

Schedule-dependent Synergistic Cytotoxicity of Arabinofuranosylcytosine with Adriamycin or 3,6-Bis(5-chloro-2-piperidinyl)-2,5-piperazinedione in Cultured Cells¹

Potu N. Rao, Suneeta Mahagaokar, Emil J Freireich, Ti Li Loo, and Jeffery A. Gottlieb

Department of Developmental Therapeutics, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

SUMMARY

The purpose of this study was to demonstrate that *in vitro* cell culture systems could serve a useful purpose in providing some guidelines in formulating drug schedules for combination cancer chemotherapy in the clinic. Using monolayer cultures of Chinese hamster ovary cells as the test system, we screened the combinations of 1- β -D-arabinofuranosylcytosine (ara-C) + adriamycin, ara-C + 3,6-bis(5-chloro-2-piperidinyl)-2,5-piperazinedione, melphalan + 5-fluorouracil, and melphalan + ara-C as to the effect of drug sequence on the plating efficiency of these cells. Schedule-dependent therapeutic synergism was observed only when ara-C treatment was followed by either adriamycin or 3,6-bis(5-chloro-2-piperidinyl)-2,5-piperazinedione but not *vice versa*. No synergistic effects were observed in the combination of melphalan with 5-fluorouracil or ara-C. The basis for this synergism appeared to be that ara-C was primarily acting as a synchronizing agent to set up the cells in S phase so that subsequent treatment with an S-phase-specific drug, such as adriamycin or 3,6-bis(5-chloro-2-piperidinyl)-2,5-piperazinedione, would produce the maximum cell kill. On the other hand, the lack of synergism in the combinations of melphalan with 5-fluorouracil or ara-C was due to the lack of S-phase specificity for melphalan. On the basis of these data, we postulate that schedule-dependent synergism could be expected if the first agent renders the cells more sensitive (for example, by selectively blocking cells in one of the phases of the cell cycle) to the action of the second drug.

INTRODUCTION

The basic aim of combination cancer chemotherapy in man is to decrease the body's tumor burden hopefully to a greater degree than that produced with single agents used

alone. It is hoped that such therapy will increase not only the degree of antitumor response seen and the proportion of patients responding but also the quality of life of the patient, *i.e.*, the toxicity of the regimen will not offset the therapeutic gains achieved. In formulation of such combinations, several principles are taken into consideration including the use of drugs that possess different mechanisms of actions, different types of toxicity and, sometimes, the ability to enter different parts of the body's cavities. The therapeutic effects of most of these combinations are usually additive or subadditive and, rarely, even antagonistic depending largely upon the mode of action of each drug. However, the full importance of scheduling and of adjusting the sequence of drug administration to patients is just now being realized. From the practical point of view it is difficult, if not impossible, to conduct large-scale trials with patients to screen for all the permutations of a given drug combination. Therefore, it would be ideal to have a model system to screen the various combinations for proper schedule or sequence and to evaluate the therapeutic contribution of each drug in the combination. With this object in mind, we are trying to develop an *in vitro* system where a number of combinations can be screened as to the effect of drug sequence on the survival (plating efficiency) of HeLa or CHO² cells (12). The *in vitro* system, in spite of its differences from the *in vivo* situation, in particular, the absence of (a) degradative or activating enzymes, (b) problems in the transport of drugs to the tumor tissue, and (c) the nonproliferating fraction, could still provide us with some guidelines in selecting proper drug schedules in the clinic.

The purpose of this study was to evaluate some drug combinations that either are being used in the clinic or are of potential therapeutic value with special emphasis placed on the role of drug sequence, in an attempt to improve the effectiveness of treatment, as measured by the plating efficiency or cell survival. The combinations under investigation include: ara-C + ADR, ara-C + BCP, melphalan +

¹This research was supported in part by Grants CA-16480, CA-14528-02, and CA-11520 and by Contract NO 1-CM-61156 from the National Cancer Institute, NIH, USPHS.

Received May 30, 1975; accepted July 23, 1975.

²The abbreviations used are: CHO, Chinese hamster ovary; ara-C, 1- β -D-arabinofuranosylcytosine; ADR, adriamycin; BCP, 3,6-bis(5-chloro-2-piperidinyl)-2,5-piperazinedione; 5-FU, 5-fluorouracil.

5-FU, and melphalan + ara-C. The 1st 2 combinations were selected because of our clinical trials with these agents in acute leukemia, while the latter 2 were being studied in solid tumors, particularly breast cancer. The results of this study revealed schedule-dependent synergism in the combinations of ara-C with ADR or BCP but not in those of melphalan with 5-FU or ara-C.

MATERIALS AND METHODS

Cells. CHO cells-used in this study were grown in Falcon plastic dishes as monolayer cultures in McCoy's Medium 5A supplemented with 16% heat-inactivated fetal calf serum and 1% penicillin (10,000 units/ml):streptomycin (10,000 $\mu\text{g/ml}$). These cells have a cell cycle time of approximately 12 hr and a high plating efficiency (about 95%).

Drugs. ara-C (NSC 63878), ADR (NSC 123127), BCP (NSC 135758), 5-FU (NSC 19893), and melphalan (NSC 8806) were supplied by the Drug Development Branch of the Division of Cancer Treatment, National Cancer Institute, NIH. Stock solutions of these drugs, 10 mg/ml, were freshly prepared just before use and then diluted with culture medium to give the desired concentrations.

Drug Treatment and Determination of Plating Efficiency. The procedures for drug treatment and the determination of plating efficiency were described in an earlier publication (12). CHO cells in exponential growth, which were trypsinized and plated in a number of dishes 1 day before the experiment, were exposed to various concentrations of a single drug or 2 drugs in sequence by replacing the old medium with fresh medium containing the drug. At the end of the treatment, the medium containing the drug was removed and cells were rinsed twice with drug-free medium, trypsinized, and plated for colonies. The duration of treatment was 4 hr with ara-C or 5-FU but 1 hr with ADR or melphalan.

Radioautography. For the purpose of determining labeling index, the control and treated cells were incubated with [^3H]thymidine (1.0 $\mu\text{Ci/ml}$; specific activity, 11.0 Ci/mole) for 30 min. After pulse-labeling, the cells were washed twice with regular medium, trypsinized, and deposited on clean slides by the use of a cytocentrifuge. The centrifugal force generated by the apparatus makes it possible to make a monolayer of cells on the slide in a relatively small area while the fluid is absorbed by the filter pad. These cells were fixed in absolute methanol:glacial acetic acid (3:1) and then extracted 3 times with cold (4 $^\circ$) trichloroacetic acid (5%). Then the slides were dipped in NTB-2 nuclear track emulsion, dried, and stored in light-tight boxes. After 48 hr the slides were developed in D-19 developer and fixed in Kodak fixer. The cells were stained with Giemsa and then scored for labeled and unlabeled cells. The background has a grain count of 3 or 4/cell. Cells with 5 or more grains were scored as labeled and expressed as a percentage of total number of cells scored.

RESULTS

Synergistic Effects in Combinations of ara-C with ADR or BCP. Since the combination of ara-C and ADR is undergo-

ing clinical trials at our institution, we decided to examine *in vitro* the effects of the sequence of drug administration on the therapeutic potential of this combination as measured by the plating efficiency of CHO cells. The drug sequences were: (a) ara-C alone, 0.8 $\mu\text{g/ml}$ for 4 hr; (b) adriamycin alone, at concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.6 $\mu\text{g/ml}$ for 60 min; (c) ADR for 1 hr followed by ara-C for 4 hr; and (d) ara-C for 4 hr followed by ADR for 1 hr. The percentage of cell survival as a function of treatment is shown in Chart 1. From this chart, it is clear that ara-C followed by ADR is more effective in killing cells than in its reciprocal sequence. For example, at a dose of 0.2 μg of ADR per ml, the surviving fractions for the above sequences, namely, b, c, and d, were 0.33, 0.18, and 0.01, respectively.

The Combinations of ara-C and BCP. BCP (structural formula shown in Chart 2) (1) has recently been introduced for clinical investigation in our department (9). It is also being tried in combination with other drugs (J. A. Gottlieb and F. M. Schabel, Jr., personal communication). Preliminary studies with this drug indicated that in CHO cells the duration of S phase was considerably prolonged (an increase of 60 to 100%) but had no effect on the duration of G₁. However, there was a slight increase (15 to 25%) in the duration of G₂ period, which appeared to be dose dependent (13). Since we know from our previous studies (12) that ara-C induced S-phase synchrony in these cells, we wanted to test whether we could induce synergistic effects by changing the sequence of administration of ara-C and BCP. The experimental protocol was the same as that for the combination of ara-C and ADR except that ADR was replaced by BCP. The results of this experiment are shown in Chart 3. Once again, the sequence in which ara-C was

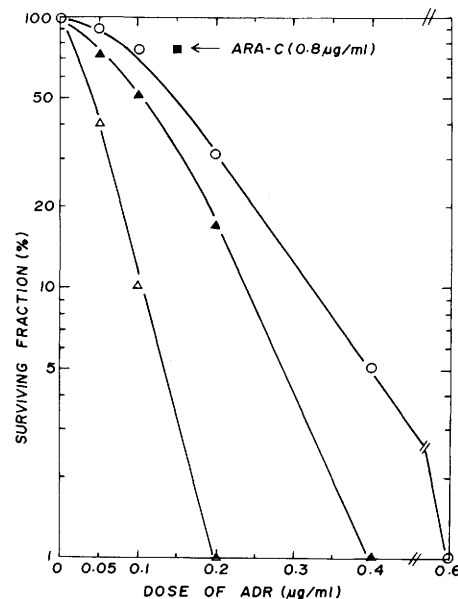


Chart 1. Effect of ara-C and ADR combinations on the plating of CHO cells. CHO cells were treated with ara-C alone for 4 hr at a concentration of 0.8 $\mu\text{g/ml}$ (■), ADR alone for 1 hr, (○), ara-C for 4 hr followed by ADR (Δ), ADR for 1 hr followed by ara-C, 0.8 $\mu\text{g/ml}$, for 4 hr (▲). ara-C was used only at 1 concentration, *i.e.*, 0.8 $\mu\text{g/ml}$, while the dose of ADR was varied.

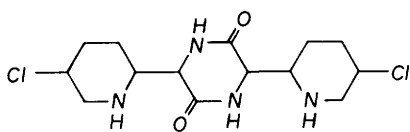


Chart 2. Structural formula of BCP.

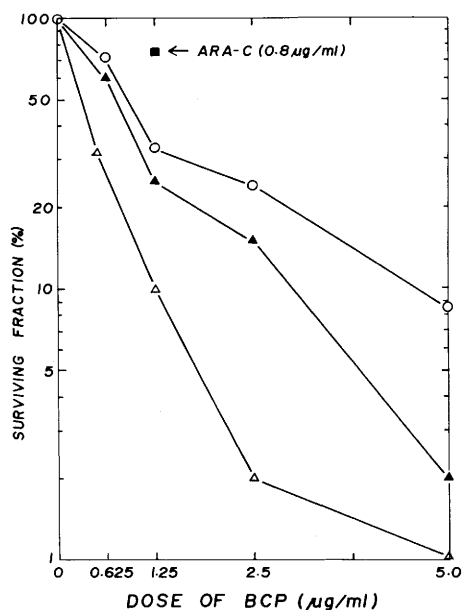


Chart 3. Effects of ara-C and BCP combinations on the plating efficiency of CHO cells. CHO cells were treated with ara-C alone for 4 hr (■), BCP for 1 hr (○), ara-C followed by BCP (△), BCP followed by ara-C (▲). ara-C was used at only 1 concentration, i.e., 0.8 µg/ml, while the concentration of BCP was varied.

followed by BCP gave the best results. In this particular sequence synergistic effects could be observed between the 2 drugs. On the other hand, the drug effects were additive only if the treatment with BCP was followed by ara-C. When 2 or more drugs, administered simultaneously or in sequence, produce a certain effect (loss of plating efficiency in this case) and if that effect is about equal to the sum of the effects of individual drug treatments, then it is considered as additive. However, the effect of the combination therapy is considered synergistic if it is significantly greater than the expected sum of the effects of individual drug treatments. The maximum difference between the 2 sequences was found at a BCP concentration of 2.5 µg/ml.

The Combination of Melphalan with 5-FU or ara-C. 5-FU remains the most effective drug for the treatment of colon and breast cancers. The purpose of this experiment was to find out whether there would be any synergistic effects if 5-FU is tried in combination with melphalan. The experimental protocol is identical to that of ara-C and ADR combination. The various combinations tested are: (a) 5-FU alone, at concentrations of 0, 0.78, 1.56, 3.12, 6.25, 12.5, or 25.0 µg/ml for 4 hr; (b) melphalan alone, at concentrations of 0, 1, 2, 3, 5, 7, or 10 µg/ml for 1 hr; (c) a followed by b; (d) b followed by a. The patterns of survival curves for melphalan and 5-FU are somewhat similar except that melphalan is more effective in reducing cell survival with

increasing dose (Chart 4). When these 2 drugs were applied in sequence, there was an additive effect on the plating efficiency but no synergistic effects were observed in either 5-FU followed by melphalan or *vice versa*.

In order to understand the cause for the lack of synergism between these 2 drugs, it would be necessary to know the effects of 5-FU on the cell cycle kinetics. Since fluorodeoxyuridine inhibits DNA synthesis (14), does 5-FU produce a similar effect that might possibly lead to a partial synchronization of cells at the G₁-S boundary? To answer this question, the following experiment was performed. CHO cells in exponential growth were exposed to various concentrations of 5-FU or ara-C for 4 or 6 hr; then the medium containing the drug was removed, and cells were washed twice with drug-free medium and reincubated for 30 min with medium containing [³H]thymidine (1.0 µCi/ml; specific activity, 11.0 Ci/mmol). After pulse labeling, the cells were washed, trypsinized, and placed on slides with the help of a cytocentrifuge. The slides were fixed and processed for radioautography and scored for the percentage of cells labeled as described under "Materials and Methods." Untreated cells as well as cells treated with ara-C, 0.8 µg/ml, for 4 or 6 hr served as controls. The data on the labeling indices shown in Table 1 indicate the accumulation of cells in the early S phase as a result of the ara-C treatment, whereas 5-FU treatment had no effect. These data suggest that 5-FU does not block DNA synthesis in CHO cells.

To determine whether there is synergism between ara-C and melphalan, various combinations of melphalan and ara-C were tested as before. The results presented in Chart 5 indicate the absence of synergism between these 2 drugs regardless of the sequence of their administration. Although

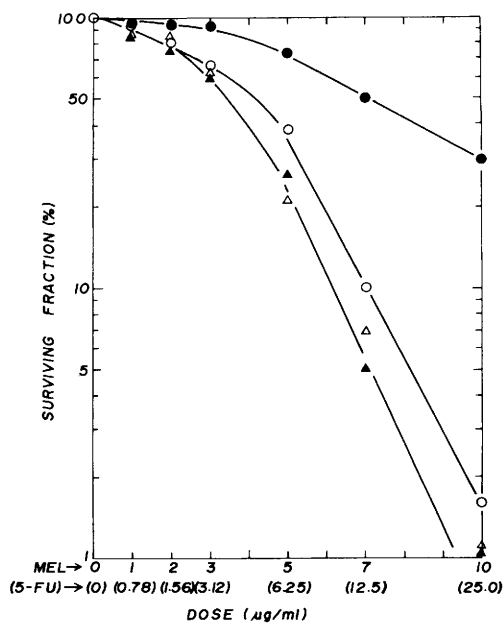


Chart 4. Effects of 5-FU and melphalan (MEL) on the plating efficiency of CHO cells. CHO cells were treated with melphalan alone for 1 hr (○), 5-FU alone for 4 hr (●), 5-FU followed by melphalan (△), and melphalan followed by 5-FU (▲).

Table 1
Effect of 5-FU treatment on labeling index in CHO cells

Dose ($\mu\text{g/ml}$)	Duration of treatment	
	4 hr	6 hr
25.0	63.0 ^a	63.2
12.5	60.9	61.0
6.25	62.2	61.9
3.125	58.6	54.2
1.56	61.2	64.1
0.78	59.8	60.0
Controls		
No drug	56.6	55.5
ara-C, 0.8 $\mu\text{g/ml}$	86.5	93.0

^a Background was less than 4 grains/cell.

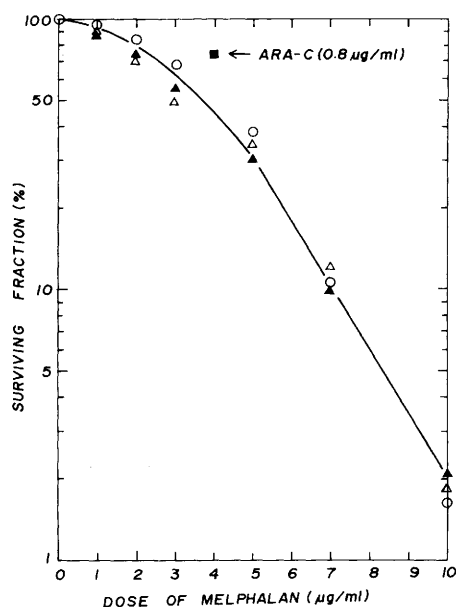


Chart 5. Effect of ara-C and melphalan on the plating efficiency of CHO cells. CHO cells were treated with ara-C alone for 4 hrs (■), melphalan alone for 1 hr (○), ara-C followed by melphalan (△); melphalan followed by ara-C (▲). ara-C was used at only 1 concentration, *i.e.*, 0.8 $\mu\text{g/ml}$, while the dose of melphalan was varied.

ara-C, unlike 5-FU, is producing S-phase synchrony, these cells are no more sensitive to melphalan than are a random population. This suggests that melphalan is not S-phase specific.

DISCUSSION

The data from this study provide examples for both the presence and the absence of schedule-dependent synergism between 2 drug treatments applied in sequence. An examination of the mechanisms of action of these various compounds may be helpful in understanding the basis for the occurrence of such a phenomenon. With regard to the

combinations of ara-C with ADR or BCP, it is obvious from this (Table 1) and other studies (8, 12) that treatment of CHO cells with ara-C, even at the lowest concentration of 0.2 $\mu\text{g/ml}$, induces accumulation of cells in S phase. In this study, the exposure of cells to ara-C, 0.8 $\mu\text{g/ml}$, for 4 hr resulted in a 25% reduction in cloning ability. In other words, we are using ara-C not for its cytotoxic effects but primarily as a synchronizing agent to set up the cells in S phase so that a subsequent treatment with an S-phase-specific drug would produce the maximum cell kill. Both ADR and BCP appear to be S-phase specific in the sense that S-phase cells are more sensitive to these drugs than those in other phases of the cell cycle. ADR has been shown to have such an effect (2, 6, 11, 16). BCP, a relatively new anticancer drug, is being studied for the mechanism of its action (Ref. 13; S. Mahagaokar and P. N. Rao, in preparation) and for its therapeutic value in the clinic (9). Our preliminary studies indicate that exposure of CHO cells to BCP, 5 $\mu\text{g/ml}$, for 1 hr prolongs the duration of S phase. The studies of Brockman *et al.* (4) have shown that BCP can cause inhibition of DNA synthesis and that it also can act as an alkylating agent. On the basis of these observations we may postulate that schedule-dependent synergism could be expected if the 1st agent renders the cells more sensitive (for example, by selectively blocking cells in 1 of the phases of the cell cycle) to the action of the 2nd drug. Utilizing another experimental system, namely, the spleen colony assay for leukemic and normal hematopoietic colony-forming units, Edelstein *et al.* (7) made similar observations for the combination of ara-C and daunorubicin.

In contrast to the above examples, no synergistic effects were observed for the combinations of melphalan with 5-FU or ara-C. The lack of synergism for these combinations was due to the lack of S-phase specificity for melphalan. Alkylating agents, in general, are characterized by toxicity to cells in all of the phases of the cell cycle (3, 5, 15). There was not much difference in cell survival when melphalan treatment was preceded by either 5-FU or ara-C. ara-C induced S-phase synchrony while, to our surprise, 5-FU did not. Later studies revealed that DNA synthesis in these cells was not blocked by 5-FU. Probably, the CHO cell line used in this study is not capable of converting 5-FU to fluorodeoxyribonucleotide, which inhibits the enzyme thymidylate synthetase, which catalyzes the methylation of deoxyuridylate to thymidylate (10). However, synchronization alone, as seen after ara-C treatment, does not include schedule-dependent synergism unless the cytotoxicity of the subsequent treatment is specific to cells in that phase.

These results, in general, would provide some basis in formulating drug schedules in the clinic. The synchronizing effects of the 1st drug treatment on the patients' tumor cells can be effectively monitored by the flow microfluorometry technique to determine the optimal time for the administration of the 2nd drug (17). By the combined application of drug scheduling and flow microfluorometry, it should be possible to improve the therapeutic index of at least some of the drug combinations that exhibit schedule-dependent synergism.

REFERENCES

1. Arison, B. H., and Beck, J. L. The Structure of Compound 593 A, a New Antitumor Agent. *Tetrahedron*, **29**: 2743-2746, 1973.
2. Bhuyan, B. H., and Fraser, T. J. Cytotoxicity of Antitumor Agents in a Synchronous Mammalian Cell System. *Cancer Chemotherapy Rept.*, **58**: 149-155, 1974.
3. Bhuyan, B. H., Scheidt, L. G., and Fraser, T. J. Cell Cycle Phase Specificity of Antitumor Agents. *Cancer Res.*, **32**: 395-407, 1972.
4. Brockman, R. W., Shaddix, S. C., Adamson, D. J., and Struck, R. F. Inhibition of Growth and of DNA Synthesis in Adenocarcinoma 755 Cells in Culture by 3,6-Bis(5-Chloro-2-Piperidinyl)-2-5-Piperazinedione (NSC-135758) and Evidence for Its Alkylating Activity. *Proc. Am. Assoc. Cancer Res.*, **16**: 35, 1975.
5. Brookes, P., and Lawley, P. D. Alkylating Agents. *Brit. Med. Bull.*, **20**: 91-95, 1964.
6. Drewinko, B., and Gottlieb, J. A. Survival Kinetics of Cultured Human Lymphoma Cells Exposed to Adriamycin. *Cancer Res.*, **33**: 1141-1145, 1973.
7. Edelstein, M., Vietti, T., and Valeriote, F. Schedule-dependent Synergism for the Combination of 1- β -D-Arabinofuranosylcytosine and Daunorubicin. *Cancer Res.*, **34**: 293-297, 1974.
8. Ernst, P., Faille, A., and Killman, S. A. Perturbation of Cell Cycle of Human Leukemic Myeloblasts *in vivo* by Cytosine Arabinoside. *Scand. J. Haematol.*, **10**: 209-218, 1973.
9. Gottlieb, J. A., Freireich, L. J., Bodey, G. P., Rodriguez, V., McCredie, K. B., and Gutterman, J. U. Preliminary Clinical Evaluation of Piperazinedione, a New Crystalline Antibiotic. *Proc. Am. Assoc. Cancer Res.*, **16**: 86, 1975.
10. Heidelberger, C. Fluorinated Pyrimidines. *Progr. Nucleic Acid Res. Mol. Biol.*, **4**: 1-50, 1965.
11. Hittelman, W. N., and Rao, P. N. The Nature of Adriamycin-induced Cytotoxicity in Chinese Hamster Cells as Revealed by Premature Chromosome Condensation. *Cancer Res.*, **35**: 3027-3035, 1975.
12. Rao, P. N., Freireich, E. J., Bodey, G. P., Gottlieb, J. A., and Smith, M. L. *In vitro* Evaluation of 1- β -D-Arabinofuranosylcytosine- β -2'-Deoxythioguanosine Combination Chemotherapy. *Cancer Res.*, **34**: 2539-2543, 1974.
13. Rao, P. N., and Mahagaokar, S. The Nature of Piperazinedione-Induced Cytotoxicity in Cultured Cells. *Proc. Am. Assoc. Cancer Res.*, **16**: 74, 1975.
14. Rueckert, R. A., and Mueller, G. C. Studies on Unbalanced Growth in Tissue Culture. 1. Induction and Consequences of Thymidine Deficiency. *Cancer Res.*, **20**: 1584-1591, 1960.
15. Schabel, F. M., Jr., Skipper, H. E., Trader, M. W., and Wilcox, W. S. Experimental Evaluation of Potential Anticancer Agents. XIX. Sensitivity of Nondividing Leukemic Cell Populations to Certain Classes of Drugs *in vivo*. *Cancer Chemotherapy Rept.*, **48**: 17-30, 1965.
16. Tobey, R. A. Effects of Cytosine Arabinoside, Daunomycin, Mitramycin, Azacytidine, Adriamycin, and Camptothecin on Mammalian Cell Cycle Traverse. *Cancer Res.*, **32**: 2720-2725, 1972.
17. Yataganas, X., Strife, A., Perez, A., and Clarkson, B. D. Microfluorimetric Evaluation of Cell Kill Kinetics with 1- β -D-Arabinofuranosylcytosine. *Cancer Res.*, **34**: 2795-2806, 1974.