studies with this compound indicate a plasma half-life of 5–10 min following IP administration (Dr. Robert Shoemaker, unpublished data). Given that the active form of this compound is highly cytotoxic to a broad range of human tumor types in the human tumor colony formation assay, localized infusion of the compound coupled with rapid hepatic metabolism may serve to improve efficacy and to limit systemic toxicity in patients.

This study utilizing a hepatocyte:tumor cell co-culture system has provided useful information concerning in vivo and in vitro activity for a limited number of experimental compounds with activity in the human tumor colony formation assay. The hepatocyte:tumor cell-co-culture methodology provides a simple and objective means of distinguishing metabolic activation/inactivation of a given compound from other pharmacokinetic and pharmacodynamic factors with a minimum amount of material. As such, the methodology may be particularly useful in the identification of compounds which (a) require metabolic activation or (b) are highly cytotoxic to tumor cells yet lacking in systemic toxicity due to metabolic inactivation. In addition, the co-culture methodology, which is amenable to large-scale screening, may provide a basis for resolution of descrepancies between in vivo and in vitro data and may provide additional insight concerning development of new agents.

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