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Cytotoxic and Cytokinetic Effects of 1-β-D-Arabinofuranosylcytosine, Daunorubicin, and 6-Thioguanine on HeLa Cells in Culture[1]

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ABSTRACT

Exponentially growing HeLa cells were exposed to 1-β-D-arabinofuranosylcytosine, daunorubicin, and 6-thioguanine singly and in combination for 21 hr. Survivals were determined by a colony-counting assay, and cytokinetic effects of the drugs were evaluated by flow cytometry. When used in two-drug combinations (concurrent exposures), 1-β-D-arabinofuranosylcytosine was found to protect cells against both 6-thioguanine- and daunorubicin-induced lethality, the former to a greater extent than the latter. Protection against 6-thioguanine appeared to be related to the degree of inhibition of DNA synthesis by 1-β-D-arabinofuranosylcytosine, but this was not the case with daunorubicin. When cells were exposed to the three drugs simultaneously, survivals were in agreement with those predicted from the various two-drug combinations. This implies that no additional drug interactions occurred when a third drug was added to the two-drug combinations.

INTRODUCTION

In the treatment of acute nonlymphocytic leukemia, it has been reported that use of the 3-drug combination of ara-C,[3] DNR, and TGua can result in higher remission rates than are attainable with any of these drugs used alone (1, 5). In addition, remission can be induced relatively rapidly with the 3-drug combination, thereby reducing the probability of cytopenia-induced mortality (1). Thus far, it is not clear whether the favorable therapeutic response to the 3-drug combination is due to synergistic drug interaction against the tumor cells themselves or simply to greater sensitivity of tumor *versus* normal cells to the individual drugs of the combination.

The objectives of our investigation were to determine in a model system in culture whether the drugs act independently or synergistically; if the latter, we wished to determine whether there is a kinetic basis for this effect. Of course, the responses of the cells to the drugs and drug combinations *in vivo* may be very different from those observed in a continuous cell line in culture. Nevertheless, observations made in culture may provide clues to the mechanisms operating *in vivo* and may be helpful in formulating experiments to determine the relevance of these mechanisms to the *in vivo* drug response.

When using drug combinations, the number of possible combinations and permutations of drug concentrations, exposure times, and schedules is too large to permit the testing of more than a small fraction. Our approach was to initially investigate single drugs, 2-drug combinations, and 3-drug combinations, with cells exposed to the drugs simultaneously for 21 hr. Future work will utilize different durations of exposure and sequential drug treatment.

As will be shown below, synergistic drug effects were not observed under any of the tested conditions. On the contrary, ara-C was found to protect cells from the cytotoxic effects of both TGua and DNR. Protection against TGua was a function of ara-C concentration below 10^{-6} M ara-C; this seemed to be related to the degree of inhibition of DNA synthesis (as inferred by flow cytometry) by the latter drug. On the other hand, protection against DNR cytotoxicity was nearly independent of ara-C concentration.

MATERIALS AND METHODS

Cell Line and Growth Conditions. HeLa S3 cells were obtained from the American Type Culture Collection as CCL 2.2. Stock cells were grown without antibiotics in Ham's F-12 medium supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). They were incubated at 37° in a 5% CO_2 atmosphere and were subcultured twice weekly. All experiments were carried out using cells in the exponential growth phase.

Drugs, Drug Exposures, and Assay of Viability. ara-C (Cytosar) was obtained from The Upjohn Company, Kalamazoo, Mich. It was dissolved and diluted in distilled water prior to adding to the dishes containing the cells. DNR was obtained as daunorubicin hydrochloride from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. It was diluted in sterile 0.9% NaCl solution. TGua was a gift from Burroughs Wellcome Co., Research Triangle Park, N. C. It was dissolved in 0.012 N NaOH and diluted in 0.006 N NaOH before adding to the dishes containing the cells. Prior to drug addition, exponentially growing cells were trypsinized using 0.05% trypsin, washed, and replated in 5.0 ml medium in 60-mm-diameter Petri dishes. One hr later, when cells had reattached, a total of 0.3 ml of drug solution was added. In cases in which not all 3 drugs were added, an equivalent volume of distilled water, 0.9% NaCl solution, and/or 0.006 N NaOH was added. The NaOH added did not alter the pH or the viability of the cell cultures. After incubating the cells for the desired time interval in the presence of drug, plates were rinsed 3 times with fresh prewarmed medium and reincubated. Cell survival was determined by a colony-counting assay. In this assay, most plates were stained with crystal violet 10 days after plating; in some cases, especially those exposed to the higher drug concentrations, additional samples were incubated 13 days before staining. Colonies were counted for determination of survival (relative to controls) using a Biotran II automated colony counter (New Brunswick Scientific Co., Inc.). Most points correspond to the mean values (± S.D.) from 3 or more experiments.

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[3] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; DNR, daunorubicin; TGua, 6-thioguanine; AML, acute myelocytic leukemia.

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Flow Cytometry and Data Analysis. Propidium iodide (Calbiochem-Behring Corp., San Diego, Calif.) was prepared at a concentration of 0.05 mg/ml in 0.1% sodium citrate containing 0.1% Triton X-100. Cells were prepared for flow cytometry using our previously described method for staining monolayer cells without using trypsin (4).

Lymphocytes from patients with chronic lymphocytic leukemia were mixed with each sample to serve as an internal reference standard. When appropriate, positions of histograms were shifted prior to data analysis in order to correct for drift or instrumental fluctuation among samples. Fluorescence distributions of these lymphocytes were omitted from the figures in order to simplify the illustrations.

Cell fluorescence was measured on a Model 4802 Cytofluorograf (Ortho Instruments, Inc., Westwood, Mass.) using an argon ion laser at 488 nm. The photomultiplier tube voltage was stabilized by a separate d.c. power supply (Hewlett-Packard, Model 6515A). Data were collected on a Northern Model NS-602 multichannel pulse height analyzer (Northern Scientific, Inc., Middletown, Wis.) and transmitted to a PDP 11/70 via a telephone line for data storage, retrieval, and analysis. Analysis of data into phases of the cell cycle was by our recently modified method (3).

RESULTS

Single-Drug Exposure

ara-C. Chart 1 (*top*) shows the survival, relative to controls, of cells exposed to ara-C for 21 hr. Survival remained above 60% up to 10^{-3} M ara-C, but a relative minimum occurred at 10^{-5} M. Although there was some variability in survival between experiments as indicated by the *error bars*, the minimum occurred at this concentration in every experiment.

Chart 2 (*left*) shows the effect of ara-C concentration on cell progression after 21 hr of exposure. Even at the lowest concentration, 10^{-8} M, ara-C inhibited cell progression through S

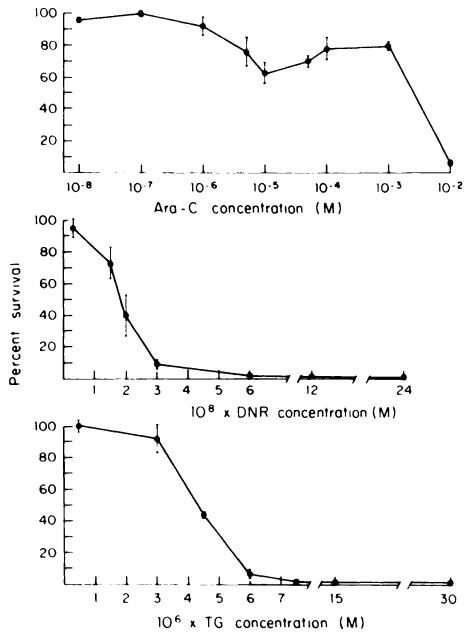

Chart 1. Survivals, relative to untreated controls, of cells exposed to ara-C (*top*), DNR (*center*), or TGua (*TG*) (*bottom*) for 21 hr. *Bars*, S.D.

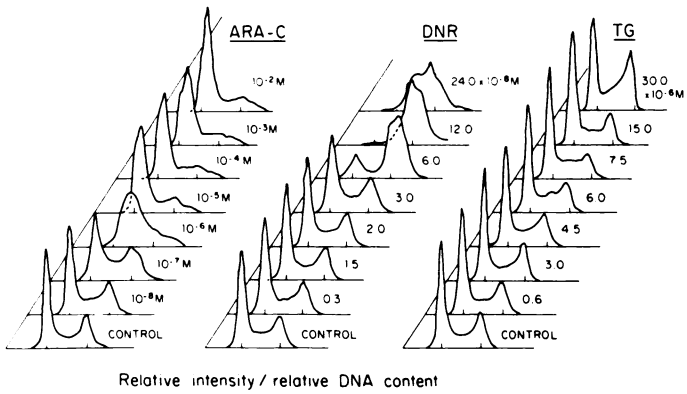

Chart 2. DNA histograms of cells exposed to ara-C (*left*), DNR (*center*), or TGua (*TG*) (*right*) for 21 hr at the concentrations indicated. These histograms were obtained in a single experiment and are representative to those obtained in additional experiments. *Tick marks* at the base of each histogram show G_1 and G_2-M peak locations of the control as an aid to determining whether cells have become arrested in early S phase or at other points in the cell cycle. Lymphocytes from patients with chronic lymphocytic leukemia were included in every sample as an internal reference standard, but these are not shown in the chart. Percentages of cells in G_1, S, and G_2-M phases are listed in Table 1.

Table 1

Flow cytometrically derived percentages of cells in G_1, S, and G_2-M phases of the cell cycle after exposure to ara-C, DNR, or TGua for 21 hr

Data were from the same experiment as in Chart 2.

	% of cells in following cell cycle phase		
	G_1	S	G_2-M
ara-C (M)			
0	48	31	21
10^{-8}	38	43	19
10^{-7}	13	64	23
10^{-6}	2	87	11
10^{-5}	22	70	8
10^{-4}	17	74	9
10^{-3}	16	75	9
10^{-2}	16	75	9
DNR (× 10^8 M)			
0	49	31	20
0.3	48	32	20
1.5	47	31	22
2.0	47	31	22
3.0	37	39	24
6.0	13	23	64
12.0	2	31	67
24.0	1	68	31
TGua (× 10^6 M)			
0	48	32	20
0.6	49	31	20
3.0	47	32	21
4.5	46	36	18
6.0	49	36	15
7.5	48	38	14
15.0	51	32	17
30.0	35	37	28

phase slightly. This is also seen in Table 1; 43% of the cells were in S phase, compared with 31% for the control. At 10^{-7} M, many cells were piling up in early S phase (64% in S), and almost all had accumulated in early to mid-S phase at 10^{-6} M. At 10^{-5} M, most cells were slowed or arrested near the G_1-S border, and many of those originally in G_2 had presumably divided and reentered G_1. Little further change in the cell cycle phase distribution was noted for ara-C concentrations above 10^{-5} M; and, presumably, cell cycle progression into and through S phase was very slow at those levels.

DNR. The relationship of survival to DNR concentration for cells exposed to this drug for 21 hr is shown in Chart 1 (*center*).

Although the survival decreased almost linearly with increasing concentration between 0.3 and 3 × 10^{-8} M, a small fraction (1%) of cells survived even at concentrations as high as 24 × 10^{-8} M.

Flow cytometry (Chart 2; Table 1) shows that cells exposed to as much as 2 × 10^{-8} M DNR did not differ significantly in cell cycle phase distribution from the control, and even at 3 × 10^{-8} M, the difference was not large. After 21 hr at 6 × 10^{-8} M, however, most of the cells were in G_2-M or late S phase, and at the highest concentration (24 × 10^{-8} M), more had accumulated in S phase than in G_2-M. These results indicate that the decrease in survival between 0.3 and 3.0 × 10^{-8} M cannot be attributed to drug-induced changes in the distribution of cells through the cycle.

TGua. The survival of cells as a function of TGua concentration decreased steeply with increasing drug concentration between 3 and 6 × 10^{-6} M (Chart 1, *bottom*). As in the case of DNR, a small percentage of cells was resistant to the highest concentration used in this study (30 × 10^{-6} M).

Flow cytometric analysis (Chart 2; Table 1) showed that the percentage of cells in S phase tended to increase at the expense of G_2-M as the TGua concentration increased. At 30 × 10^{-6} M, cells had accumulated in late S phase and G_2-M. The only TGua concentrations which severely perturbed cell progression during the first day of exposure were those lethal to virtually the entire population.

Drug Combinations

ara-C plus DNR. Chart 3 shows the survival of cells exposed for 21 hr to DNR (1.5 or 2.0 × 10^{-8} M) and ara-C as a function of ara-C concentration. Also shown are survivals after exposure to ara-C or DNR individually. It is seen that ara-C provided significant protection against the lethal effects of DNR; in the terminology of Valeriote and Lin (13), there was "interference" between the cytotoxic effects of the 2 drugs. Except at 10^{-5} M (and in one case, at 10^{-4} M), the survival of cells exposed to the combination was greater than that of cells exposed to DNR alone. Because survival of cells exposed to the combination was in all cases lower than when exposed to ara-C alone, it is improbable that DNR provided any protection against ara-C.

DNA histograms of cells exposed to DNR in combination with ara-C at 10^{-8} and 10^{-6} M are shown in Chart 4 (*middle row*).

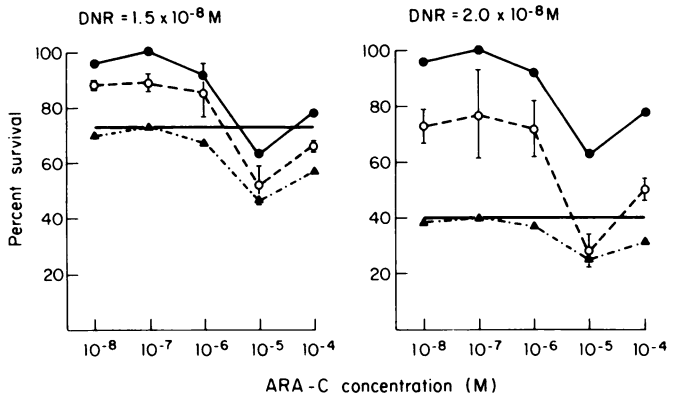

Chart 3. Survivals of cells exposed to ara-C and DNR in combination for 21 hr, as a function of ara-C concentration. Survivals of cells exposed to ara-C alone and DNR alone are also shown for comparison. ●, ara-C alone; ——, DNR alone; ○, ara-C plus DNR (observed); ▲, ara-C plus DNR (expected, assuming independent drug toxicities). *Bars*, S.D.

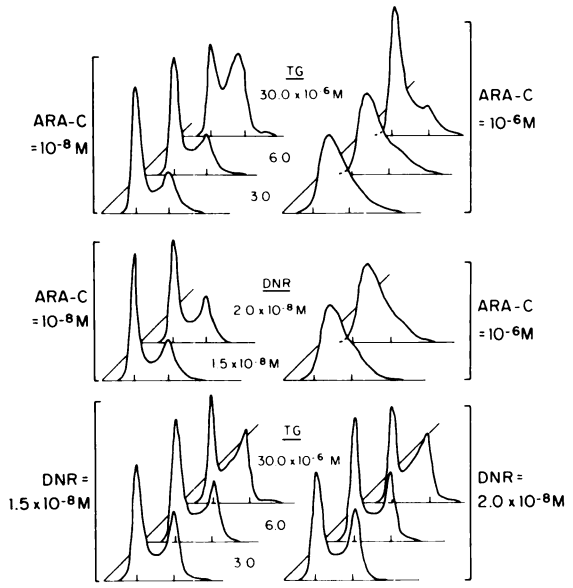

Chart 4. DNA histograms of cells exposed to various 2-drug combinations for 21 hr. These represent typical results and are similar to those obtained in repetitions of the experiment. As in Chart 2, chronic lymphocytic leukemia lymphocytes were used as reference standards, but they are not included in the figure.

DNA distributions of cells exposed to 1.5 or 2.0 × 10^{-8} M DNR, at the same ara-C concentration, were virtually identical, although the survivals (Chart 3) were different. On the other hand, DNA histograms corresponding to 10^{-8} or 10^{-6} M ara-C differed considerably, although the survivals, at the same DNR concentration, were the same. These results imply that changes in the DNA distribution do not correlate with changes in survival for this drug combination. They do not exclude the possibilities, however, that a very modest decrease in DNA synthesis rate (induced even at 10^{-8} M ara-C, as inferred from flow cytometry) was sufficient to partially protect cells against DNR lethality and that a greater degree of inhibition (at the same DNR concentration) provided no additional protection.

ara-C plus TGua. Chart 5 demonstrates the effect of ara-C on TGua-induced cytotoxicity. At the lowest TGua concentration, 3 × 10^{-6} M, at which TGua alone was only slightly toxic, the survival of cells exposed to the combination was additive. Note that TGua did not protect the cells from toxicity due to ara-C. This is consistent with the results at higher TGua concentrations, where the survival of cells treated with the combination was always less than or equal to that of cells exposed to ara-C alone.

At the 2 higher TGua concentrations shown in Chart 5, ara-C largely reversed the cytotoxic effects of TGua, except at the lowest ara-C concentrations. ara-C was therefore relatively more effective against TGua-induced toxicity than against that due to DNR.

Cytokinetic effects of the drugs are shown in Chart 4 (*top row*). At 10^{-8} M ara-C and at TGua concentrations of 3.0 or 6.0 × 10^{-6} M, the DNA histograms were similar both to each other and to the controls (Chart 2). At 30 × 10^{-6} M TGua, however, the histogram was nearly identical to that of TGua alone, as was the survival (Chart 5). At 10^{-6} M ara-C, combined with TGua at 3.0 or 6.0 × 10^{-6} M, both the DNA histograms and the survivals were similar to those of ara-C alone (Charts 2 and 5). Only at 10^{-6} M ara-C plus 30.0 × 10^{-6} M TGua was

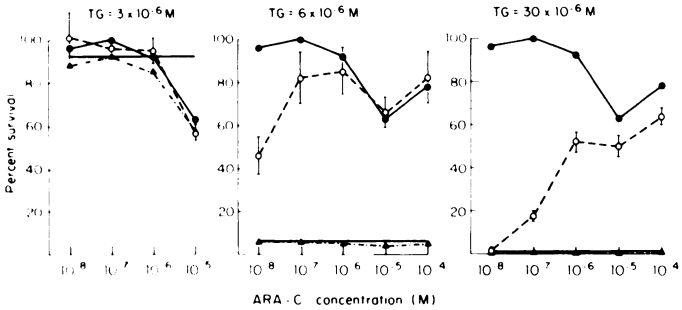

Chart 5. Survival of cells exposed to ara-C and TGua (*TG*) in combination for 21 hr, as a function of ara-C concentration. Also shown are survivals of cells exposed to ara-C or TGua alone. ●, ara-C alone; ——, TGua alone; ○, ara-C plus TGua (observed); ▲, ara-C plus TGua (expected, assuming independent drug toxicities). *Bars*, S.D.

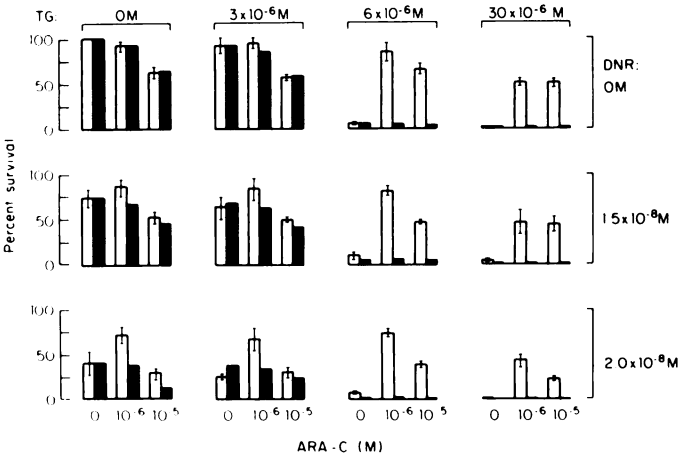

Chart 6. Survival of cells exposed to the 3-drug combination of ara-C, DNR, and TGua (*TG*) at selected concentrations (□). Also shown are survivals predicted from those for the individual drugs, assuming that the drugs act independently (▨). *Bars*, S.D.

the histogram significantly different from that of ara-C alone (it was also different from that of TGua alone). In this case, ara-C provided only partial protection from the toxic effect of TGua.

DNR plus TGua. As can be determined from Chart 6, DNR and TGua in combination appeared to act independently with respect to survival. Cytokinetic effects of the individual drugs and of the combination were small except at 30 × 10^{-6} M TGua; at this concentration, the DNA histograms of cells exposed to the combination were nearly the same as for TGua alone (Charts 2 and 4).

ara-C plus DNR plus TGua. We have shown that ara-C protects HeLa cells from the lethal effects of both DNR and TGua, the latter to a much greater extent than the former. An important additional question is whether the survivals observed when cells were exposed to the 3 drugs simultaneously were consistent with results from the 2-drug combinations; *i.e.*, were the protective effects of ara-C enhanced, reduced, or unchanged when a third drug was added?

Chart 6 shows the survivals of cells exposed to ara-C at 10^{-6} and 10^{-5} M; to DNR at 1.5 and 2.0 × 10^{-8} M; and to TGua at 3, 6, and 30 × 10^{-6} M for 21 hr in all combinations. Also shown are the values predicted from the single-drug survivals, under the assumption that each drug acted independently.

It is clear from this chart that drug interference occurred both when cells were exposed to the drugs in pairs and in 3-drug combinations. This phenomenon occurred at both ara-C concentrations but not with DNR-TGua pairs. As in the case of the 2-drug combinations, the protective effect of ara-C was more pronounced against TGua than against DNR.

To determine whether the survivals of cells exposed to the 3-drug combinations could be predicted from those of the 2-drug pairs, we utilized the latter data to predict the survivals when the 3 drugs were combined. In these calculations, the following expression was used.

$$S(A, D, T) = S(A, D) \times S(A, T) \div S(A)$$

where $S(A, D, T)$ is the predicted survival of cells exposed to the 3-drug combination of ara-C, DNR, and TGua; $S(A, D)$ is the observed survival of cells exposed to ara-C and DNR; $S(A, T)$ is the observed survival of cells exposed to ara-C and TGua; and $S(A)$ is the survival of cells exposed to ara-C alone. In deriving the above expression, we have assumed that DNR and TGua have independent cytotoxic effects as implied by the data.

Chart 7 shows the observed survivals of cells exposed to the 3 drugs in combination together with the values calculated using the above formula. It is evident from the chart that no additional drug interactions occurred when the 3 drugs were combined beyond those observed with the 2-drug pairs.

DISCUSSION

When ara-C, DNR, and TGua are administered as 2- or 3-drug combinations for induction therapy of acute nonlymphocytic leukemia, remission rates may be significantly higher than with any of the drugs used alone. Complete remission rates of about 55% can be obtained using ara-C and TGua (6), while rates in the mid-60% range (up to 80% for younger patients) have been reported for ara-C plus DNR (11, 18). When all 3 drugs were combined, complete remission rates of 80% (independent of age) were reported by Gale and Cline (5). An additional advantage of the 3-drug combination is that earlier remissions can often be achieved, reducing the duration of pancytopenia (1).

In apparent contrast to these clinical results, we have shown that simultaneous exposure of HeLa cells to the 3 drugs resulted in decreased rather than synergistic cytotoxicity. Over a wide range of concentrations which were themselves only slightly toxic, ara-C in most cases nearly reversed the lethal effects of TGua and protected cells to a significant extent from DNR-related cytotoxicity. For the 2-drug combination of ara-C

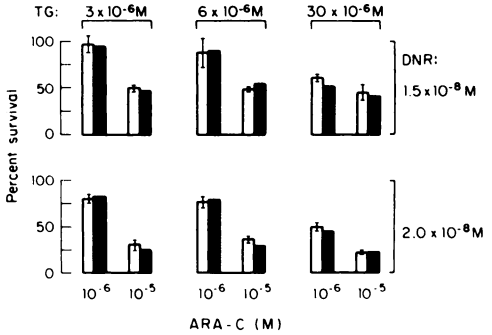

Chart 7. Survivals of cells exposed to the same 3-drug combinations as in Chart 6 (□). In this chart, the predicted survivals (■) are those calculated from the results of various 2-drug combinations. The objective in this case was to determine whether any additional drug interactions occurred (enhancement or interference) beyond those observed for the 2-drug pairs (see text). *Bars*, S.D.

and TGua, these observations agree at least qualitatively with those reported by others.

Using the mouse spleen colony technique, Valeriote *et al.* (14) found that, when ara-C and TGua were administered simultaneously, survivals of both leukemic and normal colony-forming units were greater than those expected if the drugs had acted independently. They interpreted their results as being due principally to both drugs being active against the same target (S phase) cells. Only at very high ara-C concentrations (5% survival with ara-C alone) were the lethal effects of TGua effectively reversed. The authors attributed this reversal to the effect of ara-C in inhibiting the incorporation of TGua into DNA as proposed by LePage and Kaneko (9).

LePage (8) pointed out that, while TGua is incorporated into both RNA and DNA and also inhibits purine synthesis, only its incorporation into DNA correlates with cytotoxicity. Use of a second agent to inhibit DNA synthesis, *e.g.*, cortisone or ara-C, reduced the incorporation of TGua into DNA and hence reduced its cytotoxicity (9). Presumably, then, TGua can be classified as an S-phase-specific drug at least with respect to lethality. LePage and White (10) suggested that the favorable clinical results obtained in the treatment of AML with ara-C and TGua may be due to a high cell kill by ara-C combined with the lethal incorporation of TGua into the DNA of ara-C-resistant tumor cells.

While this explanation appears plausible, we have found with the aid of flow cytometry that, in untreated AML, generally 90% or more of the leukemic blasts are in G_1 (or G_0). During induction of remission with the 3-drug combination, the relatively small percentage of cells in S phase invariably disappears, and few, if any, G_1 cells appear to be recruited to proliferate at least during the first week or 2 of induction therapy (1). These clinical results suggest that many of the G_0-G_1 tumor cells may in fact be lethally affected by the 3-drug combination.

Of the 3 drugs in this combination, DNR is probably the one to inflict lethal damage to non-S-phase cells. If the effectiveness of the combination on remission induction in AML was due principally to the cytotoxic effect of DNR on the G_0-G_1 cells, then DNR alone should be nearly equally effective.

In fact, it has been reported that DNR alone can achieve 40 to 50% complete remissions when an optimal dose and schedule are used (15, 16). The improved response obtained when ara-C or ara-C plus TGua are added to the regimen may then be due primarily to their effects on proliferating cells. ara-C, while exerting its own cytotoxic effect, may also provide partial protection from TGua and DNR but to a lesser extent for tumor than normal bone marrow cells. Of course, other factors undoubtedly play important roles in the responses of the cells to the 3 drugs. In particular, the various clinical protocols using these agents differ from the simultaneous exposure used in our experiments. It is probable that the sequence and treatment durations of the individual drugs of the combination are important factors in determining the relative responses of the tumor and normal cells to treatment. Although we considered only simultaneous drug exposures in this paper, these experiments can serve as a basis for further work exploring the effects of sequential exposure to these agents.

The colony-forming method we have used in these studies involves the trypsinization and replating of cells 1 hr prior to drug exposure. We used this procedure rather than postexposure trypsinization in order to reduce the probability of artifactual results due to: (*a*) potentially lethal or sublethal damage being converted to lethal damage by the trypsinization-subculturing procedure (12); and (*b*) the possibility that damaged but potentially clonogenic cells would be rendered unable to reattach after trypsinization. If, on the other hand, the cells were allowed to recover for 1 to 2 days prior to trypsinization, then cells sustaining the least damage would have divided one or more times, thereby increasing the apparent survival.

Barranco *et al.* (2) found, using Chinese hamster ovary cells, that trypsinization prior to ara-C exposure did not affect cell survival, although survival was altered for some other drugs, including Adriamycin. However, these investigators used drug concentrations that were lethal to the cells within the 1-hr exposure period, and, in contrast to our procedure, they also trypsinized the cells again immediately after drug exposure. In our studies, 2-hr exposures to the drugs at concentrations used in the 2- and 3-drug exposures were not toxic (not shown), and it appears improbable that the trypsinization procedure significantly affected survival.

The relative minimum that we observed in the survival curve of cells exposed to ara-C (Chart 1, *top*) may have a kinetic basis. A possible explanation of this phenomenon may be that, as the ara-C concentration increases above 10^{-5} M, those mechanisms tending to increase lethality are counterbalanced by the self-limiting effect of the increased inhibition of progression of cells into and through S phase. As seen from Chart 1 and Table 1, the percentage of cells surviving ara-C at concentrations between 0.5×10^{-5} M and 10^{-3} M is approximately equal to the percentage (69%) initially in G_1 plus G_2. However, the precise changes in survival within this concentration range cannot be determined simply from the flow cytometric data but probably reflect the competing factors of ara-C concentration, rate of DNA synthesis, length of time cells were in S phase during exposure, and their positions within S phase. At 10^{-2} M ara-C, survival decreased to 6%, suggesting that even cells not in S phase were lethally affected. An alternative possibility is that most of the cells in G_1 and G_2 at the end of the exposure period may have been in S phase during at least a portion of that period and had been lethally affected at that time.

It is evident from Chart 2 (*left*) and Table 1 that the movement of cells through the cycle becomes progressively inhibited with increasing ara-C concentration, at least up to 10^{-5} M. At higher ara-C concentrations, our flow cytometric data are not sufficiently sensitive to detect increased inhibition if it occurs. The most striking alteration of the DNA distribution occurred at 10^{-6} M ara-C. At this concentration, progression of cells through S phase was slowed sufficiently so that most cells initially in G_1 and G_2 had entered S phase before those initially in the latter phase had entered G_2. At lower concentrations, inhibition of DNA synthesis was apparently too slight to greatly affect the DNA distribution, while at higher concentrations, inhibition was presumably so great that cells initially in G_1 could move only into early S phase during the 21-hr exposure period; most cells initially in S phase could probably proceed only a short distance through this phase. While most of the cells initially in G_2 and M phases had probably reentered G_1 during this period, the small percentage of cells in G_2-M at the end of the exposure (at 10^{-5} M and above) probably represents cells which were initially in late S phase and which entered G_2 during the subsequent 21 hr but which were unable to divide. This

interpretation is consistent with observations we have made using Chinese hamster ovary cells.[4]

In the studies reported here, we found that ara-C need not arrest DNA synthesis totally in order to protect HeLa cells against TGua or DNR toxicity. However, concentrations of ara-C which inhibited DNA synthesis only slightly provided little or no protection against the most toxic TGua level. Thus, at 30 $\times 10^{-6}$ M TGua, ara-C concentrations of 10^{-6} M or higher were required to provide substantial protection (Chart 5). This was the lowest ara-C concentration which greatly perturbed the DNA synthesis and cell progression rates (Chart 2). Possibly, such inhibition enabled the endogenous dGTP pool to enlarge to the point where it could compete effectively with the TGua deoxynucleotides for incorporation into DNA.

Very high TGua concentrations (*e.g.*, 30 $\times 10^{-6}$ M) caused the cells to accumulate in G_2 and late S phase and, to a lesser extent, in G_1. To reconcile the non-S-phase accumulation with the evidence that TGua is effective primarily against cells progressing through S phase and with the fact that the observed survival at 30 $\times 10^{-6}$ M TGua was about 1%, we surmise that cells arrested in G_2 had been exposed to a lethal concentration of TGua while in late S phase and that some of the cells apparently in G_1 had actually been arrested in very early S phase after incorporating a lethal amount of TGua into their DNA. An alternative explanation could be that, at this high TGua concentration, lethality occurs in non-S-phase cells because of the effect of the drug on protein synthesis or due to its incorporation into RNA. On the other hand, if this were the principal mechanism of lethality, it would appear improbable that ara-C could reverse it.

Investigations into the cell cycle specificities of DNR by other workers have yielded somewhat conflicting results. Kim *et al.* (7) found that HeLa cells were much more sensitive to DNR in S phase than in G_1 and G_2. On the other hand, Wilkoff *et al.* (17) concluded that, in the L1210 system, DNR is not cell cycle phase specific, although the drug is more effective against proliferating than nonproliferating cells. In view of the mechanism of binding of DNR to the cells (intercalation into the DNA double helix), it would appear improbable that its lethal action would be restricted solely to cells in S phase. Nevertheless, it is possible that the relatively greater sensitivity of S-phase cells to DNR in some systems is due either to a greater uptake of drug into the cells in this phase or to the increased accessibility to DNA by DNR for cells in S phase, resulting in a larger number of bound molecules.

When the 3 drugs were used in combination, survivals were consistent with those observed using the 2-drug combinations. This implies that, when attempting to predict the effects of other concentrations of the 3 drugs or when examining in more detail the mechanisms responsible for the observed protective effects of ara-C, we need consider only the interactions between the 2-drug combinations. Of course, we have only shown this to be the case for simultaneous exposure in one cell line. Additional work will be required to determine whether this result holds true for other cell types, different drug combinations, sequential exposures, or periods longer than 21 hr. The use of synchronous cell populations would help to clarify some of the uncertainties regarding cell cycle phase specifities of drug interaction.

[4] J. Fried, A. G. Perez, J. M. Doblin, and B. D. Clarkson, manuscript submitted for publication.

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