Synergistic Activity of Doxorubicin and the Bisdioxopiperazine (+)-1,2-Bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF 187) against the Murine Sarcoma S180 Cell Line

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ABSTRACT

The bisdioxopiperazine (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)-propane (ICRF 187) abrogates doxorubicin cardiotoxicity in every mammalian species tested, but its effect on doxorubicin antitumor activity remains poorly understood. In order to better define the anthracycline-bisdioxopiperazine interaction, the ability of murine sarcoma S180 cells to form colonies in soft agar and their capability to proliferate in microtiter wells were assayed after exposure to drug at varying doses and schedules. Incubation of cell suspensions for 1 h with doxorubicin, 0.1 µg/ml, with or without (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane, 80 µg/ml, produces additive cytotoxicity for the combination. Prolonged incubation (24 h) with the same drugs produces synergistic cytotoxic and antiproliferative effects at 1- and 2-log order reductions in dose. These studies indicate that the antineoplastic activity of the single agents doxorubicin and (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane is enhanced when the drugs are used in combination, and that this phenomenon is highly dose and schedule dependent.

INTRODUCTION

The utility of the anthracycline antibiotic, doxorubicin, is limited by its capacity to produce a dose-dependent and potentially life-threatening cardiomyopathy. Although this complication occurs only in a small subset of treated patients, current practice is to limit the cumulative dose of doxorubicin to 550 mg/m². While this diminishes the risk of cardiac toxicity, it also attenuates the benefit of the drug for many patients who had responded favorably or its use as an adjuvant chemotherapeutic agent (1).

One clinical strategy to circumvent this problem has been the development of potential cardioprotective agents to be administered concomitantly with doxorubicin. The two most promising agents appear to be the bisdioxopiperazine ICRF 159 and its (+)-enantioromer ICRF 187. Because ICRF 159 is water insoluble, parenteral formulation is problematic, and p.o. administration results in erratic gastrointestinal uptake. Hence, our investigations have centered on the water-soluble and hence more reliably administered analogue ICRF 187 (2).

ICRF 187 has totally abrogated anthracycline-associated cardiac toxicity in every animal species tested (3-7). ICRF 187 does not appear to alter anthracycline actions in noncardiac tissues. Alopecia in dogs and miniature pigs following doxorubicin administration occurs despite pretreatment with the bisdioxopiperazine. Likewise, daunorubicin-induced myelosuppression is unaffected (8). Using the L1210 murine leukemia cell line implanted in animals, two groups of investigators have found that ICRF 159 not only fails to diminish the cytotoxicity of doxorubicin but potentiates its antitumor effect as measured by prolonged host survival (9, 10).

The biochemical basis for the bisdioxopiperazine-anthracycline interaction remains unexplained. ICRF 187, a potent chelating agent, may inhibit the ability of tumor cells to dispose of doxorubicin (11, 12) or to repair doxorubicin-induced DNA damage (13), may increase the availability of transition metal cations for participation in free-radical generating oxidation-reduction reactions (14), or may stabilize an intermediate in doxorubicin-mediated DNA degradation (15, 16).

To better define the nature of the bisdioxopiperazine-anthracycline interaction, we have examined the effect of the combination of ICRF 187 and doxorubicin on the murine sarcoma S180 cell line in vitro using both the clonogenic assay, the traditional measure of drug effectiveness, and a nonclonogenic assay, which examines the drug effect on the entire cell population. As dose and schedule may have a role in determining the interaction of the two agents, the effects of different concentrations and incubation times were investigated.

MATERIALS AND METHODS

Cell Culture. The S180 cell line, originally isolated from the murine CFW strain in 1955 (17), 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% HI horse serum (GIBCO) in 25-cm² polystyrene tissue culture flasks (Becton Dickinson, Oxnard, CA). No antibiotics were added to the culture medium. Cells were cultured in the dark at 37°C in a humidified atmosphere with 5% CO₂ and 95% air by the method of Fisher and Sartorelli (18). The doubling time for this cell line is 16.2 h.

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°C.

Clonogenic Assay. Cells in midexponential growth were resuspended in RPMI 1640 with 10% HI horse serum and incubated in the presence or absence of drug (doxorubicin, ICRF 187, or both) in polystyrene tissue culture flasks (Becton Dickinson) in the dark at 37°C for varying durations. Doses of doxorubicin and ICRF 187 used for the 1-h incubations...
were 0.1 and 10 μg/ml, respectively. Doses of doxorubicin used for the 24-h incubation were 0.01, 0.05, and 1.0 μg/ml, and the dose of ICRF 187 was 1 μg/ml. Cells were then washed in Dulbecco’s phosphate-buffered saline (GIBCO), resuspended in RPMI 1640 medium with 15% HI horse serum, twice diluted 1:10, and adjusted to 50–100 cells/ml. In adjusting cell number, 400 cells capable of excluding trypan blue were counted on a hemocytometer. Cells were then added to a suspension of RPMI 1640 medium with 15% HI horse serum and Noble agar (10:1, v/v) in round-bottomed 16- x 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, either 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 a final colony counts per control tube of 70–140 colonies. After adding cells to the agar-medium suspension, tubes were gently rotated, chilled at 4°C for 3 min, allowed to adjust at room temperature for 15 min, and incubated at 37°C for 10–12 days. Tubs 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 2–7 times.

Microtiter Plate Assay. In order to determine drug effect on the entire cell population, a nonclonogenic assay, modified from the method of Grunwald (19), was used. Drugs were diluted in normal saline and then diluted to appropriate concentrations in medium prior to administration to test cultures.

A 96-well, Falcon Microtest III microplate (Falcon Plastics, Oxnard, CA) was used. Cells in late exponential growth were diluted to 44,444 cells/ml. Ninety μl of the cell suspension in RPMI 1640 medium with 10% HI horse serum were added sterilely to each well with an Oxford micropipet with sterile disposable tips (Sherwood Medical, St. Louis, MO) to give a final cell number of 4000 cells/well on Day 0. Cells were allowed to enter exponential growth over 24 h in the dark at 37°C in an atmosphere of 5% CO₂:95% humidified air. On Day 1, 5-μl aliquots of each drug solution in RPMI 1640 medium with 10% HI horse serum (full medium) medium or full medium alone were added to give a final volume of 100 μl and final cell concentration of 4000 cells/well. In experiment 1, shown in Fig. 5, the doses of doxorubicin were 0.01, 0.038, and 0.075 μg/ml, and the doses of ICRF 187 were 0.1, 0.5, and 1.0 μg/ml. In Experiment 2, Fig. 6, the doses of doxorubicin were 0.01, 0.05, and 0.60 μg/ml, and the doses of ICRF 187 were 0.1, 0.5, and 1.0 μg/ml. Each drug concentration was tested in 5–8 replicates. Drugs were added in a “checkerboard” pattern with increasing doses of one drug along the “X” axis of the plate and of the second drug along the “Y” axis. Outside wells were not used in order to avoid problems of evaporation along the borders of the plate. When more than one plate was used, replicates of each experiment were performed in both plates. After it was filled, the plate was covered with its own snug-fitting lid, wrapped in Saran Wrap (Dow Chemical, Indianapolis, IN), and incubated in the dark at 37°C for an additional 36–48 h. On Day 4, when cells were in late exponential growth, microtiter wells were vacuumed with a micropipet, washed, and revaccuumed twice. Cell suspensions were resuspended in 2.5 ml Ham’s isotonic diluent (Fisher Scientific, Springfield, NJ) in 16 x 50-mm shell vials (VWR Scientific, South Plainfield, NJ) and counted by Model ZBI Couter Counter (Couter Electronics, Hialeah, FL).

Data Analysis. Statistical analysis of paired groups was performed by Student’s t test. Cell counts from the microtiter plate assay were plotted as a scattergram. Polynomial regression analysis was used to check for departures from linearity up to the fifth order (20). The significance of the difference of the correlation coefficient zero from each curve was determined by t test. To compare the correlation coefficients of the two slopes, Fisher’s r to z transformation was used (21).

Analysis of the combined drug effect was performed by the fractional product method. Synergism was defined by the equation

\[ i_2 > i_1 + i_2 - i_2 \]

where \( i \) is the inhibitory effect of each drug used singly (22).

RESULTS

Effect of ICRF 187 on S180 Cell Survival. The dose-response effect of a 1-h incubation with ICRF 187 on the S180 cell population as determined by clonogenic assay is depicted in Fig. 1. Above 1 μg/ml, cloning efficiency decreases linearly with flattening of the curve at higher concentrations (>100 μg/ml). The IC50 for a 1-h incubation is 45 μg/ml.

The effect of prolonged incubation with ICRF 187 on S180 cell survival is depicted in Fig. 2. Incubations with 10 μg/ml for durations from 4–24 h produce a linear decrease in cell survival with a single log decade of kill reached at 16 h and no further increment in cell kill thereafter, suggesting that the maximal drug
Effect of Doxorubicin on S180 Cell Survival. The dose-response effect of a 1-h incubation with doxorubicin on S180 cell survival as determined by clonogenic assay is depicted in Fig. 1. Above 0.1 μg/ml, exponential kill is noted. The IC50 is 0.175 μg/ml for a 1-h incubation. To determine the effect of prolonged exposure to doxorubicin on the S180 cell population, cells were incubated with doxorubicin, 0.075 μg/ml, for varying durations up to 36 h. As shown in Fig. 2, cell kill increased linearly up to 24 h, at which point a plateau was reached, suggesting that maximal drug effect occurs with exposures equal to 1.5 times the cell cycle duration.

Effect of the ICRF 187-Doxorubicin Combination on S180 Cell Survival. To determine whether the addition of ICRF 187 to doxorubicin enhances cell kill, S180 cells were incubated with each drug singly or in combination. As shown in Fig. 3, incubation for 1 h with a clinically achievable dose of doxorubicin (0.1 μg/ml) or ICRF 187 (10 μg/ml) diminished colony formation by 20% (P < 0.05) and 44% (P < 0.001), respectively. When cell suspensions were incubated for 1 h with both drugs simultaneously, colony formation decreased by an additional 15% compared with ICRF 187 alone (P < 0.001), suggesting an additive effect.

Because 1-h drug exposure may be inadequate to evaluate drug effectiveness for some drugs or combinations (23), S180 cell suspensions were incubated with ICRF 187 and/or doxorubicin for durations which produced maximal effect when each drug was used singly. As shown in Table 1, 24-h incubation with ICRF 187, 1 μg/ml, alone produced a single log decade of kill. The addition of doxorubicin at doses which alone produced no cell kill, a single log decade of kill, and 2 log decades of kill to ICRF 187 for 24 h resulted in synergistic inhibition of colony-forming efficiency as defined in "Materials and Methods."

To determine if the combination of ICRF 187 and doxorubicin is active at doses at which neither drug is active alone, S180 cells were incubated with microdoses of doxorubicin (0.01 μg/ml) or ICRF 187 (0.1 μg/ml) for 24 h. While neither drug alone produced any significant decrease in colony-forming activity, the combination produced a 64% reduction in colony formation as compared with control (P < 0.05), again suggesting a synergistic interaction.

Effect of the Combination of ICRF 187 and Doxorubicin on S180 Cell Proliferation. The growth curves for S180 cells incubated in microtiter wells at 4000 cells/well in 0.1 ml resemble the growth curves in tissue culture flasks with the exception of an initial lag phase (Fig. 4). At Day 3, growth curves for populations of S180 cells varying in number from 500–4000 cells/well are nonoverlapping and easily distinguishable.

To determine the effect of ICRF 187 and/or doxorubicin on the total cell population, S180 cells in early exponential growth were continuously exposed to drug in microtiter wells for 48 h. Because ICRF 187 is hydrolyzed in cell populations within 12 h, this represents continuous exposure to the parent compound for only 8–12 h; however, it is likely that it is the hydrolyzed, polar, chelating species which represents the active form of the drug within the cell (24). Fig. 5 depicts the dose response of S180 cell populations to ICRF 187 in the absence or presence of doxorubicin at three different doses using cell proliferation as end point. ICRF 187 alone produces no effect on cell proliferation at 0.1 μg/ml. At higher doses, proliferation is inhibited. In the continuous presence of doxorubicin at 0.01 μg/ml, 0.0375 μg/ml, and 0.075 μg/ml, the curves are shifted downward, indicating a dose-response effect. Not only are the curves reset lower, however, but the initial region of the ICRF 187 dose-response curve in which ICRF 187 had no effect alone now reveals an additive antiproliferative effect of the two drugs.

An identical phenomenon is noted for the doxorubicin dose-response curve as shown in Fig. 6. In the initial portion of the
ICRF 187 and doxorubicin synergy

Fig. 5. Dose-response effect of ICRF 187 at increasing doses on cell proliferation of S180 cells, incubated in microtiter wells for 3 days in the absence (•) or presence of doxorubicin, 0.01 μg/ml (△), 0.0375 μg/ml (▲), or 0.075 μg/ml (○). Points, number of cells/well, average of 4 replicates; bars, SE.

Fig. 6. Dose-response effect of doxorubicin at increasing doses on cell proliferation of S180 cells incubated in microtiter wells for 3 days in the absence (•) or presence of ICRF 187, 0.1 μg/ml (△), 0.5 μg/ml (▲), or 1.0 μg/ml (○). Points, number of cells/well, average of four replicates; bars, SE.

curve, there is no antiproliferative effect of doxorubicin at 0.01 μg/ml used alone. At higher doses, a modest antiproliferative effect is noted. In the continuous presence of ICRF 187 at 0.1, 0.5, or 1.0 μg/ml, the initial flat portion of the doxorubicin dose-response curve slopes downward.

This initial region represents exposure to ineffective concentrations of drugs. In the presence of an ineffective drug dose, the addition of a second drug would be expected to shift the curve downward without alteration in the slopes. Alteration in the slope of the curve thus suggests a biological interaction of the two drugs allowing a dose-dependent potentiation of one drug by the other.

To confirm the biological interaction suggested by the elimination of the initial region of the dose-response curve, the dose range for ICRF 187 between 0.0 and 0.1 μg/ml was examined in more detail, using 11 intermediate doses in the presence or absence of doxorubicin, 0.15 μg/ml. As shown in Fig. 7, at doses of ICRF 187 from 0.0025–0.10 μg/ml, there was no effect on cell proliferation. Cell proliferation at any dose was not significantly different from proliferation in the absence of drug, and the slope of the curve (−2593 cells/μg/ml, r = −0.052) is not significantly different from zero by t test (P = 0.361).

Using the same doses of ICRF 187 in the presence of a constant dose of doxorubicin, 0.15 μg/ml, cell proliferation was suppressed in increasing increments. In addition to a doxorubicin treatment effect in the absence of ICRF 187, there is now noted a further inhibition of cell proliferation with increasing doses of ICRF 187. The curve as fitted by polynomial regression analysis shows no significant departures from linearity up to the fifth order, suggesting a progressive ICRF effect at each dose level. The slope of the line, −13,735 cells/μg/ml (r = 0.781), is significantly different from zero (P = 0.00049). The slope of the curve produced by the combination is significantly different from the slope of the curve produced by ICRF 187 alone by Fisher’s r to z transformation (z = −2.113, P = 0.0174).

DISCUSSION

Intracellular cations, such as calcium and iron, may modulate the antitumor effects of doxorubicin by a variety of mechanisms, including alteration in the intracellular disposition of doxorubicin (25, 26) or by participating in free radical generating reactions (18). Agents which alter intracellular cation concentrations may either augment (11, 12) or block (27) the lethal effects of the drug.

The bisdioxopiperazine ICRF 187, a derivative of EDTA, is a potent chelating agent which may alter the intracellular cationic milieu (8). Possibly because of its ability to interfere with the formation of reactive oxygen radicals by chelating transition metals such as Cu(II) and Fe(III) (13), ICRF 187 has been shown to diminish the cardiotoxic effects of doxorubicin in mammalian tissue (3–7).

Because the utility of the bisdioxopiperazines as cardioprotective agents depends on their capacity not to interfere with the cytotoxic properties of the anthracyclines, we have examined the ability of ICRF 187 to alter the antitumor effects of doxorubicin in vitro in the murine sarcoma S180 cell line.

In the present studies, the combination of the bisdioxopiperazine ICRF 187 with doxorubicin was markedly synergistic against the murine sarcoma S180 cell line in vitro. This effect was noted with physiologically achievable doses of drug for prolonged exposures (24 h). With 1-h exposures using higher doses of drug, an additive effect was noted without marked synergy. Of
interest were the synergistic antiproliferative and cytotoxic effects of the combination when one or both drugs were administered at doses which produced no effect on cell proliferation or lethality.

This synergistic effect was noted in both the traditional measure of drug effectiveness, the clonogenic assay, and a nonclonogenic assay, total cell proliferation in microtiter wells, which looks at early damage to the entire cell population. The latter assay offers several advantages: (a) the relative simplicity of the technology allows simultaneous screening of a wide range of drug concentrations with multiple replicates; (b) differences in the number of viable proliferating cells determined several generation times following drug exposure may give an equally valid estimate of drug effectiveness compared to the colony-forming assay; and (c) measurement of the short-term fate of the entire cell population may be more predictive of drug effect in some instances than the clonogenic assay, which looks at the fate of only a small fraction of total tumor cells (28, 29). The major disadvantage of this assay is that it does not distinguish cytocidal effects from cytostatic drug activity.

The in vitro synergistic effect of the doxorubicin-ICRF 187 combination by both clonogenic and growth assays noted in this paper confirms an in vivo anthracycline-bisdioxopiperazine synergy noted by Woodman et al. (10). C57BL/6 x DBA/2 F2 mice inoculated i.p. with 10^6 ascitic L1210 leukemia cells or s.c. with 10^6 L1210 cells were treated with doxorubicin and ICRF 159 (the racemate of ICRF 187) alone or in combination on various dose and treatment schedules. There was no clear dose-response effect for either drug, single bolus therapy was the least effective treatment regimen, and neither agent at any dose tested was markedly effective by itself, producing at most a 139% maximal increased life span. When both agents were administered simultaneously every 3 h for 24 h, however, there was an almost 3-fold increase in life span as compared to single agent therapy using doxorubicin, 0.25 mg/kg/injection, and ICRF 159, 30 mg/kg/injection, and there was a greater than 9-fold increase in life span over single agent therapy using doxorubicin, 2.0 mg/kg/injection, and ICRF 159, 25 mg/kg/injection, with 6 of 8 long-term survivors in the latter experiment.

The schedule dependency of the bisdioxopiperazine tumoricidal effect in vivo suggested by Woodman’s experiments has been elegantly confirmed using cytotoxic aberrations as an end point by Wheeler (30), who serially examined bone marrow cell cycle distribution in humans following treatment with ICRF 187 on three different schedules: a single dose of ICRF 187, 1.0 g/m^2; a daily treatment course at 1.5 g/m^2/day for 3 days; and a continuous 48-h treatment course at 1.0 g/m^2/48 h. Following a single bolus dose, partial G_s-M accumulation was evident at 24 h, fully reversioning to normal distribution at 48 h. During multiple bolus dose therapy, there were a partial accumulation of cells at G_s-M at 24 h, accumulation of cells at an abnormal octoploid peak at 48–72 h, and then reversion to normal distribution at 120 h, approximately 60 h following the last bolus. An almost identical effect was seen during and subsequent to a 48-h infusion with ICRF 187.

The enhancement of doxorubicin activity by ICRF 187 following incubations of 16 h or more confirms the findings of Kovacs (31) and Taylor (32) using ICRF 159, that prolonged exposure may be necessary to exploit the full synergistic effect of the bisdioxopiperazines in combination with other agents.

The mechanisms responsible for synergy between ICRF 187 and doxorubicin remain obscure, but they may involve the ability of ICRF 187 to augment free-radical generation by doxorubicin (14, 33–35), to inhibit the ability of cells to repair free-radical induced damage (13), to enhance doxorubicin-stabilized topoisomerase II-mediated DNA cleavage (15, 16, 36–38), or to alter the intracellular disposition of the anthracycline (11, 12, 39–41).

In summary, we have found that the combination of the anthracycline antibiotic, doxorubicin, and the bisdioxopiperazine, ICRF 187, has synergistic cytotoxic and antiproliferative effects against the murine sarcoma S180 cell line in vitro. This synergy is highly schedule and dose dependent, occurring at nanogram dose levels of both doxorubicin and ICRF 187 with prolonged exposures (24 h). The therapeutic implications of these findings must be tempered with the knowledge that, in addition to enhanced antineoplastic effects, combinations of anthracyclines and bisdioxopiperazines in vivo have been found to also produce increased bone marrow and other toxicities (42).

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REFERENCES

13. Huang, Z. X., May, P. M., Quinlan, K. M., Williams, D. R., and Creighton, A. Metal binding by pharmaceuticals. Part 2. Interaction of Ca(ll), Cu(ll), Fe(ll), Mg(ll), Mn(ll), and Zn(ll) with the intracellular hydrolysis products of the anti-tumor agent ICRF 159 and its inactive homologue ICRF 192. Agents Actions, 12: 536–540, 1982.
15. Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., and Chen, G. L. Change of

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