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LETHAL AND SUBLETHAL EFFECTS OF THE COMBINATION OF DOXORUBICIN AND THE BISDIOXOPIPERAZINE, (+)-1,2,-BIS (3-5-DIOXOPIPERAZINYL-1-YL) PROPANE (ICRF 187), ON MURINE SARCOMA S180 *IN VITRO**

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Abstract—Doxorubicin and the bisdioxopiperazine, ICRF 187, synergistically inhibit proliferation of murine sarcoma S180 cells *in vitro*. Cell cycle analysis was employed to help discriminate cytokinetic from lethal effects of the drug combination. Twenty-four-hour incubation with either agent produced dose-dependent partial G_2M arrest. At high doses, ICRF 187 produced partial G_2M arrest, inhibition of cell division, and continued DNA synthesis at a higher ploidy, resulting in a second G_2M arrest of an 8n population. The addition of ICRF 187 to doxorubicin resulted in enhancement of cell cycle blockade at G_2M. The combination also produced enhanced lethality as measured by reduced colony-forming efficiency of drug-treated S180 cells. Measurement of [^{14}C]doxorubicin accumulation in, and effux from, ICRF 187 pretreated cells failed to reveal an effect of pretreatment with the bis-dioxopiperazine on anthracycline disposition by S180 cells, suggesting that the enhanced cytotoxic and cytostatic effects do not result from increased intracellular concentrations of doxorubicin. The positive interaction between the two drugs may represent site-specific enhancement of the anthracycline effect by ICRF 187 at an intracellular target site.

The bisdioxopiperazine ICRF 187¶ is a potent intracellular chelating agent which protects mammalian cardiac tissue against the toxic effects of doxorubicin [1–6]. In experimental tumor cell systems, however, ICRF 187 and its racemate, ICRF 159, not only fail to reverse the antineoplastic effects of doxorubicin but actually enhance these effects in a dose- and schedule-dependent fashion [7–8]. Employing the murine sarcoma S180 cell line, we have demonstrated previously that the addition of ICRF 187 to doxorubicin synergistically inhibits the ability of tumor cells to proliferate in liquid medium [9].

Inhibition of tumor cell proliferation may result from either cumulative cell death secondary to lethal drug effects, or generalized slowing of cell cycle traverse secondary to sublethal drug-induced cell damage, or both [10]. Discriminating between the cytotoxic and cytokinetic effects of antineoplastic agents allows a more accurate assessment of the mechanism of action of a single agent and of the mechanisms of interaction between drugs employed in combination, and thus contributes to a more rational design for future clinical protocols [11].

To evaluate the effects of the combination of doxorubicin and ICRF 187 on S180 cell cycle traverse, we have now employed flow microfluorometry to analyze cell DNA content following 24-hr exposure to drug. Unlike measurements of [^{3}H]thymidine uptake and mitotic index, measurement of DNA content offers a more accurate assessment of drug effect in arrested or partially arrested populations [12]. To determine whether the antiproliferative effects of the drug combination represent primary lethal events or sublethal cytostatic effects, the results of flow cytometry are compared with colony-forming efficiency.

METHODS

Cell culture. The S180 cell line, originally isolated from the murine CFW strain in 1955 [13], was obtained from Dr. Thomas Tritton, Yale University School of Medicine, New Haven, CT.

Cells were grown in RPMI 1640 medium with glutamine (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated (HI) horse serum (GIBCO Laboratories) in 25 cm^2 polystyrene tissue culture flasks (Becton Dickinson, Oxnard, CA). No antibiotics were added to the culture medium. Cells were cultured in the dark at 37° in a humidified atmosphere with 5% CO_2 and 95% air by the method of Fisher and Sartorelli [14]. The doubling time for this cell line is 16.2 hr.

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¶ Abbreviations: ICRF 187, (+)-1,2-bis (3,5-dioxopiperazinyl-l-yl) propane; and PBS, Dulbecco's phosphate-buffered saline.

Drugs. Doxorubicin (NSC 123127, Farmitalia, Carlo Erba, Milan, Italy) was diluted with sterile 0.15 M NaCl and protected from light. ICRF 187 (NSC 169780) was constituted from the lyophilized powder in sterile, preservative free saline. Stock solutions were stored at −85°. [^{14}C]Doxorubicin ([14-^{14}C]doxorubicin HCl; 13.4 μCi/mg) was obtained from SRI International (Menlo Park, CA). The radiopurity was greater than 95% as determined by thin-layer chromatographic assay.

Flow cytometry. S180 cells in early exponential growth were incubated with either doxorubicin or ICRF 187 or the combination at doses to be specified for 24 hr in RPMI 1640 medium with 10% HI-horse serum. Cells were washed twice in RPMI 1640 medium and stained with propidium iodide (PI) by the method of Krishan [15]. In brief, PI (Calbiochem, San Diego, CA) was made up at 0.05 mg/ml in a 0.1% (w/v) solution of sodium citrate with 0.5% (v/v) Triton-X-100 detergent (Amersham, Arlington Heights, IL). Cell pellets were resuspended in the PI:hypotonic citrate solution at 10^6 cells/ml solution and then allowed to sit at room temperature in the dark for 20 min. After vigorous pipetting to rupture cell membranes and remove cytoplasm and RNA, the cells were centrifuged at 200 *g* for 5 min. The supernatant fraction was removed, and the pellet was resuspended in 100 μl of RNA:se (Worthington Biochemical Corp., Freehold, NJ), 1 mg/ml in magnesium, calcium-free Hanks' Balanced Salt Solution (HBSS) (GIBCO) at room temperature for 30 min to digest remaining RNA. Following RNA digestion, 2 ml of the PI solution was added, the nuclei were dispersed, and the samples were placed on ice in preparation for cell cycle analysis.

DNA histograms were generated by an Ortho Diagnostics (Westwood, MA) 50H Cytofluorograf which employs a tuneable 5W argon ion laser. Excitation and emission wavelengths were 488 and 590 nm respectively. For each sample a total of 100,000 nuclei were examined and plotted as red fluorescence versus forward scatter and DNA fluorescence versus cell number. Coefficients of variation in control populations ranged from 7 to 9%. Chick red blood cells were added as an internal standard for early runs but were eliminated after less than a 2-channel shift was noted over a 600-channel analysis.

Data analysis. DNA histograms were analyzed according to the method of Fried. Dr. Fried's program, adapted to the Data General 2151 by Dr. Reinhold Mann of Oak Ridge National Laboratories, Oak Ridge, TN, was made available to us by Mr. Leo Burke, Ortho Diagnostics. A full description of the methodology and computer program is provided in Refs. 16–18.

Each histogram was analyzed over 450–750 channels. The Fried program was employed with the followed constraints:

(1) The $G_{1/0}$ mean in each perturbed population was within 1% (4–7 channels) of the $G_{1/0}$ mean in the control population.

(2) The S-phase configuration of the control population was broad and flat with the left side equal to or higher than the right; shoulders were absent. In addition, individual compartments within the S-phase were required to be within 20% of the neighboring compartments in control populations.

(3) The G_2M mean = ($G_{1/0}$ mean) ± 1% (4–7 channels).

(4) The visual fit of the generated curve was as nearly identical to that of the histogram as possible within the limits of the first three constraints.

Applying all four parameters, only the most perturbed populations could not be analyzed by this method. Experiments were performed two to four times and expressed as the mean ± SE of the percent of the population.

Clonogenic assay. Cells were cloned by the method of Robinson *et al.* [19]. Cells in early exponential growth were resuspended in RPMI 1640 medium with 10% HI-horse serum and incubated in the presence or absence of drug in polystyrene tissue culture flasks (Becton Dickinson) in the dark at 37° for varying durations. Cells were then washed in PBS (GIBCO) resuspended in RPMI 1640 medium with 15% HI-horse serum, twice diluted 1:10, and adjusted to 50–100 cells/ml. Viable cells as measured by trypan blue exclusion were counted on a hemocytometer. The cells were added to a suspension of RPMI 1640 medium with 15% HI-horse serum and Noble agar (10:1, v/v) in round-bottomed 16 × 125 mm plastic tubes (Becton Dickinson, Cockeysville, MD) to give a final cell number of either 100, 150 or 200 cells per tube in each of four tubes for each test point. Thus, for each test point, 400, 600 or 800 cells were plated. For experiments in which multiple log orders of kill were anticipated, either a single dilution or no dilutions were made, and either 2,000 or 20,000 cells were added to each of four tubes for each test point. Cell adjustments were made to give final colony counts per control tube of 70–140 colonies. After adding cells to the agar/media suspension, tubes were gently rotated, chilled at 4° for 3 min, allowed to adjust at room temperature for 15 min, and incubated at 37° for 10–12 days. Tubes were selected in a blinded fashion, and colonies were counted under low power magnification against a black background. A colony was defined as an aggregate containing 20 or more trypan blue-excluding cells. Questionable colonies, determined from experience, were removed and examined under higher magnification, and viable cells were counted. Cloning efficiency was 70 ± 16%. Experiments were performed from three to seven times. Statistical analysis of paired groups was performed by Student's *t*-test.

Doxorubicin flux. Cells in early experimental growth in RPMI 1640 medium with 10% HI-horse serum (GIBCO) were incubated with ICRF 187, 1.0 μg/ml, for 24 hr. Cell suspensions were then centrifuged at 200 *g* for 5 min, resuspended in fresh medium at a concentration of 2 × 10^6 cells/ml, and incubated at 37° for 10 min. A concentration of 1.0 μg/ml of ICRF was maintained in the pretreated group throughout the experiment. To determine the rate of influx of drug, cell suspensions were incubated with [^{14}C]doxorubicin, 1.0 μg/ml for 1 hr. Drug uptake was determined by removing 0.5-ml aliquots from each tube at 1, 7, 13, 19, 25, 40 and 60 min time points to a microcentrifuge tube containing 0.5 ml of

Table 1. Effect of incubation with doxorubicin with or without ICRF 187 (0.1 μg/ml) for 24 hr on cell cycle distribution of S180 cells

	Dose (μg/ml)	G_1	S	G_2M
Control		39.6 ± 2.5*	31.0 ± 2.7	29.0 ± 1.8
+DOX	0.01	32.0 ± 2.0	34.5 ± 1.5	33.0 ± 0.0
	0.05	32.4 ± 0.0	28.4 ± 0.0	39.2 ± 0.0
	0.10	30.5 ± 13.5	19.5 ± 5.5	49.5 ± 7.5
	0.20	22.0 ± 15.0	17.0 ± 12.0	60.0 ± 2.0
ICRF	0.1	38.0 ± 2.0	30.0 ± 3.0	31.5 ± .5
+DOX	0.01	38.5 ± 8.5	30.0 ± 5.0	29.5 ± 3.5
	0.05	28.5 ± 5.5	22.5 ± 11.5	48.5 ± 6.5
	0.10	21.5 ± 8.5	12.5 ± 7.5	65.5 ± 0.5
	0.20	NA†	NA	NA

Following drug exposure, cells were washed, stained with propidium iodide (0.05 mg/ml) in hypotonic sodium citrate with 0.5% Triton-X detergent, and then incubated with RNA:se (2 mg/ml). Cell cycle analysis was performed on the Ortho Diagnostics 50H Cytoflourograf and analyzed by the Fried methodology as described in Methods.

* Percent (mean ± SE).

† Not available.

ice-cold PBS (GIBCO), immediately centrifuging at 12,000 *g* for 30 sec, and then washing once with PBS.

At 60 min, the remaining cells were centrifuged at 200 *g* for 5 min, washed once in ice-cold normal saline, and resuspended in warm RPMI 1640. A 0.5-ml aliquot of cells was removed, and the remaining cells were placed in a 37° water bath. To determine the rate of drug efflux, 0.5-ml aliquots were removed at 4, 8, 15, 20, 30 and 60 min time points. Each aliquot was added to a microcentrifuge tube containing 0.5 ml of ice-cold PBS. Tubes were immediately centrifuged at 12,000 *g* for 1 min and washed once. The cell pellets were solubilized with 0.2 N of NaOH (0.5 ml) for 12 hr at 60°. The tube contents were then neutralized, scintillation flour was added, and radioactivity was determined.

RESULTS

Flow cytometry. To determine the effect of single agent doxorubicin on S180 cell cycle traverse, cells incubated with doxorubicin at various concentrations for 24 hr were analyzed by flow cytometry. As shown in Table 1, incubation with doxorubicin produced progressive accumulation of cells in G_2M and progressive depletion of cells in S-phase in a dose-dependent fashion. Depletion of $G_{1/0}$ occurred at higher doses of doxorubicin, 0.10 and 0.20 μg/ml.

To determine the effect of single agent ICRF 187 on S180 cell cycle traverse, flow microfluorometry was performed on cells following incubation for 24 hr. As shown in Fig. 1, incubation with ICRF 187 produced a different pattern of cytokinetic aberrations from doxorubicin. Incubation with 0.1 μg/ml produced virtually no effect on DNA content, whereas at a higher dose, 1.0 μg/ml, there was partial arrest at G_2M. With an increase in the dose of ICRF 187 to 10 μg/ml, a subpopulation of cells continued to synthesize DNA, without dividing, again becoming blocked in a second G_2M with an octaploid DNA content noted at Channel 480.

To determine the effect of the combination of doxorubicin and ICRF 187 on cell cycle traverse of S180 cells, cell cycle analysis was performed following 24-hr incubation of cell suspensions with both drugs at concentrations to be specified below. As shown in Table 1, at the lowest doses studied (ICRF 187, 0.1 μg/ml; and doxorubicin, 0.1 μg/ml) there was no effect of the combination on cell cycle traverse. With increasing doses of doxorubicin, from 0.05 μg/ml to 0.2 μg/ml, the presence of ICRF, 0.1 μg/ml, which produced no effect on cell cycle traverse when employed singly, enhanced the doxorubicin treatment effect with augmented accumulation of cells in G_2M and more thorough depletion of $G_{1/0}$ and S-phase. Fried analysis of cell cycle distribution, shown in Table 1, confirms that, at a dose which was itself ineffective in altering cellular DNA content, ICRF 187 increased the ability of doxorubicin to arrest cells in G_2M with subsequent depletion of the $G_{1/0}$ and S-compartments.

At 1.0 μg/ml, 24-hr incubation with ICRF 187 produced partial blockade of cell cycle traverse, which was not augmented by the presence of doxorubicin at 0.01, 0.05 and 0.1 μg/ml (Table 1). With 0.2 μg doxorubicin/ml, however, the addition of ICRF, 1.0 μg/ml produced augmentation of the blockade at G_2M with virtually total emptying of S-phase, suggesting a nearly complete blockade of cycling cells at G_2M; the residual cell component in $G_{1/0}$ may represent the presence of a non-cycling cell population (Table 2).

Clonogenic assay. To compare the cytokinetic aberrations induced by the doxorubicin–ICRF 187 combination with the cytotoxic effects, S180 cells were cloned in soft agar following 24-hr exposure to the drugs. As shown in Fig. 2, 24-hr incubation with doxorubicin at 0.01, 0.05 and 0.1 μg/ml resulted in 0-, 1-, and 2-log decades of kill, respectively, demonstrating a logarithmic dose–response effect at doses employed. The addition of ICRF 187, 1.0 μg/ml, for 24 hr, which alone produced a single log decade of kill, resulted in enhancement of cell kill at each doxorubicin dose level with consistently greater than additive effect.

To ensure that the lethal effects of the combination

Table 2. Effect of incubation with doxorubicin with or without ICRF (1.0 μg/ml) for 24 hr on cell cycle distribution of S180 cells

	Dose (μg/ml)	G_1	S	G_2M
Control		37.4 ± 1.9*	32.8 ± 2.2	29.5 ± 1.6
+DOX	0.01	31.1 ± 1.1	35.0 ± 1.2	32.0 ± 0.7
	0.05	30.3 ± 1.4	27.0 ± 4.9	38.0 ± 5.8
	0.10	30.5 ± 6.3	26.8 ± 6.0	40.0 ± 6.4
	0.20	22.0 ± 15.0	17.0 ± 12.0	60.0 ± 2.0
ICRF	1.0	28.8 ± 2.5	24.0 ± 3.9	46.8 ± 2.7
+DOX	0.01	35.0 ± 4.6	17.9 ± 5.5	46.5 ± 5.9
	0.05	26.7 ± 4.7	20.3 ± 9.7	50.0 ± 3.2
	0.10	32.5 ± 6.9	18.0 ± 8.2	49.3 ± 3.4
	0.20	25.0 ± 13.0	0.0 ± 0.0	75.0 ± 13.0

See legend of Table 1 for details.
* Percent (mean ± SE).

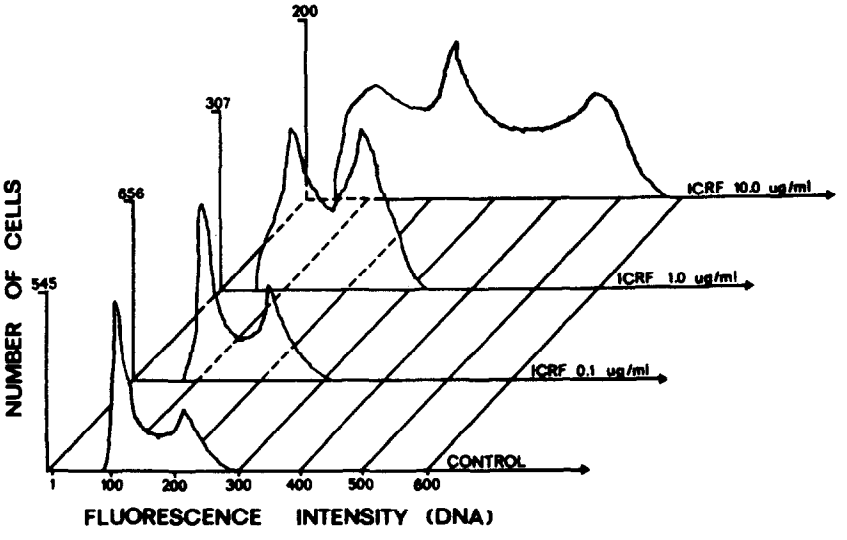

Fig. 1. Red (DNA) flourescence histogram of 1 × 10^5 PI-stained S180 cells following 24-hr incubation with ICRF 187. Abscissa: fluorescence intensity (DNA) over 600 channels. Channel number was expanded to 600 in order to encompass a chick red blood cell internal standard at channel 55 in histograms with an 8n peak. Ordinate: relative number of cells. A partial G_2M arrest with concomitant depletion of $G_{1/0}$ and S-phase is noted at 1.0 μg/ml. At 10 μg/ml, partial G_2M arrest is noted at a 4n peak (channel 240) with continued DNA synthesis at higher ploidy and second arrest at an 8n peak (channel 480). See Methods for details of histogram analysis.

did not result from acute membrane or global metabolic damage following drug exposure, cells were counted in the presence of trypan blue, and the proportion of cells which excluded the dye was calculated. The drug combination produced no diminution of the ability of treated cells to exclude the dye.

Doxorubicin flux. To determine whether the enhancement of the doxorubicin treatment effect on cell cycle traverse and cell survival by ICRF 187 was a consequence of the ability of ICRF 187 to augment cellular accumulation of the anthracycline, intracellular levels of doxorubicin were measured during continuous exposure to the drug (influx) and following loading with the drug (efflux) in cells that had been incubated previously with ICRF 187. As shown in Fig. 3, preincubation of S180 cells with ICRF 187, 1.0 μg/ml, for 24 hr produced a diminution in uptake of [^{14}C]doxorubicin. Neither the difference in rate of uptake nor total uptake at any time point was statistically significant by Student's *t*-test or by repeated measures analysis of variance. Efflux curves were virtually superimposable, allowing for the difference in anthracycline uptake.

DISCUSSION

Antineoplastic agents may inhibit the growth of tumor cell populations, either by inducing lethal cellular events or by altering the growth cycle kinetics of individual tumor cells without inducing cell death [10]. With a few exceptions these effects appear to occur independently of each other, although the biological relationship between them remains to be

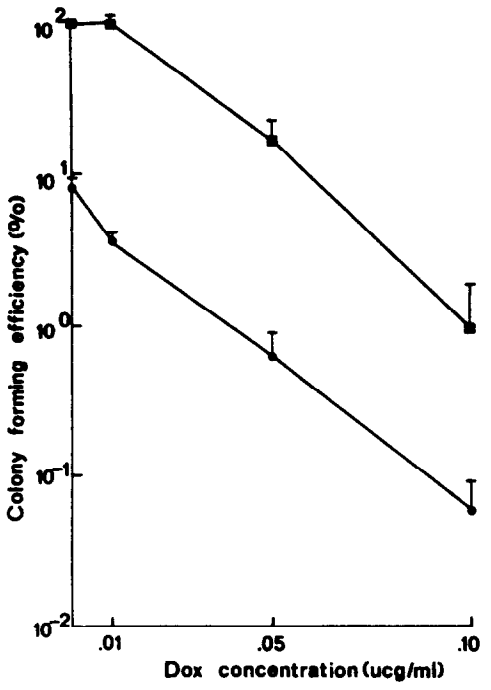

Fig. 2. Colony-forming ability of S180 cells following 24-hr exposure to doxorubicin, ICRF 1.0 μg/ml, or the combination as percent of control. Bars: mean ± SE. Key: ICRF, 1. μg/ml (■) or the combination (●). Dox, 0.01 μg/ml, vs combination: $P < 0.001$; dox, 0.05 μg/ml, vs combination: $P < 0.05$; dox, 0.10 μg/ml, vs combination: $P < 0.025$; ICRF vs ICRF + dox (0.01 μg/ml): $P < 0.001$; ICRF vs ICRF + dox (0.05 μg/ml): $P < 0.025$; ICRF vs ICRF + dox (0.10 μg/ml): $P < 0.05$ by Student's *t*-test.

clearly defined [20, 21]. Thus, knowledge of both the cytostatic and cytocidal effects of an antineoplastic agent or combination may help to elucidate the mechanisms of antitumor activity and provide useful information for the rational design of chemotherapeutic regimens [11, 22, 23].

The bisdioxopiperazine, ICRF 187, has been shown to augment the antiproliferative effects of the anthracycline antibiotic, doxorubicin, against the murine sarcoma S180 cell line *in vitro* in a dose- and schedule-dependent fashion [9]. As antiproliferative effects may reflect both lethal events and sublethal inhibition of cell growth, we have employed flow cytometry to discriminate between these effects in order to elucidate the nature of the interaction between these two drugs [10].

The Fried methodology offers advantages over the commonly employed planimetric methods [24–26] for analyzing perturbed cell populations. The results are highly reproducible when employed within the constraints noted in Methods. These constraints are necessitated by the fact that perturbed populations are analyzed according to parameters pre-established by analysis of control populations. Rapidly doubling cell populations with a large proportion of cells in S-phase present problems for planimetric methods, which may, for example, rely on the presence of a nearly linear downslope of $G_{1/0}$ or flat upper boundary of S-phase to define the limits of the S-phase population [27]. When parameters, noted above, for defining the shape of the S-phase are employed and when restrictions on variations on the size of S-phase compartments are employed, the Fried program is capable of reproducibly defining the proportion of cells in S-phase in moderately perturbed populations.

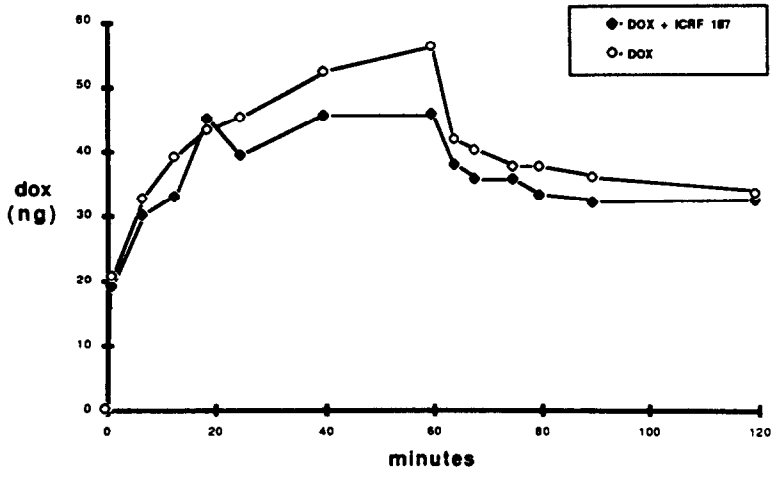

Fig. 3. Influx and efflux of [^{14}C]doxorubicin following 24-hr preincubation with (◆) or without (◇) ICRF 187, 1.0 μg/ml. Following preincubation, cells were loaded with [^{14}C]doxorubicin, 1.0 μg/ml, for 1 hr. Aliquots were removed at indicated time points, washed, solubilized in 0.2 N NaOH, and counted in a scintillation counter. Following loading, remaining cells were washed at 4° in normal saline and resuspended in drug-free medium with or without ICRF 187, 1.0 μg/ml. At indicated time points, aliquots were removed, washed, solubilized and counted. Points: means of three experiments. Bars denoting standard error are omitted: all bars fall within the point. Between-group differences were compared by Student's *t*-test and analysis of variance and were not statistically significant.

In populations with exaggerated perturbations, such as ICRF-treated cells with large octaploid populations, however, we were not able to analyze successfully the DNA histograms employing this methodology and, thus, for these heavily perturbed populations have relied on planimetric methods.

Our results indicate clear cytokinetic differences between ICRF 187 and doxorubicin. Both agents produce a G_2M arrest; however, in contrast to the cytokinetic effects induced by doxorubicin, a subpopulation of cells treated with ICRF 187 escape blockade at G_2M, undergo mitosis as evidenced by cyclic chromatin condensation, but fail to undergo cell division [28, 29]. These cells continue to synthesize DNA and are again arrested in a second G_2M peak with an 8n DNA content as illustrated in Fig. 1. These findings are consistent with those of Wheeler *et al.* [30], employing the cultured Burkitts lymphoma P3J cell line, who found that cells exposed to ICRF 187, 1 μg/ml, for 4, 8, 14, and 20 hr are partially arrested in G_2M at all time intervals tested, but that cell populations incubated in the presence of 5 μg/ml for the same time intervals are partially arrested at a 4n distribution at 4 and 8 hr, and then were secondarily blocked at an 8n peak at 14 and 20 hr. Likewise, Traganos *et al.* [29] exposed L1210 and Friend leukemia cells to ICRF 187 at either 1.0 or 10 μg/ml for 16–24 hr and noted accumulation of cells at an 8n peak at the higher dose, but not the lower dose.

In contrast to ICRF 187, incubation of cell suspensions with doxorubicin produces a dose-dependent G_2M block, which has been shown to correlate with cytotoxicity [10], although, as the data presented here indicate, on a much reduced scale: thus, at doses which produce 80% cell kill, block at G_2M increases by only 6%, and at doses which produce two log decades of cell kill, block is enhanced by only 16%. Our results do not indicate selective depletion of $G_{1/0}$ as has been found with prolonged, high dose drug exposure employing cultured human lymphoblasts [24], but indicate depletion of *both* $G_{1/0}$ and S-phase cells. As we have previously demonstrated only a modest antiproliferative effect of doxorubicin at doses similar to those employed in the experiments now reported [9], and as the trypan-blue exclusion data presented here suggest no acute loss of membrane integrity among cells treated with doxorubicin, the depletion of the $G_{1/0}$ and S-phase compartments most likely resulted from a dose-related sublethal cytostatic effect as opposed to selective lethality for $G_{1/0}$ and S-phase cells. Furthermore, as no accumulation of cells was noted in S-phase at higher doses of doxorubicin as had been reported previously [24], this suggests the presence of a single dose-related cytostatic effect rather than invoking a second cytostatic mechanism at higher doses.

The addition of ICRF 187 to doxorubicin for 24 hr produced a marked positive interaction with augmented arrest of cells in G_2M and a more thorough depletion of $G_{1/0}$ and S-phase cells than when either agent was employed alone. For example, in the presence of ICRF 187, 0.1 μg/ml, a concentration which by itself produced no cytostatic effects following 24-hr incubation, accumulation of cells in G_2M following 24-hr exposure to doxorubicin at 0.1 and 0.05 μg/ml was equivalent to accumulation following 24-hr exposure to single agent doxorubicin at twice the concentration: 0.2 and 0.1 μg/ml respectively. Drewinko and Barlogie [31] and Tobey *et al.* [12] have reported that cells in S and G_2 are more sensitive to the effects of treatment with doxorubicin, whereas cells in G_1 are less sensitive. Thus, the positive interaction between doxorubicin and ICRF 187 may result from the ability of ICRF 187 to increase the proportion of the cell population in G_2 making them relatively more accessible to the anthracycline effect and to deplete the G_1 population, which is relatively anthracycline resistant.

As mentioned previously, cytostatic effects, which occur within 1–2 doublings following drug exposure as measured by cell cycle analysis, and lethal events, which are manifested after 8–10 doublings as assayed by colony-forming efficiency, generally result from separate biological mechanisms and are rarely predictive for each other [32, 33]. Doxorubicin [12, 31] and ICRF 187 [34] as single agents represent two exceptions; hence, it is not surprising that, for the combination, cytokinetic aberrations predict for lethal events, as our results indicate.

Because the mechanism of action of ICRF 187 remains poorly understood, any attempt to define a common cause for the cytostatic and cytotoxic effects of the anthracycline–bisdioxopiperazine combination remains highly speculative. Based on our results, however, certain conclusions may be drawn. In the absence of a decrease in trypan-excluding cells following drug treatment, it is unlikely that the link between the cytotoxic and cytostatic effects produced by the drug combination is the production of global metabolic cell damage, as acute poisoning of the cell would most likely be reflected in alteration of membrane integrity and damage to energy-dependent excretory function [35, 36]. Furthermore, in the absence of enhanced doxorubicin accumulation following pretreatment with ICRF 187, it is unlikely that the common cause for both cytotoxic and cytostatic effects of the anthracycline–bisdioxopiperazine combination is exposure of cell target sites to increased doxorubicin levels.

It has been postulated that drug-induced chromosome damage resulting in decreased synthesis of proteins critical for cells to enter mitosis may account for G_2 arrest [37]. While ICRF 187, a potent chelating agent, most likely does not itself bind to DNA or cause chromosome damage, the potentiation of the doxorubicin treatment effect may result from the ability of ICRF to augment doxorubicin activity at the genome level: to inhibit repair of doxorubicin-induced DNA damage [38], to increase the availability of transition metal cations for participation in free-radical generating oxidation-reduction reactions [39–42], or to stabilize an intermediate in doxorubicin-mediated DNA degradation [43–45]. Thus, by enhancing doxorubicin-mediated DNA damage, ICRF 187 may potentiate *both* the cytotoxic and cytostatic effects of the anthracycline.

Various *in vitro* tests have been employed previously to evaluate cellular damage following exposure to chemotherapeutic agents, including dye exclusion, chromium release, incorporation of radio-

active DNA precursors, and cell proliferation in short-term cultures [36, 37, 46, 47]. Colony-forming ability in soft agar has been the traditional standard for measurement of drug lethality. We now report that for the combination of ICRF 187 and doxorubicin, perturbation of S180 cell cycle traverse as measured by flow cytometry following 24-hr drug exposure is also predictive for drug lethality. While the combination synergistically inhibits colony formation, as defined by the fractional product method [9], no such criteria are available for defining synergy as measured by cell cycle analysis. Nevertheless, a positive interaction between ICRF 187 and doxorubicin is suggested by cell cycle analysis which demonstrates augmented accumulation of drug-treated cells in G_2M with the combination, as compared with either agent employed singly, and supports previous findings of an anthracycline–bisdioxopiperazine synergy [7, 8]. Absence of an increase in trypan-blue excluding cells and failure of ICRF 187 to augment doxorubicin accumulation in S180 cells suggest that ICRF 187 does not produce a global increase in doxorubicin activity, but enhances doxorubicin activity in a site-specific fashion, presumably at the genome level, which results in both lethal and cytokinetic effects.

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