

***In Vitro* Drug Sensitivity of Tumor Cells Is Correlated with Drug-Induced Inhibition of DNA Synthesis**

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The objective of this study was to develop a rapid *in vitro* method for predicting the response of human tumors to anticancer drugs. In this study an attempt was made to correlate the drug effects on the relative incorporation of (³H) thymidine (ThdR) into DNA with the sensitivity of tumor cells to that drug. The results of the study indicate that following treatment of the cells with adriamycin (ADR) or 1-(2-chloroethyl)-3-(4-methyl cyclohexyl)-1 nitrosourea (MeCCNU), there was a significant inhibition of DNA synthesis in the drug-sensitive cells. However, the inhibition was relatively small in the drug-resistant cells. Following cytosine arabinoside (Ara-C) treatment, a dramatic recovery in the rate of DNA synthesis was seen in Ara-C-resistant cells but not in cells sensitive to Ara-C. Thus, the method described in this study appears to be capable of distinguishing whether a tumor cell line is sensitive or resistant to a given drug.

INTRODUCTION

Although there is no panacea for all neoplastic diseases, a variety of agents that possess antineoplastic activity have been discovered. Unfortunately, patients' responses to a given drug vary over a wide range [1]. Thus, a patient may or may not respond well to a particular treatment or may require several trial therapies before an effective treatment is found.

A solution for avoiding ineffective and unnecessary chemotherapeutic regimens would be to develop a rapid *in vitro* method that would predict the best drugs to use for a given patient based on the drug sensitivity of that patient's tumor cells. Most of the anticancer agents in use today not only inhibit cell division but also cause damage to DNA, evidenced as chromosome lesions. A method that was developed to screen for mutagenic agents on the basis of their ability to inhibit DNA synthesis in HeLa cells [2-4] may also be useful to predict the response of human tumors to anticancer drugs. The object of this study was to ascertain whether or not this method can indeed distinguish tumor cell lines of known sensitivity to a specific drug from those that are resistant to the drug.

MATERIALS AND METHODS

Cells

Mouse leukemia cell lines, P-388/O (P-388 mouse leukemia cells sensitive to all drugs listed in this study), P-388/ADR (adriamycin-resistant mouse leukemia cells),

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and P-388/Ara-C (cytosine arabinoside-resistant mouse leukemia cells) were kindly supplied by Dr. Lee Wilkoff of Southern Research Institute, Birmingham, Alabama. The P-388/ADR resistant cell line is actually a vincristine-resistant cell line found to be cross-resistant to 0.02–2.0 $\mu\text{g/ml}$ concentrations of adriamycin as determined using a semisolid medium plating assay. *p* values of <0.0001 were obtained for all drug concentrations within the 0.02–2.0 $\mu\text{g/ml}$ range when comparing P-388/ADR and P-388/O cell survival following drug treatment [5]. Both P-388/ADR and P-388/Ara-C drug-resistant cell lines were established by treatment of (C57BL $\delta \times$ DBA/2 δ (BDF₁) mice bearing ascites tumor with vincristine or Ara-C, respectively, over a number of transplant generations [5]. The cell lines were maintained in culture as was previously described [5]. The three cell lines were routinely grown in Falcon 75 cm² tissue culture flasks (Falcon Plastics, Oxnard, CA) at 37°C in a humidified CO₂ incubator in RPMI-1640 medium with L-glutamine (Grand Island Biological Co., Santa Clara, CA) supplemented with 16 percent heat-inactivated fetal calf serum and a 1 percent antibiotic mixture (10,000 U penicillin/ml and 10,000 μg streptomycin/ml).

Two human colon cancer cell lines, HT-29 (MeCCNU-resistant), originally isolated by Fogh and Trempe [6], and the cell line BE (MeCCNU-sensitive) were kindly supplied by Dr. Kurt Kohn of the National Institutes of Health, Bethesda, Maryland. The BE and HT-29 cell lines were determined to be sensitive and resistant, respectively, using both *in vivo* and *in vitro* tests [13]. We also performed an *in vitro* study on the sensitivity and resistance of the two cell lines. Following a one-hour exposure to 2.5, 5, and 10 $\mu\text{g/ml}$ concentrations of MeCCNU, the HT-29 drug-treated cells, when compared to untreated cells, had a 100 percent relative plating efficiency at all concentrations tested, whereas drug-treated BE cells had a 11.3 percent, 2.25 percent, and 0 percent relative plating efficiency, respectively, when compared to untreated cells. These two cell lines were routinely grown as monolayer cultures in Falcon plastic dishes at 37°C in a humidified CO₂ incubator in McCoy's modified medium 5-A (Grand Island Biological Co., Santa Clara, CA), supplemented with the amounts of fetal calf serum and antibiotics used in the RPMI-1640 medium.

Drugs

Ara-C (Upjohn) was dissolved in specially provided bacteriostatic water for injection with benzyl alcohol 0.9 percent weight per volume to give a stock solution of 20 mg/ml. ADR (Adria Laboratories, Inc., Columbus, OH) was dissolved in 0.9 percent NaCl to give a stock solution of 2 mg/ml. MeCCNU (NSC-95441) was dissolved in specially provided diluent to give a stock solution of 25 mg/ml. Stock solutions of all the drugs were prepared just before use, and the desired concentrations were obtained by serial dilution with complete culture medium.

Measurement of Drug Effects on DNA Synthesis in Tumor Cells

Human colon cancer cell lines, i.e., BE and HT-29, were plated in a number of 35 mm plastic dishes (Lux, Newbury Park, CA) one or two days before the experiment. The experiment was begun by adding (¹⁴C) ThdR (0.01 $\mu\text{Ci/ml}$; specific activity 30 Ci/mM) to all the dishes and incubating them for 24 hours. At the end of this incubation (¹⁴C) ThdR was removed; the cells were washed with fresh medium and then exposed to different drug concentrations for 30 minutes. Cells incubated in medium containing no drug served as a control. The cells were washed free of drug and reincubated in regular medium. At 0, 0.5, and 1.5 hours after the removal of the

drug, the cells were pulsed with (^3H) ThdR ($3 \mu\text{Ci/ml}$) for ten minutes and then washed three times with ice-cold SSC (0.15 M sodium chloride– 0.015 M sodium citrate). The cells were then scraped with a rubber policeman into cold 4 percent perchloric acid (PCA), transferred to a filter paper, washed three times with cold PCA, and dehydrated by washing serially with 70 percent, 95 percent, and 100 percent ethanol. The filter paper with cells was dried under a heat lamp and then transferred to a scintillation vial containing 10 ml Scinti Verse (Fisher Scientific Co.). The scintillation vial was shaken vigorously until the filter paper disintegrated, at which time the radioactivity incorporated into the cells was counted in a Packard liquid scintillation spectrometer.

The P-388 cell lines that grow as suspension cultures were maintained in T75 culture flasks. These cells were first labeled with (^{14}C) ThdR for 24 hours, as in the case of monolayer cultures. Following the removal of labeled medium by centrifugation, the cells were washed, resuspended in fresh medium, and distributed into a number of Falcon 25 cm^2 tissue culture flasks to facilitate treating the cells with different drug concentrations for a period of 30 minutes at 37°C . After the drug treatment, the cells were washed, resuspended in fresh medium in T25 flasks in a total volume of 10 ml for each different drug concentration, and further incubated. From these flasks, 3 ml cell samples (approximately $2 \times 10^6/\text{ml}$) were drawn at 0, 0.5, and 1.5 hours after treatment, pulsed with (^3H) ThdR for 10 minutes, and processed for scintillation counting as described above. The (^3H) and (^{14}C) counts for each sample were obtained. The (^3H)/(^{14}C) ratio for each sample was calculated, expressed as a percentage of the control, and plotted as a function of time.

RESULTS

The Correlation Between the Drug-Induced Inhibition of DNA Synthesis and Drug Sensitivity of Tumor Cells

The effects of adriamycin on the rates of DNA synthesis in ADR-sensitive (P-388/O) and ADR-resistant (P-388/ADR) mouse leukemia cell lines were compared. In P-388/O cells treated with three different concentrations of ADR, a dose- and time-dependent inhibition of DNA synthesis was observed (Fig. 1A). No inhibition of DNA synthesis was observed in P-388/ADR cells treated with 0.1 or $1.0 \mu\text{g/ml}$ ADR (Fig. 1B). However, at a higher concentration ($5 \mu\text{g/ml}$) of ADR, the inhibition of DNA synthesis in P-388/ADR was almost as great as in P-388/O cells.

Similarly, the effects of Ara-C on DNA synthesis were compared in Ara-C-sensitive (P-388/O) and Ara-C-resistant (P-388/Ara-C) cell lines (Fig. 2). In Ara-C-resistant cells there was a dramatic increase in the incorporation of label following the removal of the drug. However, in P-388/O cells, practically no incorporation of (^3H) ThdR was seen 1.5 hours after Ara-C treatment.

The effects of MeCCNU on the incorporation of (^3H) ThdR into sensitive (BE) and resistant (HT-29) cell lines of human colon cancer are shown in Figs. 3A and 3B, respectively. The pattern of labeling kinetics observed in this set of experiments appears to be similar to that of ADR effects in P-388 cells shown in Figs. 1A and 1B. The MeCCNU treatment caused a significant dose-dependent decrease in the incorporation of (^3H) ThdR into BE cells, while it had only a slight effect on the HT-29 cells.

The contrast between the response of sensitive and resistant cell lines to a given drug becomes even more obvious when the difference between the rates of incorporation of (^3H) ThdR at zero and 1.5 hours after the drug treatment is taken into

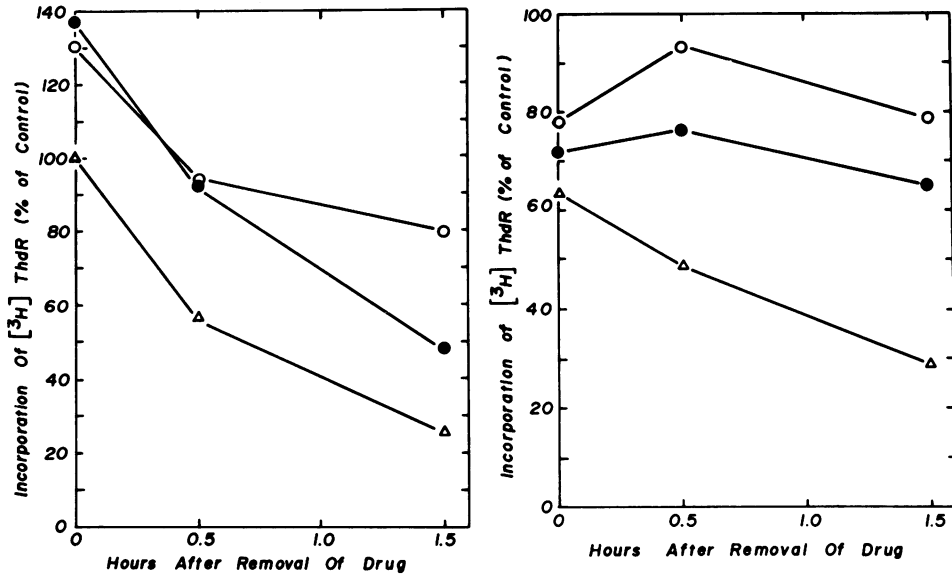


FIG. 1. Effect of ADR on the incorporation of (^3H) ThdR in the ADR-sensitive P-388/O and the ADR-resistant P-388/ADR mouse leukemia cells. A ten-minute pulse of (^3H) ThdR was given at 0, 0.5, and 1.5 hours after the removal of the ADR from the medium. The cells were treated with ADR for 30 minutes at concentrations of 0.1 $\mu\text{g}/\text{ml}$, \circ ; 1.0 $\mu\text{g}/\text{ml}$, \bullet ; and 5.0 $\mu\text{g}/\text{ml}$, \triangle . **A** (left). ADR-sensitive P-388/O mouse leukemia cells. **B** (right). ADR-resistant P-388/ADR mouse leukemia cells.

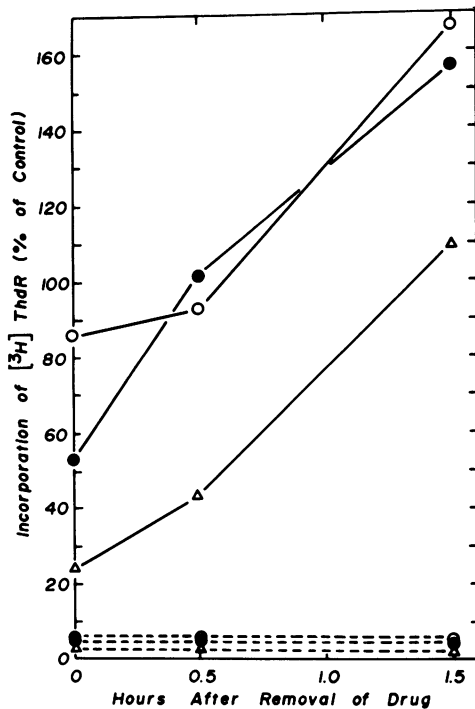


FIG. 2. Effect of Ara-C on the incorporation of (^3H) ThdR in Ara-C-sensitive and resistant cell lines. The cells were treated with Ara-C for 30 minutes at concentrations of 1 $\mu\text{g}/\text{ml}$, \circ ; 10 $\mu\text{g}/\text{ml}$, \bullet ; and 50 $\mu\text{g}/\text{ml}$, \triangle . The solid lines represent Ara-C-resistant P-388/Ara-C cells and the dashed lines represent Ara-C-sensitive P-388/O cells.

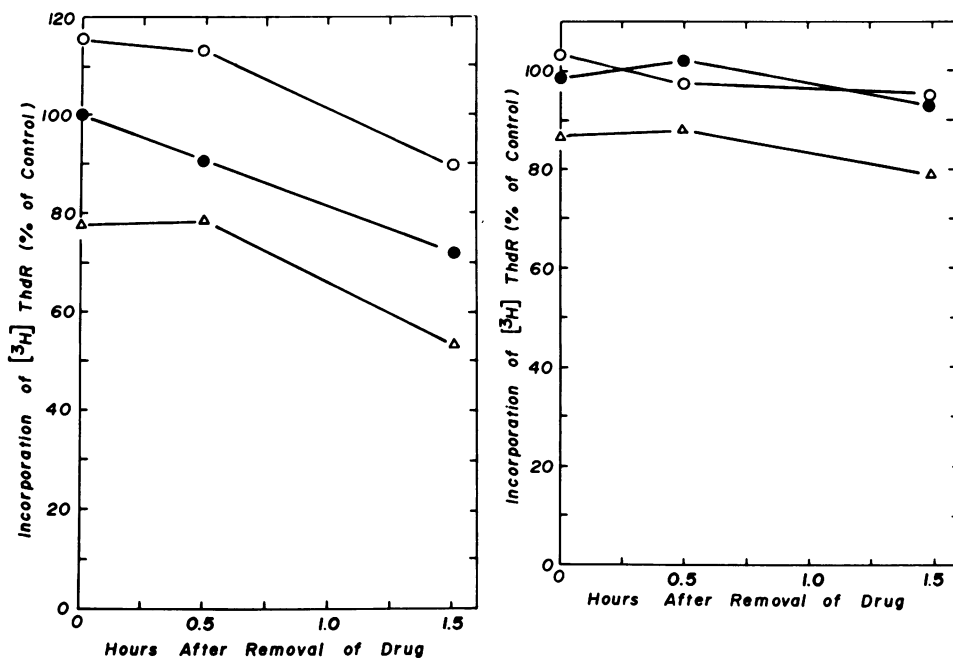


FIG. 3. Effects of MeCCNU on the incorporation of (^3H) ThdR in MeCCNU-sensitive BE cells and MeCCNU-resistant HT-29 cells. The cells were treated with MeCCNU at concentrations of 1 $\mu\text{g}/\text{ml}$, \circ ; 10 $\mu\text{g}/\text{ml}$, \bullet ; and 100 $\mu\text{g}/\text{ml}$, \triangle . **A** (left). MeCCNU-sensitive BE cells. **B** (right). MeCCNU-resistant HT-29 cells.

consideration. This difference, which indicates the degree of inhibition of DNA synthesis in each treatment, is calculated as follows:

$$\% \text{ inhibition} = \frac{V_i - V_f}{V_i} \times 100 \quad [\text{a}]$$

where V_i is the incorporation of (^3H) ThdR (expressed as percentage of control) at zero hour after drug treatment and V_f is the same parameter taken 1.5 hours later.

Using this formula, we calculated the percentage of inhibition of DNA synthesis for ADR-sensitive and resistant P-388 cells from the data presented in Figs. 1A and 1B, respectively (Fig. 4A). Particularly at a low concentration of ADR the inhibition in P-388/ADR cells was less than 10 percent, whereas it ranged from 37 percent to 75 percent in the ADR-sensitive cells. A similar treatment of the data from Figs. 3A and 3B brings into sharper focus the differences in the response of BE and HT-29 cells to MeCCNU (Fig. 4B). Increasing the concentration of MeCCNU had no significant effect on the percentage of inhibition of DNA synthesis in the resistant HT-29 cells; it remained less than 10 percent. In the sensitive BE cells the percentage of inhibition increased with dose, i.e., from 23 percent to 33 percent.

Since there was an increase in the rate of incorporation of (^3H) ThdR into P-388/Ara-C cells following treatment with Ara-C, the percentage of increase in DNA synthesis was calculated as follows:

$$\% \text{ increase} = \frac{V_f - V_i}{V_i} \times 100 \quad [\text{b}]$$

where V_i and V_f are defined as in formula [a].

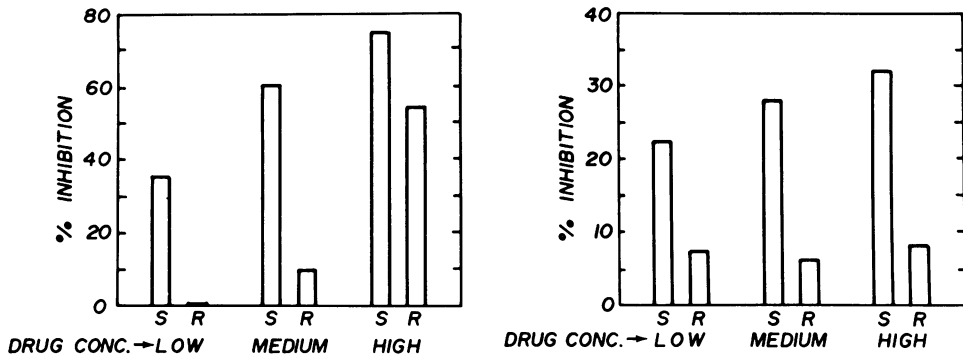


FIG. 4. Relative rates of inhibition of DNA synthesis in drug-sensitive (S) and resistant (R) cells at 1.5 hours after the drug treatment. **A (left).** Effect of ADR on P-388/O (S) and P-388/ADR (R) cells. Drug concentrations used were 0.1 µg/ml (low), 1.0 µg/ml (medium), and 5 µg/ml (high). **B (right).** Effect of MeCCNU on DNA synthesis in BE (S) and HT-29 (R) human colon cancer cells. The concentrations of MeCCNU were 1 µg/ml (low), 10 µg/ml (medium), and 50 µg/ml (high). The formula for estimating the percentage of increase is given in the Results section.

Application of this formula to the data from Fig. 2 provides a means to detect a dose-dependent increase in the rate of incorporation of (³H) ThdR into Ara-C-resistant cells (Fig. 5). There was practically no incorporation in the Ara-C-sensitive cells.

DISCUSSION

The method of estimating the effects of anticancer drugs on the rate of DNA synthesis presented in this study appears to be capable of distinguishing drug-sensitive cell lines from those that are resistant. When the percentage inhibition formula [a] was used, inhibition of DNA synthesis in ADR-sensitive P-388/O cells at 0.1 and 1.0 µg/ml of ADR was seen to be significantly greater than in the ADR-resistant cells (Fig. 4A). It should be noted that for the P-388/ADR cell line, the 1.5 hour time point regarding (³H) ThdR incorporation was not significantly different from that of the sensitive cell line at all three ADR concentrations tested (Figs. 1A and 1B). However, when the difference in (³H) ThdR incorporation is observed over time, it becomes easy to distinguish the sensitive cell line from the resistant cell line (Fig. 4A). The observed difference is related to the mechanism of resistance that the P-388/ADR cell line maintains.

The possible factors that render P-388/ADR cells resistant to ADR include de-

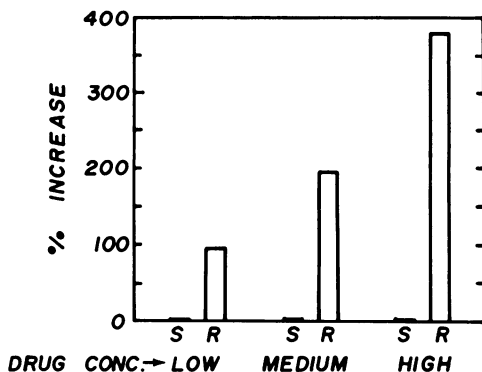


FIG. 5. Relative rates of increase in the incorporation of (³H) ThdR in P-388/O (S) and P-388/Ara-C (R) cells at 1.5 hours after the drug treatment. The concentrations of Ara-C used were 1 µg/ml (low), 10 µg/ml (medium), and 50 µg/ml (high). The formula for estimating the percentage of increase is given in the Results section.

creases in (i) permeability to the drug, (ii) drug retention by cells, and (iii) binding affinities of the target molecules [5]. Recent reports suggest that decreased drug retention by the cell via an enhanced efflux process is the most likely factor responsible for drug resistance in P-388/ADR cells [7,8].

The data shown in Figs. 1A and 1B are best explained by an enhanced efflux mechanism of resistance. At time zero, assuming that ADR treatment stimulates the efflux mechanism, P-388/ADR drug-treated cells should have a lower cellular concentration of (^3H) ThdR available for incorporation into DNA when compared to the cellular (^3H) ThdR levels of P-388/ADR cells not receiving the drug treatment, yielding the results shown in Fig. 1B. It would appear that at ADR concentrations greater than 1 $\mu\text{g}/\text{ml}$, the efflux system is overloaded as ADR greatly depressed the incorporation of (^3H) ThdR over time when the 5 $\mu\text{g}/\text{ml}$ concentration of ADR was used (Fig. 1B).

In contrast, HT-29 cells seem to be resistant to MeCCNU even in higher concentrations. An increased dose of MeCCNU did not significantly increase the inhibition of DNA synthesis. In MeCCNU-sensitive BE cells, the drug effects were dose-dependent. But even at the highest doses tested, the inhibition of (^3H) ThdR uptake in BE cells was only 33 percent, compared to 9 percent in the HT-29 cells. In spite of the narrow differences, the pattern of DNA synthesis inhibition in drug-sensitive cells is quite distinct from that observed in a resistant cell line.

These results confirm the observations of Painter and Painter and Howard [2-4], who used this method to distinguish DNA-damaging compounds by measuring their effects on DNA synthesis. They also reported that agents such as hydroxyurea and cycloheximide, which inhibit DNA synthesis without causing any damage, show a reverse effect, i.e., increase in the uptake of (^3H) ThdR as a function of time. In our study, we observed that P-388/O cells, which are sensitive to Ara-C, did not recover at all following Ara-C treatment (Fig. 2). It appears that this cell line is extremely sensitive to this drug. Even in the resistant line (P-388/Ara-C), incorporation of (^3H) ThdR immediately after the treatment exhibited a sharp decrease with increasing drug concentration (Fig. 2). However, the recovery was dramatic and dose-dependent (Fig. 5). The greater the inhibition at zero hours after treatment, the higher the recovery 1.5 hours later. Thus, it is possible that the recovery of DNA synthesis following treatment with other metabolic inhibitors such as hydroxyurea, 5-fluorouracil, or methotrexate may distinguish resistant cell lines from non-recovering or poorly recovering sensitive cell lines. This, however, remains to be tested.

The dramatic recovery of DNA synthesis in the P-388/Ara-C cells that eventually exceeded the rate of (^3H) ThdR incorporation of the untreated control is difficult to explain because of the complex and opposing effects that Ara-C has on ThdR incorporation [12]. However, a recent report demonstrated that reinitiation of DNA synthesis occurred in some of the DNA segments that replicated earlier in the S phase as a result of a 60-minute exposure of GK cells (EB virus-transformed human peripheral lymphocyte cell line) to Ara-C [9]. This aberrant double DNA replication appears to be the consequence of interruption of DNA replication rather than some special property of the Ara-C molecule. Possibly when the Ara-C block is removed, the number of replicating units synthesizing DNA increased in a dose-dependent manner in comparison to the untreated control (Fig. 2).

Since we are able to distinguish drug-sensitive cell lines from the resistant ones, as demonstrated in our study, we believe that this method could be applied to screen human tumors rapidly for their drug sensitivity so that therapeutic regimens may be designed on an individual basis. The possibility of applying such *in vitro* methods to

predict the response of human tumors to anticancer drugs appears to be very encouraging [10,11].

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