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A RAPID *IN VITRO* METHOD FOR THE EVALUATION OF POTENTIAL ANTITUMOR DRUGS REQUIRING METABOLIC ACTIVATION BY HEPATIC S9 ENZYMES

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Abstract—Metabolic activation is a prerequisite for the antitumor activity of certain drugs such as cyclophosphamide. *In vitro* assays require systems for metabolic activation to reveal the toxicity of such compounds for tumor cells. Although a number of methods utilizing systems for the *in vitro* metabolic activation of drugs have been published, practical assays applicable to large scale screening for such agents have been lacking. We, therefore, now report that incorporation of a liver subcellular fraction (S9) into a recently established cell growth inhibition assay (microculture tetrazolium assay) significantly increased the cytotoxicity of cyclophosphamide. Under optimal conditions, the 50% growth inhibitory concentration was decreased in the presence of S9 from more than 600 μg/ml to less than 4 μg/ml, depending upon the cell line. The method also proved suitable for studies investigating metabolic detoxification (enzymatically or non-enzymatically) by conjugation reactions. For example, glutathione (5 mM) markedly reduced the cytotoxicity of activated cyclophosphamide. In contrast, the addition of UDP glucuronate (10 mM) in the presence of the UDP-glucuronosyltransferase activator UDP-*N*-acetylglucosamine (10 mM) had little effect on cyclophosphamide toxicity.

The development of new models and strategies for the discovery of anticancer drugs plays a central role in the "disease-oriented" drug-screening program currently being established by the National Cancer Institute (NCI). In this project an *in vitro* assay, termed microculture tetrazolium assay (MTA§), has been used to screen the toxicity of a test compound against a number of human tumor cell lines [1–3]. Differential cytotoxicity or selective toxicity toward cell lines derived from particular tumor types represents the criterion for the initial identification of compounds to be selected for further studies. The test procedure comprises the cultivation of cells in microculture wells in the presence of appropriate concentrations of test compounds. Following drug exposure, the number of viable cells is estimated by exploiting their ability to reduce a tetrazolium salt (MTT) to a colored formazan [1, 4]. However, certain drugs such as cyclophosphamide (CPA), which require metabolism by hepatic cytochrome P-450 dependent monooxygenases [5–7], not surprisingly lack cytotoxic activity in this assay system. To provide the necessary *in vitro* conditions to study such drugs, preincubation or co-cultivation of the agents with liver cellular [8] or subcellular fractions [9–12] as means for the *in vitro* activation has been described by several authors. Most of these studies have been carried out with liver S9, a sub-cellular fraction that is also used in mutagenicity assays. In addition to a variety of monooxygenase activities, S9 also contains numerous enzymes for conjugation reactions, including glutathione-*S*-transferases, and UDP-glucuronosyl-transferases and -sulfotransferases. The activities of both the conjugating enzymes and the monooxygenases are dependent upon the presence of the corresponding cofactors. By the selective addition of various cofactors to the S9 fraction, particular conjugation reactions can be differentially activated. Such a system can then be used to investigate whether a particular conjugation reaction participates in the inactivation of a potential antitumor agent.

In the present study we investigated the feasibility of whether a system for metabolic activation can be integrated into the MTA. Of specific importance was the desirability for simplicity, enabling the ready use of the modification for special applications to complement a large scale drug-screening program. Furthermore, the effect of cofactors for conjugating enzymes on the cytotoxicity of activated cyclophosphamide was examined.

METHODS

Animals. Liver subcellular fractions were prepared from male Sprague–Dawley rats (180–200 g; Animal Production Area, NCI-Frederick Cancer Research Facility). The animals were given Purina Laboratory Chow and water *ad lib.* until 16 hr before sacrifice when the food was removed. Liver enzymes were

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§ Abbreviations: CPA, cyclophosphamide; GSH, glutathione; IC_{50}, 50% growth inhibitory concentration; MTA, microculture tetrazolium assay; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

induced by a single i.p. injection of Aroclor 1254 (500 mg/kg) diluted in sesame oil (200 mg/ml) 5 days prior to killing the rats.

Chemicals. All drugs were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Aroclor 1254, was obtained from Analabs Inc.; glucose-6-phosphate dehydrogenase (type IX), NADP, glucose-6-phosphate, glutathione, uridine-5′-diphosphoglucuronic acid, and uridine-5′-diphospho-*N*-acetylglucosamine were purchased from the Sigma Chemical Co.

Cell lines. Samples of lung adenocarcinoma (A549) [13], lung large cell carcinoma (A427) [13] and bronchiolo-alveolar carcinoma (NCI-H322) [14], continuous cell lines obtained from Dr Isaiah Fidler, M.D. Anderson Hospital, Houston, TX; Dr Dominic Scudiero, NCI-Frederick Cancer Research Facility, Frederick, MD; and Dr Adi Gazdar, NCI-Navy Medical Oncology Branch, Bethesda, MD, respectively, were grown as bulk cell culture monolayers in growth medium (RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine) in the absence of antibiotics. Each cell line was verified to be mycoplasma free and to be of human origin by chromosomal and isoenzyme analysis.

S9 Mix. The 9000 *g* supernatant fraction was prepared by centrifugation of rat liver homogenate (S9) according to Maron and Ames [15]. Portions (1 ml) of S9, containing about 40 mg/ml protein, were stored frozen at −70°. Immediately before addition to the culture wells liver S9 was thawed, diluted with growth medium, and supplemented with NADP (4 mM) and glucose-6-phosphate (4 mM).

Microsome mix. Rat livers were homogenized in 3 vol. of cold Tris–KCl buffer, pH 7.3 (0.15 M KCl; 20 mM Tris). The homogenate was centrifuged at 9000 *g* for 20 min. Microsomes were prepared by centrifugation of the resulting supernatant fraction at 105,000 *g* for 1 hr. The microsomal pellet was washed once with Tris–KCl buffer, and the microsomes were resuspended in RPMI-1640 (protein concentration of approximately 10 mg/ml) filtered through a 0.45 μm Millipore filter and stored frozen in portions of 1 ml at − 70°. Before each experiment microsomal suspensions were diluted with growth medium and supplemented with NADP and glucose-6-phosphate as described above. In addition, glucose 6-phosphate dehydrogenase was added (2 mUnits/ml microsome mix).

Protein determination. Protein concentrations were determined using the BIO-RAD standard assay procedure.

Microculture tetrazolium assay (MTA). The sensitivity of cell lines toward the test compounds was determined using a modification [1] of the MTT assay [4]. Cells in exponential growth phase were harvested by trypsinization and replated in 96-well flat-bottom microculture plates at 1000 cells per well in 100 μl growth medium. Culture plates were maintained in an incubator at 37°, 5% CO_2 and 100% relative humidity. Twenty-four hours later 50 μl of growth medium containing various dilutions of test drug and 50 μl of S9 mix, microsome mix or growth medium were added to each well. Twenty-four hours later the drug/S9 mix containing medium was removed by aspiration and replaced with 200 μl growth medium. Following continuous cultivation for several days (3-7), 50 μl MTT tetrazolium salt solution (5 mg/ml PBS, 5-fold diluted with RPMI-1640 medium before use) was added to the wells and the cells were incubated for a further 4 hr. Culture medium was then aspirated from the wells and the intracellularly generated formazan was solubilized in 150 μl dimethyl sulfoxide per well. After shaking briefly, the optical density (O.D.) at 570 nm was measured on a Dynatech MR-600 plate reader. Formation of colored formazan by reduction of the tetrazolium salt is proportional to the number of viable cells [4]. Mean values and standard deviations of triplicate determinations were calculated. The standard deviations were generally less than 15%.

RESULTS

The cytotoxic effect of CPA on A549 cells in the presence of increasing concentrations of S9 protein was evaluated using an S9 preparation from Aroclor 1254 induced rats and is shown in Fig. 1. After replacing the drug/S9 containing medium with growth medium, the cultivation was continued for 5 days, resulting in a total culture duration of 7 days. The amount of S9 added to each well required for optimal cell kill was 16 μg protein. Lower S9 protein concentrations did not activate CPA efficiently, whereas higher protein concentrations caused a substantial growth inhibition. Interestingly, a decline in CPA-mediated cytotoxicity was observed when the S9 protein concentration exceeded 100 μg/well.

The effect of the culture duration post-treatment with CPA/S9 was investigated subsequently (Fig. 2). There were no significant differences in the 50% growth inhibitory CPA concentration (IC_{50}) regardless of whether the cultures were terminated after 4, 5, 6 or 7 days. Further studies showed that the continuous presence of S9/CPA until the end of the cultivation did not increase the sensitivity for CPA but actually caused a substantial reduction of cell growth. When S9 was prepared from liver of uninduced rats, higher CPA concentrations were required for 50% growth inhibition, even when increased S9 protein concentrations were used (Fig. 3).

These data suggest an optimal concentration of S9 protein of 16 μg/well for the activation of CPA. The diminished cytotoxicity of CPA at high S9 protein concentrations (> 100 μg/well) could be due to an accelerated inactivation of the cytotoxic metabolites. Reaction with glutathione (GSH) has been suggested as a mechanism for detoxification of CPA metabolites [16]. Since GSH is a component of S9, increasing the S9 concentration in the culture wells increases the GSH level. Upon the microsomal activation of CPA (minus the cytosolic fraction including GSH), inactivation of the cytotoxic metabolite did not occur at higher microsomal concentrations in contrast to experiments in which S9 mix was used (Fig. 4). This could be due to the absence of GSH and/or other cytosolic components including proteins with nucleophilic sites competing for electrophilic metabolites. On the other hand, microsome mix considerably inhibited the growth of A549 cells. When adding

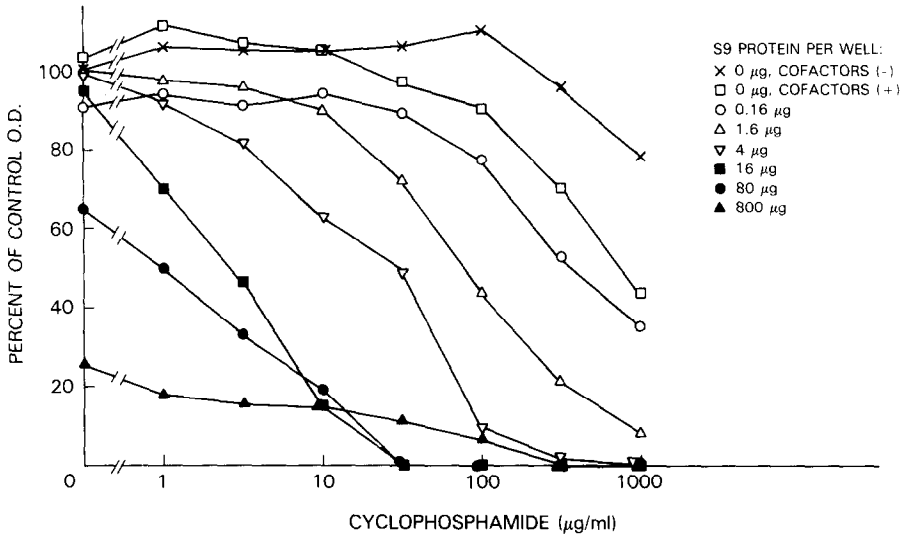

Fig. 1. Growth inhibition of A549 cells by CPA in the presence of liver S9 from Aroclor 1254 induced rats. After a one-day incubation of A549 cells (10^3 cells/well), CPA and S9 mix containing different concentrations of S9 protein were added. Twenty-four hours later, CPA/S9 containing medium was aspirated and growth medium was added. After a further 5 days, the number of viable cells was determined using a tetrazolium assay. Formation of colored formazan by reduction of the tetrazolium salt is proportional to the number of viable cells. Percent of O.D. at 540 nm is expressed relative to cultures not exposed to CPA/S9 mix. Points represent the mean of three determinations.

1 μg microsomal protein per well together with the components of the NADPH-generating system, this inhibition amounted to 25–40% of the untreated control. This inhibitory effect could not be reduced by further lowering the amount of added microsomal protein. Careful examination of the data obtained with the S9 mix revealed that even at an S9 concentration of 16 μg/well, showing no growth inhibitory effect in the 7-day assay (Fig. 1), a growth inhibition of up to 25% could be detected when the total culture duration was 5 days or less. Since the doubling time of the cells is extended with increasing cell densities, a slight inhibitory effect on cell growth may be missed in the 7-day assay. The growth inhibitory effect was apparently caused by the presence of the cofactors glucose-6-phosphate and $NADP^+$ in the S9 mix. Microsome mix, in addition, was supplemented with glucose-6-phosphate dehydro-

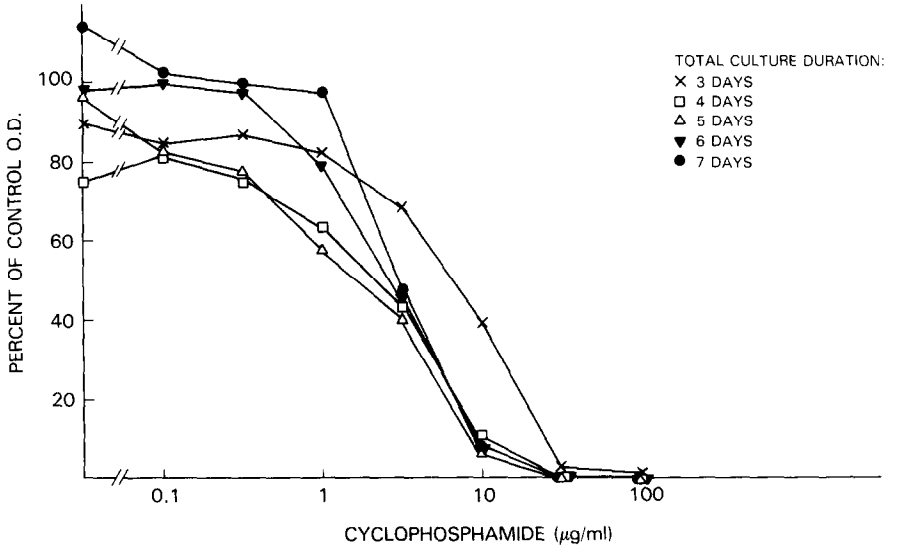

Fig. 2. Effect of culture duration post-treatment with CPA/S9 mix. CPA was activated by S9 mix containing 16 μg S9 protein from Aroclor 1254 pretreated rats. The time after treatment with CPA/S9 mix varied from 1 to 5 days, resulting in a total culture duration of 3–7 days. Other conditions were the same as described in the legend of Fig. 1. Percent of O.D. at 540 nm is expressed relative to cultures untreated with CPA/S9 mix. Points represent the mean of three determinations.

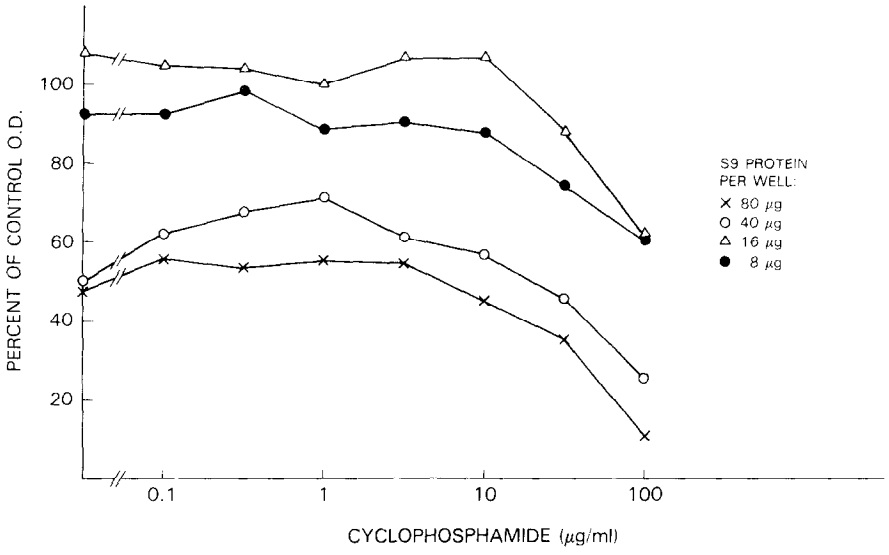

Fig. 3. Activation of cyclophosphamide by liver S9 from uninduced rats. Conditions were the same as described in the legend of Fig. 1 except that S9 from uninduced rats was used and that the total culture duration was reduced to 4 days. Percent of O.D. at 540 nm is expressed relative to cultures untreated with CPA/S9 mix. Points represent the mean of three determinations.

genase, resulting in a slightly increased growth inhibition.

To substantiate the role of GSH in the detoxification of CPA metabolites, S9 mix was supplemented with exogenous GSH. The addition of GSH led to a pronounced inhibition of the cytotoxicity caused by S9-activated CPA (Fig. 5). CPA concentrations up to 1000 mg/ml did not result in complete cell killing in the presence of 5 mM GSH. In contrast, addition of UDP-glucuronic acid had little effect on the IC_{50} for activated CPA.

The influence of S9 mix on IC_{50} values of cytotoxic drugs that are effective without metabolic activation is shown in Table 1. None of the compounds tested had lower IC_{50} values in the presence of S9 mix. According to the standard protocol used for drug screening, cell cultures are exposed continuously to test compounds which are added 24 hr after cell plating. Shorter periods of drug exposure led to slightly higher IC_{50} values, depending on the drug (Table 1).

Cell lines other than A549 may be used for the screening of substances that require metabolic activation. NCI-H322, a cell line derived from a bronchiolo-alveolar carcinoma, and A427, a lung large cell carcinoma (DNA repair deficient) cell line, showed slightly higher sensitivity to cyclophosphamide (Table 2). The growth of both cell lines, however, was inhibited considerably by S9 mix alone. The addition of S9 mix (16 μg S9 protein/well) caused

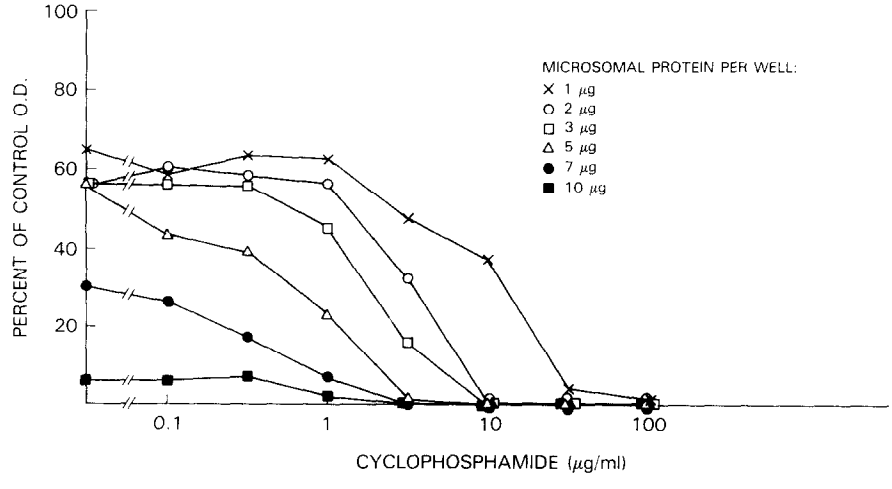

Fig. 4. Activation of cyclophosphamide by microsomes from Aroclor 1254 pretreated rats. Conditions were the same as described in the legend of Fig. 1 except that microsomes rather than S9 were used and the total culture duration was reduced to 4 days. Percent of O.D. at 540 nm is expressed relative to cultures untreated with CPA/microsome mix. Points represent the mean of three determinations.

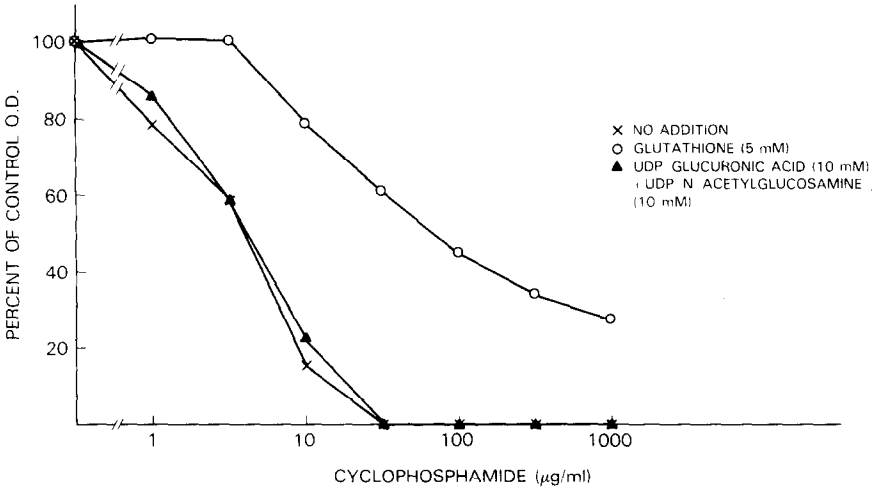

Fig. 5. Inactivation of cytotoxic metabolites of CPA. S9 mix (16 μg S9 protein/well, S9 from pretreated rats) was supplemented with cofactors for conjugation reactions to give a final concentration in the culture wells as indicated. After treatment with drug-supplemented S9 mix, cells were cultivated for 2 additional days (4 days total culture duration). Other conditions were the same as described in the legend of Fig. 1. Percent of O.D. at 540 nm is expressed relative to cultures not exposed to CPA but treated with S9 mix and cofactors for the corresponding conjugation reaction. Points represent the mean of three determinations.

Table 1. Effect of S9 mix on drug cytotoxicity in the MTT assay

	IC_{50} (μg/ml)		
	Exposure time		
	24hr		3 days
Drug	With S9 mix	Without S9 mix	Without S9 mix
Adriamycin	0.007 (88%)	0.005	0.003
Cisplatin	1.2 (89%)	1.0	0.9
Methotrexate	3 (97%)	0.09	0.02
Vinblastine	0.004 (88%)	0.003	0.002
$HgCl_2$	200 (97%)	60	30

A549 cells (10^3 cells/well) were grown 24 hr prior to application of drug and S9 mix (16 μg S9 protein/well, S9 from induced rats). Drug/S9 containing medium was either replaced with growth medium or left for the remaining cultivation. The total cultivation duration amounted to 4 days. The IC_{50} (50% growth inhibitory concentration) values were determined by linear interpolation from concentration–response curves. The percentage of control cell growth in the presence of S9 mix compared to unexposed cells is given in brackets.

Table 2. Cytotoxicity of cyclophosphamide towards A427 and NCI-H322 in the presence and absence of S9 mix

	IC_{50} (μg/ml)	
Cell line	With S9 mix	Without S9 mix
NCI-H322	1.5 (50%)	700
A427	1.3 (35%)	600

Culture conditions were the same as described in the legend of Fig. 1. CPA was activated by S9 mix (16 μg S9 protein/well, S9 from induced rats). Total culture duration was 7 days. The IC_{50} (50% growth inhibitory concentration) values were determined by linear interpolation from concentration-response curves. The percentage of control cell growth in the presence of S9 mix is given in parentheses.

a 50% or 65% growth inhibition of NCI-H322 and A427 cells respectively. This growth inhibitory effect persisted even when the S9 protein concentration was reduced to 8 μg/well. By using reduced S9 protein concentrations, however, an increase in the IC_{50} values for cyclophosphamide was noted.

DISCUSSION

In the present studies with the microculture tetrazolium assay, we have shown that cyclophosphamide was activated efficiently *in situ* by both S9 and microsomes from rat liver. Under optimal conditions S9 mix increased the cytotoxicity of CPA more than

200-fold towards all cell lines tested. The standard MTA procedure had to be expanded by only three simple steps: (1) addition of S9 mix or microsome mix, (2) aspiration of drug-containing medium, and (3) addition of fresh growth medium 1 day later. All three steps can be rapidly performed for a large number of samples when using 96-well microtiter plates.

We do not question the value of viable hepatocytes for studies concerning drug activation or inactivation [8]; however, its general application in drug screening has been hampered by the intense labor burden associated with such an approach. Preincubation of the drug with subsequent removal of microsomes either by centrifugation [17] or filtration [11] is also unsuitable when hundreds of samples have to be processed. A further disadvantage of this approach is possible loss of activity due to instability of some cytotoxic metabolites [11]. *In situ* activation of the test compound with either cofactor-fortified microsomes [18] or S9 [9, 12] circumvents time-consuming steps.

Cell growth inhibition by S9 or microsomes supplemented with cofactors has been reported to be a major disadvantage when CPA is activated *in situ* [17, 19]. The growth of different cell types has been shown to be inhibited differentially [17]. We also observed the differential inhibition of cell growth by S9, in combination with cofactors, when using various cell lines. Metabolic activation, however, could easily be detected even when S9 mix caused cell growth inhibition greater than 50%. The question of whether a compound is activated by S9 mix, therefore, can probably be answered using any cell line showing sufficient formazan formation after cocultivation with S9 mix even in the presence of substantial growth inhibition by S9 mix alone. Since a pattern of differential cytotoxicity or selective toxicity toward cell lines derived from particular tumor types is the principal criterion for the selection of a compound for further study, compatibility of S9 mix with most of the cell lines used in the screening program would be a prerequisite for further evaluation of a test compound requiring metabolic activation. Erroneous data could be obtained if the addition of S9 alone increased the sensitivity of cell lines toward test drugs, for example by membrane damage. Thus, an S9-dependent differential alteration in cell sensitivity would falsely indicate differential cytotoxicity toward the test drug. According to our data, however, the sensitivity of A549 cells for cytotoxic drugs was not increased by S9 mix. This suggests that S9 mix alone does not interfere with IC_{50} determinations by rendering the cells more susceptible to the test compound. In the case of methotrexate, substantial reduction in growth inhibitory activity was observed in the presence of S9 (Table 1 and additional experiments not shown). While we have not studied this phenomenon in detail, other investigators have shown that methotrexate can be metabolized to 7-hydroxymethotrexate polyglutamates by rat hepatocytes [20], that 7-hydroxymethotrexate is less effective as an inhibitor of dihydrofolate reductase [21], and that it interferes with methotrexate uptake into cells [22]. Thus, S9-mediated metabolism of methotrexate could be expected to reduce measured growth inhibition. Further investigations will demonstrate whether the approach for metabolic activation described here can be applied to all cell lines used in the screening program.

Growth inhibition by S9 mix may have multiple underlying mechanisms. First, an inhibitory effect which varies considerably among different cell lines and which cannot be abolished by reduction of the S9 protein concentration seems to be exerted by the added cofactors. Second, when using higher amounts of S9 protein, additional growth inhibition was observed, dependent upon the presence of both S9 protein and cofactors. This inhibitory effect may be due to the production of reactive oxygen radicals via electron transfer from NADPH to molecular oxygen. This reaction can be catalyzed by various enzymes present in S9 [23]. For all three cell lines tested, the same S9 protein concentration proved to be optimal for the activation of cyclophosphamide. We cannot exclude, however, that other cell lines have different susceptibilities to the inhibitory effects of S9 in combination with the necessary cofactors. Therefore, the optimal S9 protein concentration should be determined separately for each cell line. However, to ensure comparable levels of cytotoxic metabolites generated by S9 mix, it seems mandatory to use identical S9 protein concentrations for all cell lines used for the testing of a particular drug.

For *in vitro* drug evaluation applications, activation by S9 may be superior compared to microsomes because of its simpler preparation. Induction of cytochrome P-450-dependent monooxygenases by Aroclor 1254 substantially lowered IC_{50} values for cyclophosphamide. Although we have no data yet for other drugs, we believe that S9 preparations from such pretreated rats activate most compounds more efficiently. This hypothesis is supported by evidence from mutagenicity studies indicating improved activation of promutagens when S9 was prepared from pretreated animals [15].

The application of the method presented here, however, is not limited to the detection of compounds requiring metabolic activation. For example, additional information can readily be obtained with respect to the involvement of particular conjugation reactions in the detoxification of test compounds and/or their active metabolites. In this respect, the substantial decrease in cytotoxicity of activated CPA observed in the presence of GSH support recent data, suggesting an important role for GSH in the detoxification of reactive CPA metabolites [16]. Compounds with hydroxyl groups may be subject to conjugation with glucuronic acid. Whether a compound is detoxified by glucuronidation can be investigated by supplementing the S9 mix with UDP-glucuronic acid. This metabolic pathway is of particular importance for those compounds containing the quinone moiety, a characteristic of many antitumor drugs. Quinones can readily undergo redox cycling with the concomitant formation of toxic oxygen radicals [24–26]. Conjugation of the corresponding quinol with glucuronic acid would lead to removal of the compound from the redox cycles [27]. The present experiments demonstrate that the potential contribution of conjugations with GSH

and/or glucuronic acid can readily be detected with only minor modifications of the standard protocol.

The methods described here provide a simple and effective means for *in vitro* activation/inactivation of potential antitumor drugs. The technical ease of this methodology is compatible with its use on a relatively large scale for detailed evaluation of selected compounds. Experience with the A549, NCI-322, and A427 cell lines suggests that the simple methods described here may be applicable to a broad range of cell types. However, the unique sensitivities of individual cell lines to the toxic effects of the metabolic activation mixtures complicate the quantitative application of the method. This, coupled with the added cost associated with preparation and administration of the S9 mixture, indicates that the most appropriate use may be with one or a few of the members of the disease-oriented cell line panels to enable detection of compounds requiring metabolic activation in the primary screen and to facilitate detailed studies of such compounds.

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REFERENCES

1. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH and Boyd MR, Feasibility of drug screening with panels of human tumor cell lines using a microculture assay. *Cancer Res* **48**: 589–601, 1988.
2. Shoemaker RH, Monks A, Alley MC, Scudiero DA, Fine DL, McLemore TL, Abbott BJ, Paull KD, Mayo JG and Boyd MR, Development of human tumor cell line panels for use in disease-oriented drug screening. In: *Prediction of Response to Cancer Chemotherapy* (Ed. Hall T), pp. 265–286. Alan Liss, New York, 1988.
3. Boyd MR, Shoemaker RH, McLemore TL, Johnston MR, Alley MC, Scudiero DA, Monks A, Fine DL, Mayo JG and Chabner BA, Drug development. In: *Thoracic Oncology* (Eds. Roth JA, Ruckdeschel JC and Weisenberger TH), pp. 711–722. W. B. Saunders, New York, 1989.
4. Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. *J Immunol Methods* **65**: 55–63, 1983.
5. Brock N and Hohorst HJ, Uber die aktivierung von cyclophosphamid *in vivo* und *in vitro*. *Arzneim Forschung* **13**: 1021–1030, 1963.
6. Cohen JL and Jao JY, Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat. *J Pharmacol Exp Ther* **174**: 206–210, 1970.
7. Sladek NE, Metabolism of cyclophosphamide by rat hepatic microsomes. *Cancer Res* **31**: 901–908, 1971.
8. Alley MC, Powis G, Appel PL, Kooistra KL and Lieber MM, Activation and inactivation of cancer chemotherapeutic agents by rat hepatocytes cocultured with human tumor cell lines. *Cancer Res* **44**: 549–556, 1984.
9. Meltz ML and Winters DW, Utilization of metabolic activation for *in vitro* screening of potential antineoplastic agents. *Cancer Treat Rep* **63**: 1795–1801, 1979.
10. Shrivastav S, Stone KR, Paulson DF and Bonar RA, Activation of cyclophosphamide for *in vitro* testing of cell sensitivity. *Cancer Res* **40**: 4443–4445, 1980.
11. Alberts DS, Einspahr JG, Struck R, Bignami G, Young L, Surwit EA and Salmon SE, Comparative cytotoxicity of cyclophosphamide, its major metabolites and the new oxazaphosphorine ASTA Z 7557 (INN mafosfamide). *Invest New Drugs* **2**: 141–148, 1984.
12. Horner SA, Fry JR, Clother RH and Balls M, A comparison of two cytotoxicity assays for the detection of metabolism-mediated toxicity *in vitro*: a study with cyclophosphamide. *Xenobiotica* **15**: 681–686, 1985.
13. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H and Parks WP, *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* **51**: 1417–1423, 1973.
14. Falzon M, McMahon JB, Gazdar AF and Schuller HM, Preferential metabolism of *N*-nitrosodiethylamine by two cell lines derived from human pulmonary adenocarcinomas. *Carcinogenesis* **7**: 17–22, 1986.
15. Maron DM and Ames BN, Revised methods for the Salmonella mutagenicity test. *Mutat Res* **113**: 173–215, 1983.
16. Ohno Y and Ormstad K, Formation, toxicity and inactivation of acreolin during biotransformation of cyclophosphamide as studied in freshly isolated cells from rat liver and kidney. *Arch Toxicol* **57**: 99–103, 1985.
17. Nissen E and Tannenberger S, Letter to the editor: correspondence re: S. Shrivastav, K.R. Stone, D.F. Paulson and R.A. Bonar, Activation of cyclophosphamide for *in vitro* testing of cell sensitivity. *Cancer Res* **40**: 4443–4445, 1980. *Cancer Res* **40**: 3149–3150, 1984.
18. Dolfini E, Martini A, Donelli MG, Morasca L and Garattini S, Method for tissue culture evaluation of the cytotoxic activity of drugs active through the formation of metabolites. *Eur J Cancer* **9**: 375–378, 1973.
19. Voelker G, Draeger U, Peter G and Hohorst HJ, Studien zum spontanzerfall von 4-hydroxycyclophosphamid und 4-hydroxyperoxyphosphamid mit hilfe der dunnschichtchromatographie. *Arzneimittel Forschung* **24**: 1172–1176, 1974.
20. Rhee MS and Galivan J, Conversion of methotrexate to 7-hydroxymethotrexate polyglutamates in cultured rat hepatic cells. *Cancer Res* **46**: 3793–3797, 1986.
21. Johns DG and Loo TL, Metabolite of 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (methotrexate). *J Pharm Sci* **56**: 356–359, 1969.
22. Fabre G, Matherly LH, Fabre I, Cano JP and Goldman ID, Interactions between 7-hydroxymethotrexate and methotrexate at the cellular level in Ehrlich ascites tumor *in vitro*. *Cancer Res* **44**: 970–975, 1984.
23. Dybing E, Nelson SD, Mitchell JR, Sasame HA and Gillete JR, Oxidation of α-methyldopa and other catechols by cytochrome P-450-generated superoxide anion: possible mechanism of methyldopa hepatitis. *Mol Pharmacol* **12**: 911–920, 1976.
24. Thayer WS, Adriamycin stimulated superoxide formation in submitochondrial particles. *Chem Biol Interact* **19**: 265–278, 1977.
25. Powis G, Svingen BA and Appel P, Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells. *Mol Pharmacol* **20**: 387–394, 1981.
26. Doroshow JH, Mitomycin C-enhanced superoxide and hydrogen peroxide formation in rat heart. *J Pharmacol Exp Ther* **218**: 206–211, 1981.
27. Nemoto N and Gelboin HV, Enzymatic conjugation of benzo(*a*)pyrene oxides, phenols and dihydrodiols with UDP-glucuronic acid. *Biochem Pharmacol* **25**: 1221–1226, 1976.