Cancer Chemother Pharmacol (1987) 19: 301–306

Cancer Chemotherapy and Pharmacology

Antitumor activity of new anthracycline analogues in combination with interferon alfa*

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Summary. Combinations of recombinant interferon alfa$_2$ (IFN-α_2) with doxorubicin, 4'-epidoxorubicin, 4'-deoxydoxorubicin, or 4-demethoxydaunorubicin were tested for antiproliferative activity against a panel of human tumor cell lines in a human tumor clonogenic assay. The histologies of the cell lines were ovary, cervix, breast, and melanoma. Each of the cytotoxic compounds showed dose-dependent antiproliferative effects against each of the cell lines, and the results indicated that doxorubicin derivatives were consistently more potent than the parent drug. In all instances, 4-demethoxydaunorubicin was the most potent derivative, requiring 2–20 times less drug to inhibit 70% of tumor colony formation. Combinations of IFN-α_2, with doxorubicin or its derivatives may show additive or synergistic antiproliferative activity against certain tumor cell lines. The ovarian carcinoma cell line, BG-1, responded synergistically to each of the four compounds in combination with IFN-α_2. The cervical carcinoma cell line, CaSki, and the breast carcinoma line, MCF-7, responded to the combinations in a manner best described as additive. In the melanoma line, SK-Mel-28, the drugs were found to be subadditive or even antagonistic. While the potency of the anthracycline derivatives ranked consistently across the different cell lines, the synergistic interaction with IFN-α_2 is a cell line-specific phenomenon unrelated to sensitivity to either anthracyclines or interferon.

Introduction

Identification or development of truly new chemotherapeutic agents is extremely slow, prompting structural manipulation of existing drugs or testing of new drug combinations to improve efficacy. In this latter respect, we have previously reported in vitro potentiation of the antitumor effect of doxorubicin by alpha interferon (IFN-α) [22]. Optimal scheduling of the agents was found to be either simultaneous application of the two drugs, or pretreatment of the tumor cells with IFN-α before doxorubicin exposure.

Recently, new derivatives of doxorubicin and daunorubicin have been synthesized, with the goal of reducing the cardiac toxicity of the anthracycline antibiotic or generating a molecule with more potent antitumor effects. In the present investigation, doxorubicin, two of its synthetic analogues, and a derivative of daunorubicin were tested for antiproliferative activity against a series of four human tumor cell lines in a soft agarose clonogenic assay. The relative ranking of the efficacies of the analogue against these different cell lines was determined. In addition, the effect of recombinant IFN-α (IFN-α_2) on the antiproliferative activity of the cytotoxic agents was investigated. We were concerned to determine the degree to which the direct antiproliferative effect was associated with additive, synergistic, or inhibitory interactions with IFN-α.

Materials and methods

Drugs. All anthracyclines and their derivatives were supplied by Farmitalia Carlo Erba, Milan, Italy. The compounds tested were doxorubicin (DOX), 4'epidoxorubicin (4'-EDX), 4'-deoxydoxorubicin (4'-DDX) and 4-demethoxydaunorubicin (4-DDR). Each of these was soluble in water. In all experiments, an appropriate vehicle solvent was included as a control. The drugs were stored as lyophilized aliquots in light-protected vials.

IFN-α_{2b} was supplied by Schering Corporation, Bloomfield, NJ. The recombinantly cloned material demonstrated a specific antiviral activity of 1.9×10^8 units/mg protein. Purity was greater than 98%. Aliquots of IFN-α_2 in phosphate-buffered saline were stored at −20°C.

Human tumor cell lines. A series of human tumor cell lines was used to evaluate the antiproliferative activity of the drugs. Ovarian carcinoma (BG-1 [22], breast carcinoma (MCF-7 [19]), cervical carcinoma (CaSki [12]), and the melanoma (SK-Mel-28 [3]) cell lines were propagated as monolayers in McCoy's 5A culture media (Gibco Laboratories) enriched with 10% fetal calf serum and 0.1% penicillin and streptomycin. Cultures were routinely tested for mycoplasma contamination (Hoechst stain) and found to be negative. Cells were seeded in culture flasks and allowed to expand in logarithmic phase growth prior to initiation in soft agarose clonogenic culture.

Soft agarose tumor clonogenic assay. A modified two-layer semisolid agarose assay was used to determine changes in

* Supported by a grant from Farmitalia Carlo Erba, Milan, Italy

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the clonogenicity of tumor cells resulting from treatment with drugs [9, 14]. Briefly, underlayers of 0.8% agarose in enriched McCoy's were prepared in 35-mm culture dishes and allowed to gel at 4 °C. Single-cell suspensions of the cell lines were prepared in enriched McCoy's medium with standard supplements and then mixed with molten agarose to achieve a final agarose concentration of 0.5%. Aliquots (1 ml) were applied to the underlayers and allowed to gel. Laying the culture plates onto a chilled marble slab ensured the formation of an even, firm gel.

Drugs were diluted from reconstituted stock and applied to the two-layer agarose culture as a one-time application. Drugs were made up as 20× concentrations of the desired final strength. Aliquots of 110 µl (1:20 dilution) were applied to the upper layer. Control dishes received phosphate-buffered saline. The cultures were incubated at 37 °C, 7.5% CO_2 in humidified air for 7–10 days. Colony enumeration was done by automated image analysis [16]. Colonies were defined as cell aggregates greater than 50 µm in diameter.

Statistical analysis. All cloning results were analyzed as mean fractional colony growth (FCG) relative to untreated controls (mean no. of colonies in treated plate/mean no. of colonies in control plates). All experiments were done in triplicate. Response relationships of drug dose and colony growth were evaluated by least-squares regression analysis over the linear portion of the linear-log survival curves. Interpolation was used to determine doses of drugs which inhibited colony formation by 70% of control cultures. These values were used to approximate doses for the combination studies with IFN-α_2. Calculated ID_{70}s were used for comparison of the relative efficacies of the different anthracycline analogues.

Analysis of combination chemotherapy in vitro was done using a statistical model that tests for additivity of drug effects [22]. The product of each of the fractional colony survivals from treatment with single agents (FCG anthracycline analogue × FCG IFN-α_2) (expected FCG) was compared with the survival in cultures treated with the drugs in combination (observed FCG). This analysis also takes into consideration the variance of each series of replicate measurements. Statistical significance for synergy or antagonism is achieved by rejection of the null hypothesis for additivity ($p \leq 0.2$). Synergy is described by those results where the observed FCG is less than the expected FCG; antagonistic interactions are those where the observed FCG is greater than the expected FCG. A subadditive response is that which shows less colony inhibition in the drug combination than the better of either single agent.

Results

The dose-response relationship of different anthracycline analogues against ovarian carcinoma cell line BG-1 is shown in Fig. 1. Over the range of drug concentrations tested, an exponential decline in the colony survival curve is evident for each compound.

Each of the four cell lines was tested for sensitivity to each of the drugs. Table 1 lists the doses of each of the derivatives which inhibited the formation of 70% of the colonies for each cell line. In all cases 4-demethoxydaunorubicin was found to be the most potent compound on a per-weight basis. Considerable variation is observed in the

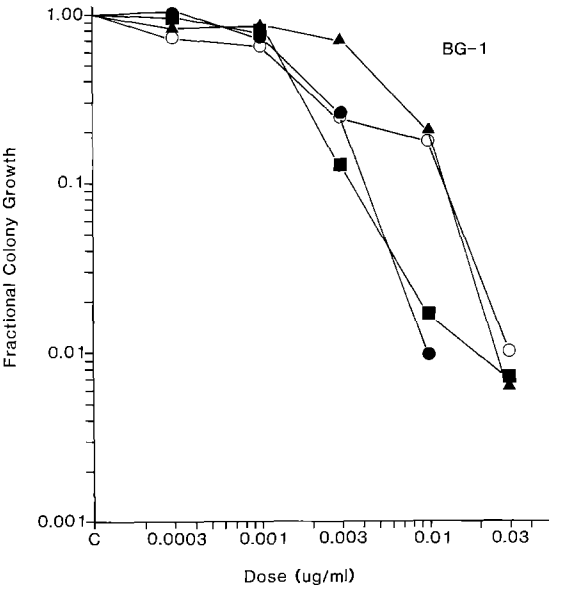

Fig. 1. Dose-response curves for BG-1 colony growth with increasing concentrations of anthracycline derivatives. ○, doxorubicin; ▲, 4'-epidoxorubicin; ●, 4'-deoxydoxorubicin; ■, 4-demethoxydaunorubicin. Standard errors of each measurement were less than 15% in triplicate cultures

ID_{70} values for the same drug between the cell lines. MCF-7 was most resistant, and CaSki was the most sensitive. Treatment of the cell lines with IFN-α_2 in combination with an anthracycline derivative resulted in cell line-specific interactions.

The ovarian carcinoma line, BG-1, was sensitive in an additive to synergistic fashion to the combination treatment (Table 2). For DOX and 4'-EDX, synergy was observed only at the highest doses of IFN-α_2, while for 4'-DDX and 4-DDR doses of 1000 units/ml IFN-α_2 proved to act synergistically. A comparison of the ratio between the expected colony growth after drug combination treatment with the observed fractional growth shows that 4'-DDX with IFN-α_2 at 10000 units/ml is 5.12 times more active than a simple additive response. Similar calculations for 4-DDR, 4'-EDX, and DOX at the same dose of interferon were 2.85, 2.52, and 1.78, respectively, each being a synergistic interaction.

IFN-α_2 is inactive as an antiproliferative agent against the breast carcinoma line, MCF-7, at the concentrations tested (Table 3). Combinations with doxorubicin or its derivatives proved somewhat effective. Doxorubicin with 1000 units/ml IFN-α_2 is 2.01 times more active than expected, but none of the other derivatives produced a syner-

Table 1. Antiproliferative efficacy of anthracycline analogues [Dose inhibiting colony growth by 70% (ng/ml)][a]

Anthracycline analogue	Cell line			
	BG-1	MCF-7	CaSki	SK-Mel-28
Doxorubicin	6.21	71.98	4.56	14.00
4'-Epidoxorubicin	7.31	60.96	3.67	8.90
4'-Deoxydoxorubicin	2.79	17.71	0.85	1.97
4'-Demethoxy-daunorubicin	2.54	4.81	0.21	0.96

[a] Calculated from linear regression of dose-response curve

Table 2. Antiproliferative effect of anthracycline analogues in combination with IFN-α_2 against BG-1

Treatment	Single agent FCG[a]	Combination treatment FCG		Interaction	*P*
		Observed	Expected		
DOX (9.0 ng/ml)	0.735 ± 0.022				
IFN-α_2 100 units/ml	0.860 ± 0.103	0.700 ± 0.084	0.630	Additive	n.s.[b]
1000 units/ml	0.720 ± 0.043	0.421 ± 0.080	0.529	Additive	n.s.
10000 units/ml	0.223 ± 0.013	0.092 ± 0.006	0.164	SYNERGY	0.03
4′-EDX (3.0 ng/ml)	0.306 ± 0.015				
IFN-α_2 100 units/ml	0.860	0.303 ± 0.042	0.263	Additive	n.s.
1000 units/ml	0.720	0.136 ± 0.030	0.220	Additive	n.s.
10000 units/ml	0.223	0.027 ± 0.010	0.068	SYNERGY	0.16
4′-DDX (1.4 ng/ml)	0.391 ± 0.047				
IFN-α_2 100 units/ml	0.860	0.234 ± 0.033	0.336	Additive	n.s.
1000 units/ml	0.720	0.070 ± 0.019	0.281	SYNERGY	0.03
10000 units/ml	0.223	0.017 ± 0.005	0.087	SYNERGY	0.02
4-DDR (1.3 ng/ml)	0.165 ± 0.043				
IFN-α_2 100 units/ml	0.860	0.089 ± 0.028	0.142	Additive	n.s.
1000 units/ml	0.720	0.058 ± 0.005	0.119	SYNERGY	0.14
10000 units/ml	0.223	0.013 ± 0.002	0.037	SYNERGY	0.09

[a] Fractional colony growth (60 μm)
[b] n.s., not statistically significant

gistic response. For 4'-DDX and 4-DDR, subadditive drug interactions were noted. The effect of the 4'-EDX analogue was additive at each IFN-α_2 dose tested.

In all trials of the doxorubicin derivatives in combination with IFN-α_2 against the cervical carcinoma line, CaSki, an additive response was found (Table 4). This cell line is more sensitive to direct antiproliferative effects of IFN-α_2 as a single agent than the other lines investigated in this study. Regardless of the dose of IFN-α_2 used, each derivative proved to have an additive effect in combination.

Table 5 demonstrates the subadditive and antagonistic interactions of IFN-α_2 with the anthracycline analogues against the melanoma cell line, SK-Mel-28. The efficacy of 4'-EDX combinations was approximately half that expected, and 4-DDR with 10000 units/ml IFN-α_2 was only 0.19 times as active as an additive interaction would have predicted.

Discussion

In vitro tumor cloning has demonstrated its effectiveness in screening new compounds for antitumor activity [18]. This technique may prove to be more appropriate than other in vitro methods in testing the antitumor efficacy of anthracycline derivatives, since it has been reported that there is no correlation between adriamycin cytotoxicity and metabolic endpoints of activity, such as incorporation of labeled thymidine or RNA precursors [19].

We used human tumor cell lines in a soft agarose clonogenic assay, and our results demonstrate that doxo-

Table 3. Antiproliferative effect of anthracycline analogues in combination with IFN-α_2 against MCF-7

Treatment	Single agent FCG[a]	Combination treatment FCG		Interaction	*P*
		Observed	Expected		
DOX (10.0 ng/ml)	0.488 ± 0.088				
IFN-α_2 10 units/ml	1.058 ± 0.053	0.282 ± 0.051	0.520	Additive	n.s.
100 units/ml	0.944 ± 0.104	0.256 ± 0.054	0.461	Additive	n.s.
1000 units/ml	1.092 ± 0.175	0.265 ± 0.019	0.533	SYNERGY	0.12
4′-EDX (11.0 ng/ml)	0.270 ± 0.035				
IFN-α_2 10 units/ml	1.058	0.244 ± 0.046	0.285	Additive	n.s.
100 units/ml	0.944	0.223 ± 0.013	0.255	Additive	n.s.
1000 units/ml	1.092	0.194 ± 0.043	0.295	Additive	n.s.
4′-DDX (3.1 ng/ml)	0.191 ± 0.052				
IFN-α_2 10 units/ml	1.058	0.283 ± 0.062	0.202	Subadditive	n.s.
100 units/ml	0.944	0.247 ± 0.017	0.180	Subadditive	n.s.
1000 units/ml	1.092	0.192 ± 0.044	0.209	Additive	n.s.
4-DDR (0.5 ng/ml)	0.471 ± 0.033				
IFN-α_2 10 units/ml	1.058	0.445 ± 0.049	0.498	Additive	n.s.
100 units/ml	0.944	0.505 ± 0.076	0.445	Subadditive	n.s.
1000 units/ml	1.092	0.609 ± 0.110	0.515	Subadditive	n.s.

[a] Fractional colony growth

Table 4. Antiproliferative effect of anthracycline analogues in combination with IFN-α_2 against CaSki

Treatment	Single agent FCG	Combination treatment FCG		Interaction	*P*
		Observed	Expected		
DOX (0.8 ng/ml)	0.943 ± 0.038				
IFN-α_2 10 units/ml	0.688 ± 0.076	0.869 ± 0.035	0.679	Additive	n.s.
100 units/ml	0.601 ± 0.078	0.534 ± 0.032	0.593	Additive	n.s.
1000 units/ml	0.313 ± 0.031	0.257 ± 0.013	0.309	Additive	n.s.
4′-EDX (1.0 ng/ml)	0.828 ± 0.140				
IFN-α_2 10 units/ml	0.688	0.765 ± 0.084	0.570	Additive	n.s.
100 units/ml	0.601	0.483 ± 0.053	0.497	Additive	n.s.
1000 units/ml	0.313	0.258 ± 0.018	0.259	Additive	n.s.
4′-DDX (0.3 ng/ml)	0.779 ± 0.210				
IFN-α_2 10 units/ml	0.688	0.712 ± 0.114	0.536	Additive	n.s.
100 units/ml	0.601	0.598 ± 0.030	0.468	Additive	n.s.
1000 units/ml	0.313	0.263 ± 0.026	0.244	Additive	n.s.
4-DDR (0.07 ng/ml)	0.949 ± 0.104				
IFN-α_2 10 units/ml	0.688	0.785 ± 0.039	0.654	Additive	n.s.
100 units/ml	0.601	0.404 ± 0.077	0.570	Additive	n.s.
1000 units/ml	0.313	0.269 ± 0.038	0.297	Additive	n.s.

Table 5. Antiproliferative effect of anthracycline analogues in combination with IFN-α_2 against SK-Mel-28

Treatment	Single agent FCG	Combination treatment FCG		Interaction	*P*
		Observed	Expected		
DOX (2.0 ng/ml)	0.421 ± 0.051				
IFN-α_2 100 units/ml	0.632 ± 0.139	0.494 ± 0.124	0.297	Subadditive	n.s.
1000 units/ml	0.650 ± 0.033	0.498 ± 0.015	0.305	Subadditive	n.s.
10000 units/ml	0.118 ± 0.028	0.128 ± 0.045	0.055	Subadditive	n.s.
4′-EDX (2.0 ng/ml)	0.417 ± 0.033				
IFN-α_2 100 units/ml	0.632	0.758 ± 0.205	0.264	ANTAGONISM	0.14
1000 units/ml	0.650	0.457 ± 0.123	0.271	Subadditive	n.s.
10000 units/ml	0.118	0.109 ± 0.017	0.049	ANTAGONISM	0.18
4′-DDX (0.2 ng/ml)	0.572 ± 0.126				
IFN-α_2 100 units/ml	0.632	0.580 ± 0.197	0.362	Subadditive	n.s.
1000 units/ml	0.650	0.333 ± 0.097	0.372	Additive	n.s.
10000 units/ml	0.118	0.101 ± 0.036	0.067	Subadditive	n.s.
4′-DDR (1.4 ng/ml)	0.667 ± 0.293				
IFN-α_2 100 units/ml	0.632	1.093 ± 0.240	0.422	Subadditive	n.s.
1000 units/ml	0.650	0.692 ± 0.159	0.434	Subadditive	n.s.
10000 units/ml	0.118	0.419 ± 0.034	0.079	ANTAGONISM	0.10

rubicin was less active than any of the three analogues tested. The ranking of activity from most potent to least was 4-demethoxydaunorubicin > 4′-deoxydoxorubicin > 4′-epidoxorubicin > doxorubicin. These potencies corroborate those reported for in vitro [8] and in vivo [4, 7, 21] studies. For the same compound in the different cell lines, the dose which inhibited tumor colony formation by 70% showed considerable variation, spanning at least an order of magnitude. Interestingly, the breast cancer cell line, MCF-7, was least sensitive to each of the derivatives, whereas one of the cervical carcinoma cell lines, CaSki, proved to be most sensitive to each of the analogues. This finding suggests that relative sensitivity or insensitivity of tumors to anthracyclines and their derivatives is a tumor cell-specific phenomenon; that is, cells are relatively sensitive or resistant to this class of compounds, irrespective of structural changes. It is of interest to note that when the cell lines are ranked by order of sensitivity to each compound the same sequence is always found. Numerically, averaging the ID_{70}'s of the four drugs, SK-Mel-28 is 3.1 ± 1.0 times less sensitive than CaSki, while BG-1 and MCF-7 are 4.7 ± 5.0 and 19.0 ± 3.4 times less sensitive. Such results suggest that modification of the structure of doxorubicin, in the ways represented by the compounds studied in this report, is insufficient to overcome relative drug insensitivity to the anthracyclines. Alternately, anthracycline derivatives show the same change in potency across cell lines of differing sensitivities.

Studying three different 4-demethoxyanthracyclines, Twentyman et al. [21] also observed that although analogues showed a range of potency relative to doxorubicin, the new compounds were unable to inhibit cells made resistant to doxorubicin. Interestingly, the degree to which a tissue accumulates adriamycin is related to the efficacy of adriamycin in clinical trials [5]. A reduction of intracellular drug levels, however, is not indicative of the cellular

basis of resistance to doxorubicin [1, 2, 13]. The relative efficacies of the analogues may be partially a function of cellular retention of the drug as well as a manifestation of differing DNA intercalation potential [6] or DNA topoisomerase binding [13] of the analogues. A cell's ability to exclude or remove adriamycin or to shield lethal targets from exposure to the drug may carry over to analogues with a similar structure. Salmon et al. [15] have presented the ranking of a series of anthracycline analogues based on their activity against seven fresh human malignancies in the tumor clonogenic assay. In their study they found that all three compounds presented in this report were more active than doxorubicin when used as single agents.

Against HeLa cells, Namba et al. [11] observed potentiation of the antitumor effects of adriamycin by interferon of the beta type (IFN-β). This potentiation was not limited to anthracyclines. Similarly, Kangas et al. [10] demonstrated some additive and synergistic combinations of cytotoxic agents in combination with interferons in a subrenal capsule assay of xenografts. This interaction was not accompanied by enhanced toxicity to normal tissue in the animals.

The potentiation of anthracycline cytotoxicity by IFN-α_2 appears to be a tumor-specific phenomenon. Whereas the relative efficacies of the analogues studied in this investigation were consistent between the different cell lines, the positive interactions with IFN-α_2 were cell line-specific. It is noteworthy that the cell line least responsive to growth inhibition by IFN-α_2, MCF-7, showed positive interactions to the drug combination (additive to synergistic), while the cell line most sensitive to colony inhibition by IFN-α_2, CaSki, was only additive in response. Alternately, in those cell lines in which synergistic and antagonistic interactions occurred between anthracycline analogues and IFN-α_2 (BG-1 and SK-Mel-28, respectively), there was not a striking difference in the inherent sensitivity of either cell line to the different compounds. Positive, antiproliferative effects of IFN-α_2 in combination with doxorubicin and its derivatives appear to be uniquely related to intrinsic interactions between the effects of the agents on the cell and cannot be predicted on the basis of the efficacy of the drugs as single agents.

Circumstances which lead to the improved use of IFN-α_2 in combination with cytotoxic agents will be difficult to define. The results of the present investigation suggest that the most profound influences on positive drug interactions are dependent on features of the target cells which appear to be unrelated to inherent sensitivity to either the interferon or the anthracycline. In vitro clonogenic assays using fresh human tumors should prove of value in determining the general nature of the drug interactions described. Additionally, it will be important to address the biological basis of the positive drug interactions using definable features of tumor cells in order to identify those patients who will ultimately benefit from such novel therapeutic modalities.

Acknowledgements. The expert technical assistance of Yvonne Dennard and Jeffrey A. Johnson is gratefully acknowledged. Appreciation is expressed to Farmitalia Carlo Erba, Milan, Italy, for research support (Dr. F. C Giuliani) and for the provision of anthracycline compounds (Dr. F. Arcamone). IFN-α_2 was kindly supplied by Dr. Paul Trotta of The Schering Corporation, Bloomfield, N. J., USA

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Received November 24, 1986/Accepted February 3, 1987