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# Potential limitations of in vitro clonogenic drug sensitivity assays

**G. Wei Dong, H. D. Preisler, and R. Priore**

Roswell Park Memorial Institute Department of Health, 666 Elm Street, Buffalo, New York 14263, USA

***Summary.*** *P388 murine leukemic cells lines which were resistant (P388R) or sensitive (P388S) to adriamycin (adr) were used to evaluate the potential utility of in vitro clonogenic assays for detecting and quantitating the number of adr-resistant cells present in a cell mixture. The progeny of P388S cells that had been exposed for 1 h or continuously to adr were as sensitive to the drug as the original P388S cells. Serial passages in the presence of adr, however, resulted in the selection of adr-resistant cells, but even these cells varied in their level of resistance. When P388S and P388R cells were mixed together and exposed to adr, the clonogenic assay consistently overestimated the proportion of P388S cells present. These studies demonstrate some of the inherent limitations of clonogenic drug sensitivity assays and may provide an explanation for the reported discrepancies between in vitro drug sensitivity and clinical responses to therapy reported in the literature.*

## Introduction

Measurement of the effects of chemotherapeutic agents upon clonogenic malignant cells has been proposed as a means of assessing the drug sensitivity of human neoplastic cells [2–5]. In the studies reported here two P388 murine leukemia cell lines, one sensitive and one resistant to adriamycin (adr), were used to assess the ability of the clonogenic drug sensitivity assay to detect drug-resistant cells when these cells were present together with drug-sensitive cells. Additionally, the adr sensitivity of the cells that survived exposure to the drug was also determined.

## Materials and methods

*Cell lines.* Two sublines of P388 cells were kindly provided by Dr A. Krishan of the University of Miami School of Medicine. One line was sensitive (P388S) and the other resistant (P388R) to adr. The P388 murine lymphoid leukemia cells were suspended at a concentration of $10^5$ cells/ml RPMI 1640 culture medium, with 10% (v/v) fetal bovine serum and 10% (v/v) $10^{-4}$ *M* 2-mecaptoethanol. The cells were cultured in a humidified atmosphere consisting of 5% $CO_2$/95% room air at 37° C. A plateau phase was reached after 72 h of growth with a cell concentration of $2 \times 10^6$ cells/ml. The cells were passaged in fresh culture medium every 72 h. To maintain the resistance of the P388R cells the subline was cultured every other passage in medium containing 5 μg adr/ml.

*Clonogenicity method*

*Plating.* One thousand cells were plated in 35-mm petri dishes containing 1 ml RPMI 1640 culture medium with 0.3% agar, 10% (v/v) $10^{-4}$ *M* 2-mecaptoethanol, 20% (v/v) fetal bovine serum, and 30% (v/v) conditioned medium. The culture dishes were placed in a humidified 37° C, 5% $CO_2$/95% room air incubator for 7 days. Triplicate plates were scored, aggregates consisting of more than 50 cells being counted as colonies. In preliminary studies, cells were removed from the liquid phase every 12 h for 72 h after seeding in liquid culture, and the cloning efficiency was found to be unaffected by the duration of growth in liquid medium.

*Conditioned medium.* Medium in which P388 cells had been grown for 72 h was collected and passed through 0.45 μm filters. 'Conditioned medium' was prepared from each P388 subline and was stored at −15° C. When each subline was cloned its own conditioned medium was used. When mixtures of the two sublines were cloned equal volumes of each of the two condtioned media were mixed and used at a final concentration of 30%.

*Drug exposure.* The following general methods of drug exposure were used:

*1) Pulse drug exposure.* Cells obtained from cultures containing $5 \times 10^5$ cells/ml (logarhythmic growth phase) were suspended in RPMI 1640 culture medium with the indicated concentration of drug. After incubation in a 37° C, 5% $CO_2$ incubator for 1 h, the cells were washed three times with 50 ml RPMI 1640 medium and resuspended for cloning.

*2) Continuous incubation of cells with drug.* Cells were cloned in semisolid culture medium in the presence of the indicated concentration of drug.

*Single colony subculture.* One hundred thousand cells were plated in a 60-mm petri dish with 3 ml RPMI 1640 culture medium with 0.9% (v/v) methylcellulose, 10% (v/v) $10^{-4}$ *M* 2-mecaptoethanol, 20% (v/v) fetal bovine serum, and 30% (v/v) conditioned medium. After 10 days' culture at 37° C in a humidified atmosphere consisting of 95% room air/5% $CO_2$, individual colonies with a diameter of approximately 0.5 mm

*Offprint requests to:* H. D. Preisler

*Abbreviations:* P388R, P388 murine leukemia cells resistant to adriamycin; P388S, P388 murine leukemia cells sensitive to adriamycin; adr, adriamycin; v/v, volume per volume

were picked up with a Pasteur pipet. Each individual colony was suspended in 0.3 ml RPMI 1640 medium, made into a single-cell supsension and then divided into two equal parts for further investigation.

*Statistics.* Means and standard error of the mean (means ± SEM) are used to express the data presented in these studies. Student's *t*-test was used to determine the statistical significance of differences between means.

## Results

### *Growth characteristics in liquid culture in the presence and absence of adriamycin*

Figure 1 shows the growth curves of P388S and P388R cells in the presence or absence of adr. While exposure of P388S cells to 0.01 μg/ml adr produced 50% inhibition of growth, exposure of P388R cells to as much as 3 μg/ml adr had little or no effect on cell growth. Table 1 provides data on the doubling time of both cell lines and on their cloning efficiency in a semisolid matrix and the $LD_{50}$s for exposure to adr under different conditions. Note that 14% of P388R cells and 24% of P388S cells produced colonies in vitro consisting of several hundred cells. Aggregates of less than 50 cells were rare.

### *Cell growth in semisolid medium – effects of adriamycin*

The effects of adr on the ability of P388 cells to produce colonies in vitro were assessed in two ways: cells were either exposed to adr for 1 h and then plated in agar (pulse exposure) or were directly plated in agar to which adr had been added (continuous exposure). Pulse exposure of P388S cells to 0.3 μg/ml adr for 1 h resulted in a 23% reduction in the number of colonies produced. Exposure to higher concentrations of drug produced greater reductions (Fig. 2), and at 10 μg/ml clonal growth was completely inhibited. In contrast, P388R cells were unaffected by exposure to as much as 30 μg/ml adr for 1 h (Fig. 2). The inclusion of adr in the agar in which P388 cells were cloned resulted in a greater decrease in the number of colonies than when the cells were pulse-exposed to the same concentration of drug (Fig. 3). These effects were more pronounced for the P388S cells than for the P388R cells.

### *Adriamycin sensitivity of cells that clone in vitro after exposure to the drug*

To assess the adr sensitivity of cells that were able to clone in vitro after exposure to the drug the following experiments were performed:

1) A 1-h exposure followed by growth in vitro for 10 days and a further 1-h exposure (Fig. 4 panels A and B). The initial exposure of P388S cells to adr resulted in an 83% decline in the number of colonies (Fig. 4A). As noted in the figure legend, 20 colonies were picked from the plates containing colonies that had been produced by adr-exposed cells, dispersed, divided into two equal aliquots with one aliquot exposed to adr (1 μg/ml) for 1 h, washed, and replated, while the other half was treated identically except that it was not exposed to adr and served as a control (Fig. 4B).

The number of colonies produced per half-control colony ranged from 14 to 560, with mean ± SEM and median colony numbers of 186 ± 29 and 160, respectively. In contrast, the mean ± SEM and median values for the 20 half-colonies that were exposed to adr for a second time were 12 ± 7 and 2, respectively, with 10 of the half-colonies failing to produce growth when recloned. Comparison of the cloning efficiencies of the P388S cells after the first and after the second exposure

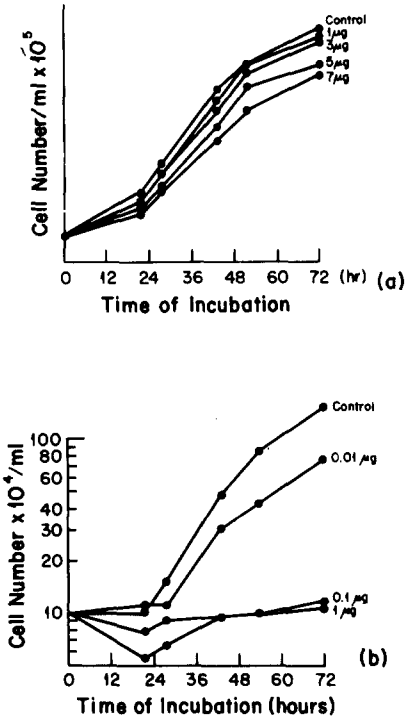


**Fig. 1a, b.** Cell growth curves for P388R **(a)** and P388S **(b)** cells in liquid culture system. Controls (no adr) and different concentrations (μg/ml) of adr

**Table 1.** Growth characteristics and adriamycin sensitivity of P388 cells

| Cell line | Doubling time (h) | Cloning efficiency[a] | LD50[c] (μg/ml adr) | | |
|---|---|---|---|---|---|
| | | | Exposure of cells to adr in liquid culture | Cloning studies Exposure conditions | |
| | | | | 1 h pulse | Continuous |
| P388R | 16.7 | 14 ± 0.01[b] | 7 | 30 | 0.321 |
| P388S | 16.7 | 24 ± 0.02 | 0.01 | 0.321 | 0.01 |

[a] Summarized from all the control groups throughout this study
[b] Mean ± SEM as percentages of cells plated. The cloning efficiencies were significantly different ($P < 0.001$)
[c] Concentration of adr which kills 50% of the cells

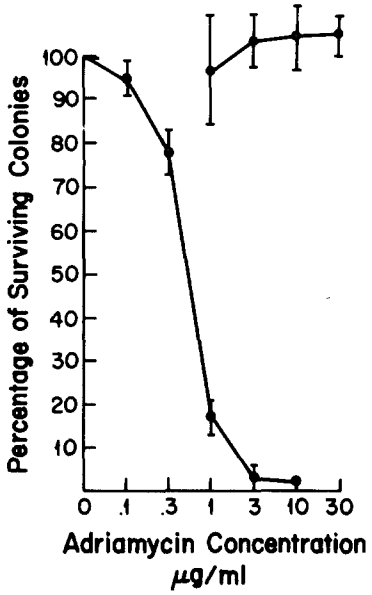


**Fig. 2.** Sensitivity of P388 cells to adr exposure at different concentrations of adr. P388S cells exhibit a progressive decrease in the percentage of cells surviving, while the P388R cells do not (*curve* in the *upper right-hand corner.* Adr concentrations for this curve are indicated by the *abscissa* values directly below the *curve* and range from 1μg/ml to 30 μg/ml). *Points,* means of three results in three experiments; *bars,* SEM

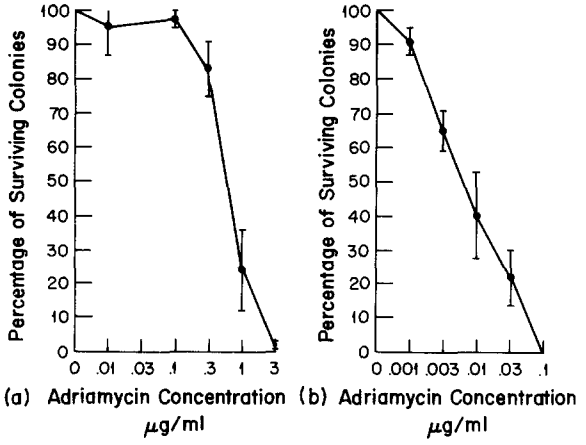


**Fig. 3a, b.** Effects of inclusion of adr in the semisolid matrix used to clone P388 cells: **a** percent survival of P388R clonogenic cells vs adr concentration; **b** percent survival of P388S clonogenic cells vs adr concentration. *Points,* means of three results in three experiments; *bars,* SEM

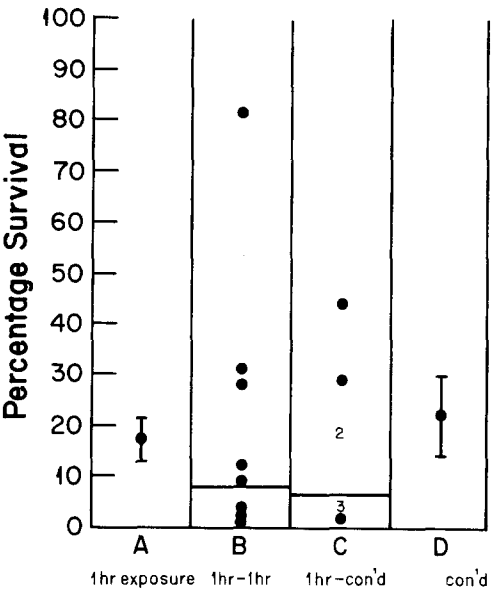


**Fig. 4.** Adr sensitivity of cells which survived pulse exposure to the drug: *A,* percent survival of cells exposed to adr (1 μg/ml) for 1 h and cloned in methylcellulose for 10 days; *B,* sensitivity of the progeny of cells which had survived 1 h exposure to adr to a seconnd 1-h exposure to adr. Cells were exposed to adr (1 μg/ml) for 1 h, washed free of the drug and cloned in methylcellulose for 10 days. Individual colonies were picked, the cells dispersed and divided into two equal aliquots. One half of the cells were re-exposed to adr for 1 h while the other half was not. Both were recloned and the number of colonies produced enumerated. Each *circle* represents the percentage of clonogenic cells of individual colonies which survived the second pulse exposure to adr. Twenty individual colonies were studied. Only those colonies with a ≥ 1% survival after a second exposure to adr are represented in the figure. The *line* indicates the mean percent surviving cells; *C,* cells were initially exposed to adr as described for panel *A,* above, cloned, and the colonies picked and dispersed. Instead of a second pulse exposure, half of the cells were cloned in the continuous presence of adr (0.03 μg/ml). As before, 20 individual colonies were studied but only those whose cloning efficiency was ≥ 1% are represented in the figure. *Bar* indicates mean survival; *D,* sensitivity of P388S cells to growth in the continuous presence of adr. Cells were placed directly into methylcellulose containing 0.03 μg/ml adr. Data are represented as mean *(point)* ± SEM *(bar).* This is the control study for the data presented in *panel C*

demonstrated that the latter was significantly less than the former ($P < 0.01$). Hence, the daughter cells of cells that produced colonies in vitro after the first exposure to adr were more sensitive to adr than the parent P388S cell line, raising the question of residual damage even after the cells have gone through several cell divisions.

2) A 1-h exposure followed by growth in vitro for 10 days and then continuous exposure (Fig. 4, panels C and D). P388S cells were exposed to adr for 1 h and then cloned for 10 days. Twenty colonies were picked from the adr-exposed plates, divided in half as described above, and then plated in methylcellulose in the presence or absence of adr (0.03 μg/ml), those without adr serving as controls. The colonies in the matched plates were counted 1 week later.

Cells from all the control half colonies grew in vitro, producing mean ± SEM and median colony numbers of 1,066 ± 189 and 693, respectively. The mean ± SEM and median values for the 20 half-colonies which were plated in the presence of adr were 56 ± 3 and 3, respectively, with 8 of the half-colonies failing to produce even a single colony. Figure 4, panel D presents the effects of cloning the parent P388S cell line directly in methylcellulose containing adr; this procedure provided an overall control for the data recorded in Fig. 4, panel D. These studies demonstrate once again that the progeny of P388S cells that grew after an initial 1-h exposure to adr were more sensitive to adr than the parent cell line.

3) Serial continuous incubation with drug (Fig. 5). P388S cells were cloned in the presence of adr (0.015 μg/ml) (passage 1). Control cells cloned in adr-free methylcellulose produced 158 ± 17 colonies with a median value of 176, while the cells cloned in the presence of adr produced only 7 ± 1

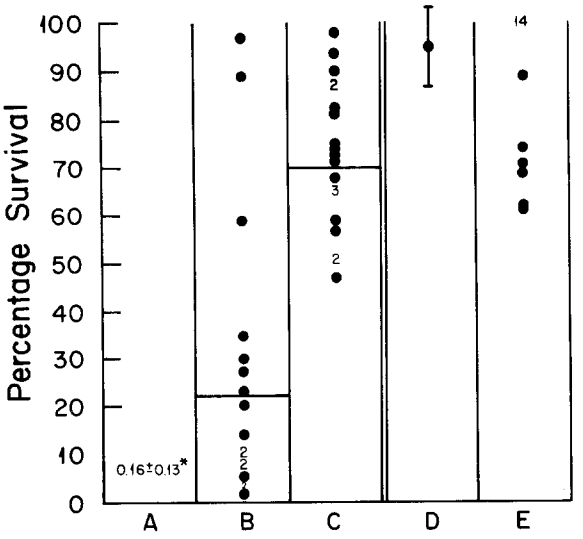


**Fig. 5.** Adr sensitivity of cells which survived continuous exposure to the drug: *A*, P388S cells were cloned in the presence or absence of adr (0.015 μg/ml) and the percentage of surviving colonies determined. Note that these studies were carried out at a different time than the studies described in Fig. 4 and that the P388S cells at this time were more sensitive to adr than were the cells used in the Fig. 4 studies. *Figures* in the panel indicate mean ± SEM cloning efficiency of cells cloned in the presence of adr; *B*, twenty colonies which grew in the continuous presence of adr were individually picked, divided in half; one half was cloned in the absence of adr and the other half in the presence of adr (0.015 μg/ml); the percentage of colony-forming cells that survived was determined. *Bar* indicates mean value. Only the 18 colonies whose cloning efficiency was ≥ 1% are represented. The mean value is 22%; *C*, same experiment as in *panel B*, with 20 colonies picked from *B* cultures of cells grown in the continuous presence of adr (0.015 μg/ml). *Bar* indicates mean value; *D*, percent surviving (± SEM; *bar*) P388R cells cloned in the presence of adr 0.15 μg/ml; *E*, individual colonies were picked from experiment *illustrated in panel D*, divided in half, and cloned in the presence or absence of adr (0.15 μg/ml)

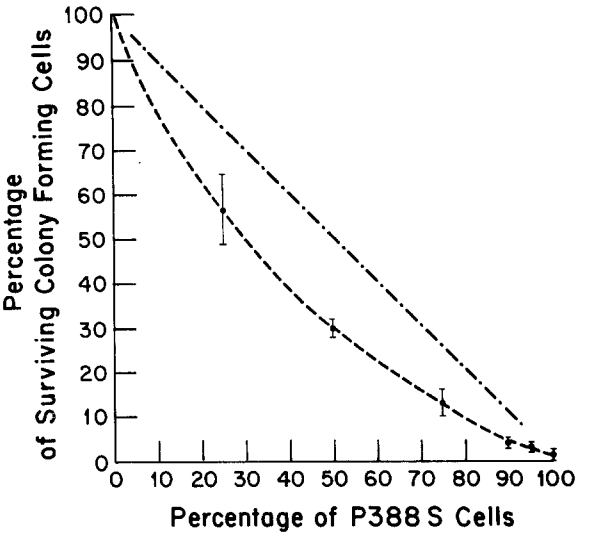


**Fig. 6.** Relationship between the percentage of adr-sensitive cells in a cell population and the proportion of clonogenic cells killed by adr. · · · · · theoretical relationship between the percent adr-sensitive cells present and the percent surviving colony-forming cells; , actual mean ± SEM of percent surviving colony-forming cells after exposure to adr; - - - - -, computer model-derived curve of the relationship between percent adr-sensitive cells present and percent surviving cells (see *Discussion* for details)

colonies, with a median of 5 colonies per plate. The colonies present in the methylcellulose containing adr were picked, divided in half, and cloned in the presence or absence of adr (passage 2). Those cloned in the absence of adr produced 512 ± 51 colonies (median = 515), while the cells cloned in the presence of adr produced 78 ± 21 colonies (median = 75). Colonies were picked once again from adr-containing plates (passage 3), and the process was repeated. The control cells produced 2,497 ± 421 colonies (median = 2,660) while the matched cells cloned in the presence of adr produced 1,865 ± 322 (median = 2,119) clones.

These data demonstrate that serial cloning in the presence of adr resulted in a step-wise increase in adr resistance with the cloning efficiency of adr-exposed cells (compared with the matched control cell values), increasing from 2.8% to 11.9% to 72% ($P < 0.001$ for the comparison of all three values). These data are illustrated in Fig. 5.

*Effects of adriamycin on mixtures of P388S and P388R cells*

P388S and P388R cells were mixed in different proportions, exposed to 1 μg/ml adr for 1 h, washed, and cloned in vitro. Figure 6, which represents the pooled data of three separate experiments, compares the percent reduction in colony number with the actual percentage of adr-sensitive cells in the mixture. At all points the percent reduction in colony number exceeded the actual percentage of P388S cells in the cell mixture. For example, when the percent reduction in colony number was 50%, the actual percentage of P388S cells was only 30%.

## Discussion

The information presented in the first part of this paper demonstrated the significant differences in the adr sensitivity of the two P388 murine leukemia cell lines used in these studies. While the growth of the P388S line in liquid culture was completely inhibited by the presence of adr at a concentration of 0.1 μg/ml, the continuous presence of adr at a concentration of 7 μg/ml inhibited the growth of the P388R line by only 50%. These differences in adr sensitivity were exploited in our studies of the characteristics of clonogenic drug sensitivity assays.

The dependence of adr effects upon both duration of exposure and adr concentration are consistent with flow-cytometric studies which demonstrated that adr uptake was both time- and concentration-dependent, and that the adr resistance of the P388R cells was due to poor uptake of the drug [1, 6]. The studies in which mixtures of drug-sensitive and drug-resistant cells were used demonstrated that for almost every mixture of sensitive and resistant cells the percent reduction in clonogenic cells produced by adr exceeded the percentage of P388S cells in the population. This phenomenon occurred because the cloning efficiency of the P388S cells was almost twice that of the P388R cells. Figure 6 presents the percent survival of clonogenic cells as a function of the percentage of

P388S cells in the mixture and also the best-fitting curve obtained by nonlinear curve fitting. The function fitted to the curve was of the form where $X$ is the percentage of P388S cells in the mixture, $a$ is the proportion of P388S cells surviving the adriamycin, $b$ is the proportion of P388R cells surviving adr, $k$ is the cloning efficiency of P388S cells relative to P388R cells, and $Y$ is the number of clonogenic cells in the mixture surviving as a percentage of the untreated control.

$$Y = \frac{akX + b\,(1-X)}{kX + (1-X)}.$$

The parameters estimated in fitting this curve to the data indicate that the cloning efficiency of the P388S cells in 2.16 times as high as that of the P388R cells, an estimate compatible with the actual cloning efficiencies of these cell lines. In view of these observations, it is clear that unless the cloning efficiency of each subpopulation of a cell population is known beforehand it is impossible to use a clonogenic drug sensitivity assay for an accurate estimate of the proportion of sensitive and resistant cells in any cell population under study. Taken to an extreme, if one or more subpopulations are incapable of cloning under the in vitro conditions being used, the drug sensitivity assay would be useless in that it could not provide an accurate picture of the drug sensitivities of all the neoplastic cells actually present. Some of the discrepancies between in vitro drug sensitivity and in vivo response to chemotherapy may reflect this methodological problem [7].

The studies conducted with P388S cells provide data which further complicate the interpretation of drug sensitivity studies when they are performed with 'unknown' specimens. These studies demonstrate that the cells which survive exposure to adr may be as sensitive to the drug as the cells that are killed. This was true even after continuous exposure to adr studies (passage 1, Fig. 5). Therefore, even the growth of colonies after exposure to or during exposure to a chemotherapeutic agent is not necessarily indicative of the presence of truly drug-resistant clones, since these cells may be killed by a second exposure to the drug. The survival of drug-sensitive cells might occur for a variety of reasons; for example, the cells may not be in a sensitive phase of the cell cycle during drug exposure or there may be stochastic reasons.

In summary, the studies described here demonstrate some of the limitations of clonogenic drug sensitivity assays. Leaving aside such problems as the fact that cells from the majority of patients will not clone in vitro and the question of whether or not the cells that do clone are neoplastic, at present the clonogenic assay is capable at best only of determining whether or not some of the clonogenic cells can be killed by the drug(s) under study.

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