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Enhancement of anthracycline and alkylator cytotoxicity by ethacrynic acid in primary cultures of human tissues*

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Summary. Ethacrynic acid [2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxyl] acetic acid, is a water-soluble diuretic agent that has been shown to potentiate the in vitro cytotoxicity of chemotherapeutic agents in established cell lines. We used the differential staining cytotoxicity (DiSC) assay to determine whether ethacrynic acid at 1 and 3.3 μM would potentiate the cytotoxicity of nitrogen mustard and/or doxorubicin in primary cultures of hematologic neoplasms from heavily pretreated patients and in normal peripheral blood lymphocytes. At 3.3 μM, ethacrynic acid was toxic to 8 of 24 (33%) tumor specimens studied. In subsequent studies, ethacrynic acid at 1 μM was toxic to only 2 of 54 (4%) tumor specimens. Significant enhancement for doxorubicin or nitrogen mustard was confined to lymphatic malignancies and to normal peripheral blood lymphocytes. Interspecimen variability was observed, with no enhancement in most individual specimens, 2-fold enhancement in some specimens, and 4-fold enhancement in occasional specimens. Clinical trials will be required to determine whether the observed in vitro activity for ethacrynic acid is associated with clinical benefit in unselected or assay-selected patients.

Introduction

Resistance to antineoplastic agents represents a major impediment to curative therapy in modern oncology. Investigators have identified several basic mechanisms of acquired drug resistance in cancer cells, including enhanced drug efflux associated with the P-glycoprotein [7, 21, 23]; enhanced repair of drug-induced injury [16, 20]; and enhanced detoxification of drug intermediates, specifically related to intracellular glutathione [2, 26, 27] and glutathione-associated enzymes [14, 28]. The latter mechanism is potentially perturbable at several points. Buthionine sulfoximine, an inhibitor of glutathione synthesis, has been used to resensitize cell lines that are resistant to alkylating agents, cisplatin, and anthracyclines [10, 12, 22]. The nitrosoureas, through carbamoylation, inhibit the enzyme glutathione reductase, thereby depleting cells of reduced glutathione (GSH). These latter agents have been investigated in alkylator-resistant cell lines [29].

The glutathione-S-transferases are a family of homo- and heterodimeric enzymes that participate in the detoxification of xenobiotics [11]. Glutathione-S-transferase amplification has been associated with resistance to antineoplastic agents in tumor cell lines [8, 13]. Ethacrynic acid, the phenoxyacetic acid diuretic agent, has been shown to bind glutathione-S-transferases [1, 15]. Based on these observations, investigators have identified potentiation of antineoplastic drug effects in tumor cell lines through co-incubation with ethacrynic acid [25, 30].

We have previously documented the ability of the differential staining cytotoxicity (DiSC) assay to detect accurately the presence of clinically acquired drug resistance in primary cultures of fresh human neoplasms [32, 33]. We have also described our preliminary results in the study of ethacrynic acid for the potentiation of alkylator and anthracycline cytotoxicity in drug-resistant human tumor specimens [19]. This report further describes the activity of ethacrynic acid in the enhancement of nitrogen mustard and doxorubicin cytotoxicity in human neoplastic cells and peripheral blood lymphocytes studied in primary culture using the DiSC assay.

Materials and methods

Drugs. Ethacrynic acid (Sodium Edecrin; Merck, Sharp and Dohme, West Point, Pa.) was prepared in 0.15 M NaCl solution at a stock concentration of 6.6 μM, then aliquoted into cryovials and stored at -70° C for later use. Nitrogen mustard (Mustargen; Merck, Sharp and Dohme) and doxorubicin (Adria Labs, Columbus, Ohio) were obtained from the hospital pharmacy, and stock concentrations were made up in 0.15 M NaCl solution, then aliquoted and stored at -70° C.

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Sample preparation. Fresh human tumor specimens were placed in Roswell Park Memorial Institute-1640 media (Flow Labs, McLean, Va.) containing 15% heat-inactivated fetal bovine serum, penicillin (100 IU/ml): streptomycin (100 µg/ml), 2 m*M* glutamine, and 15 units/ml preservative-free heparin for transport to the laboratory. Tumor cells from peripheral blood and bone marrow specimens were isolated by centrifugation over Lymphocyte Separation Medium (Organon Teknika, Durham, N. C.). Cells at the interface were aspirated off with a Pasteur pipet and washed twice in RPMI-1640 media. Lymphatic tissue containing non-Hodgkin's lymphoma were dissociated by mincing in a media-containing petri dish with scissors and forceps. Cells were collected with a Pasteur pipet and resuspended in media. Solid tissues other than lymphoma specimens were dissociated by mechanical teasing followed by a 2-h exposure to 0.8% collagenase I and 0.002% DNase I (Sigma Co., St. Louis, Mo.). Cells were collected by centrifugation, washed two times, and resuspended in fresh media. The number of viable cells in each specimen was determined using 0.4% trypan blue in 0.15 *M* NaCl in a standard hemacytometer counting chamber (Reichert Co., Buffalo, N. Y.).

Assay procedure. The DiSC assay was used as previously described [4, 32]; it involves a 4-day cell culture with continuous drug exposure in conical polypropylene microtubes. A 1-h preincubation of tumor cells (1×10^6/ml) with either ethacrynic acid (1.0 µ*M*) or control vehicle alone (0.15 *M* NaCl) was performed before the addition of other agents. After the preincubation, cytotoxic drugs were thawed and serial dilutions were prepared; 20 µl of each drug solution at the concentration to be tested was added to 80 µl of the cell suspension with and without ethacrynic acid. Control tubes contained vehicle (0.15 *M* NaCl) alone. All drug exposures were continuous, for the duration of the culture. All control and drug-treated tubes were incubated for 4 days at 37°C in an atmosphere containing 5% CO_2 in air. Following the incubation, 100 µl nigrosin: fast green dye containing 37,500 acetaldehyde-fixed duck red blood cells (DRBCs) was added to each culture tube, which was briefly vortexed. After 10 min, samples were cytocentrifuged onto glass slides and air-dried. Slides were stained with Wright/Giemsa for hematologic specimens and with hematoxylin/eosin for solid tumors. Cell survival was determined as the ratio of living tumor cells over simultaneously counted DRBCs for each slide using a Whipple disc (VWR Scientific, Los Angeles, Calif.), with cell survival of drug-treated samples being expressed as a percentage of the saline control values.

Results

Table 1 describes the histologic subtypes of the tissues studied. All tumor specimens were obtained from patients who had failed multi-agent chemotherapy; the average number of prior treatment regimens was 2 (range, 1–4). All patients had failed alkylator-based regimens and a majority had also failed anthracycline-based therapy. Peripheral blood lymphocytes were obtained from normal healthy volunteers.

Table 2 describes the results of cytotoxic drug exposure, expressed as the mean percentage of cell survival as compared with saline-treated values. Specimens in which ethacrynic acid alone was toxic (<80% control cell survival) were excluded from analysis of enhancement. Percentage of control cell survival was compared by the paired *t*-test at each of five concentrations studied for both doxorubicin and nitrogen mustard. Data provided include the mean percentage of control cell survival, the mean difference in percentage of control cell survival (D bar), and the *P* value at each concentration. Figure 1 is a plot of the percentage of control cell survival for individual studies completed in specimens from four different patients with chronic lymphocytic leukemia. It illustrates the degree of interspecimen variability of enhancement; this variability was also found in other tumor types (not plotted).

Table 1. Number of specimens according to histology

Diagnosis		Number evaluated
I.	CLL	10
II.	NHL	9
III.	ALL	7
IV.	MM	7
V.	AML	9
VI.	CML	3
VII.	ST:	7
	Small-cell lung cancer	3
	Unknown primary site	2
	Breast cancer	1
	Head and neck squamous cell cancer	1
VIII.	N.Ly	8

CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ST, solid tumors; N.Ly, normal peripheral blood lymphocytes

To evaluate the relative activity of ethacrynic acid potentiation of doxorubicin and nitrogen mustard in different histologic classes, we compared each tumor type with regard to (a) potentiation of *either* agent, (b) potentiation of *each agent alone*, and (c) potentiation of *both* agents in the same sample. For this analysis, potentiation was defined as a 2-fold or greater enhancement in the activity of the cytotoxic drug at two or more consecutive points on the dose-response curve (a shift to the left of the dose-response curve or a dose-modifying factor of ≥2). The results are provided in Table 3.

Discussion

Enhancement of antineoplastic drug activity by nontoxic modifying agents represents a potentially important direction for the management of acquired and/or de novo drug

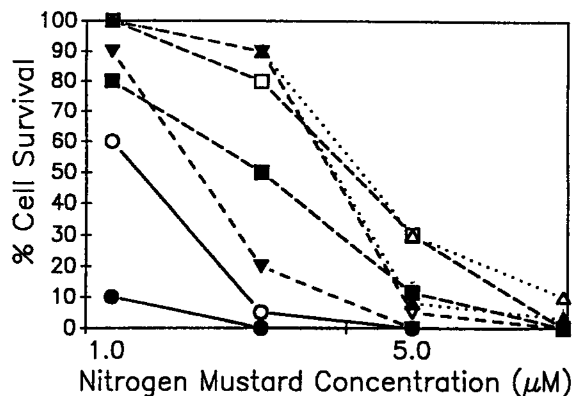

Fig. 1. Dose-response curves in specimens obtained from four patients with chronic lymphocytic leukemia, indicating individual variations in the degree of drug sensitivity and the degree of modification by ethacrynic acid. Percentage of saline-control cell survival is plotted on the y-axis; concentration of nitrogen mustard (1–7 µ*M*) is plotted on the x-axis. *Open symbols*, drug alone; *closed symbols*, drug plus 1 µ*M* ethacrynic acid

Table 2. Degree of enhancement of nitrogen mustard and doxorubicin cytotoxicity by 1 μ*M* ethacrynic acid in tumor specimens and normal lymphocytes

Diagnosis	Nitrogen mustard mean % control viability						Doxorubicin mean % control viability					
	(*n*)	(μ*M*)	NM	NM+EA	D	*P*	(*N*)	(μ*M*)	DOX	DOX+EA	D	*P*
CLL	10	28	0	0	0	>0.5	9	1.0	7.2	3.9	3.3	<0.2
	10	14	1.5	0	1.5	<0.2	9	0.5	40	18	16	<0.04
	10	7	12	3.5	8.0	<0.02	9	0.25	70	50	20	<0.03
	10	3.5	50	27	23	<0.025	9	0.13	94	76	18	<0.03
	10	1.7	71	54	17	<0.015	9	0.07	100	91	8.9	<0.03
NHL	9	28	0	0	0	>0.5	8	1.0	12	6.7	4.8	<0.15
	9	14	0	0	0	>0.5	8	0.5	21	14	6.9	<0.03
	9	7	10	3.9	6.1	<0.02	8	0.25	54	35	19	<0.05
	9	3.5	44	31	13	<0.08	9	0.13	78	67	11	>0.50
	9	1.7	73	63	10	<0.10	9	0.07	89	82	7.2	<0.05
ALL	6	28	0	0	0	–	7	1.0	0	0	0	–
	6	14	7.5	4.2	3.3	<0.2	6	0.5	4.2	0.7	3.6	<0.3
	6	7	28	19	8.3	<0.3	6	0.25	18	10	7.9	<0.35
	6	3.5	56	33	23	<0.08	6	0.13	66	34	32	<0.06
	6	1.7	70	51	19	<0.03	6	0.07	91	74	17	<0.1
MM	6	28	2.0	0	2.0	–	5	1.0	34	6.7	27	<0.2
	6	14	22	6.2	16	<0.06	7	0.5	85	64	20	<0.02
	6	7	65	38	27	<0.04	7	0.25	96	77	19	<0.2
	6	3.5	98	75	23	<0.08	6	0.13	100	88	11	<0.5
	6	1.7	100	90	10	>0.4	7	0.07	100	91	8.6	<0.5
AML	8	28	11	14	2.7	≥0.2	9	1.0	34	28	6.1	≥0.2
	9	14	24	23	1.6	≥0.2	8	0.5	51	36	16	≥0.2
	8	7	41	40	1.3	≥0.2	8	0.25	59	65	5.6	≥0.2
	8	3.5	49	46	3.3	≥0.2	8	0.13	77	81	3.9	≥0.2
	8	1.7	60	58	2.5	≥0.2	8	0.07	90	94	3.8	≥0.2
CML	3	28	0	0	0	–	3	1.0	47	40	6.7	≥0.5
	3	14	17	13	3.4	>0.4	3	0.5	53	53	0	≥0.5
	3	7	53	35	18	>0.2	3	0.25	57	67	10	≥0.5
	3	3.5	65	60	5.0	>0.2	2	0.13	70	90	20	≥0.5
	3	1.7	65	65	0	–	2	0.07	83	100	17	≥0.5
ST	6	28	3.3	0.8	2.5	<0.4	7	1.0	67	64	2.8	>0.5
	6	14	25	19	6.3	<0.3	7	0.5	71	69	2.8	>0.5
	6	7	72	66	5.8	<0.4	7	0.25	87	83	4.2	>0.5
	6	3.5	83	73	11	<0.5	6	0.13	100	100	0	–
	6	1.7	100	97	3.3	<0.4	7	0.07	100	100	0	–
N.Ly	8	28	0	0	0	–	8	1.0	6.3	7.5	1.2	>0.5
	8	14	0	0	0	–	8	0.5	23	28	5.0	>0.5
	8	7	0	0	0	–	6	0.25	43	40	3.3	<0.4
	8	3.5	5.6	4.4	1.2	<0.2	7	0.13	81	67	14	<0.05
	8	1.7	23	16	7.5	<0.025	8	0.07	93	89	3.7	<0.1

CLL, chronic lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ST, solid tumors; N.Ly, normal peripheral blood lymphocytes; NM, nitrogen mustard; EA, ethacrynic acid; DOX, doxorubicin; D, mean difference; *P*, probability value calculated using the paired *t*-test

resistance in human cancer. Recent observations in cell lines in vitro indicate activity for ethacrynic acid as a modifier of chlorambucil and carmustine cytotoxicity. The remaining question is whether these observations will translate into clinically relevant degrees of enhancement. As a first step to address this issue, we studied ethacrynic acid in several tumor types at concentrations that were, for the most part, nontoxic to the tumor cells or to normal lymphocyte controls. We found that certain tumor specimens obtained from clinically drug-resistant human neoplasms can be sensitized to the effects of the alkylating agent nitrogen mustard and the anthracycline doxorubicin through co-incubation with nontoxic concentrations of ethacrynic acid. In this study, enhancement of cytotoxicity was confined to specimens obtained from patients with lymphatic malignancies and, to a lesser degree, to normal peripheral blood lymphocytes, with the effects being most pronounced in chronic lymphocytic leukemia. This held true for both doxorubicin and nitrogen mustard at several concentrations. Non-Hodgkin's lymphoma, multiple myeloma, and acute lymphoblastic leukemia (in decreasing order) showed some enhancement. Acute and chronic my-

Table 3. Comparison of enhancement of the cytotoxicity of nitrogen mustard, doxorubicin, or both drugs by ethacrynic acid according to histology

Specimen type	(*n*)	Dox- or NM-enhanced[a]	(*n*)	Only NM-enhanced[a]	(*n*)	Only DOX-enhanced[a]	(*n*)	Dox- and NM-enhanced[a]
CLL	10	7 (70%)	10	5 (50%)	9	4 (44%)	9	2 (22%)
ALL	7	3 (43%)	6	2 (33%)	6	3 (50%)	6	2 (33%)
MM	7	2 (29%)	6	2 (33%)	6	1 (16%)	6	1 (16%)
NHL	9	3 (33%)	9	1 (11%)	9	2 (22%)	9	0
AML	9	2 (22%)	9	0	9	2 (22%)	9	0
CML	3	0	3	0	3	0	3	0
ST	7	1 (14%)	6	1 (14%)	7	0	6	0
N.Ly	8	1 (12%)	8	1 (12%)	8	0	8	0

CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ST, solid tumors; N.Ly, normal peripheral blood lymphocytes; DOX, doxorubicin; NM, nitrogen mustard

[a] The number of specimens in which a ≥ 2-fold enhancement was observed at 2 or more consecutive points on the dose-response curve (dose-modifying factor, ≥2) after the addition of 1 μM ethacrynic acid

elogenous leukemia specimens and solid tumors revealed only minimal, if any, enhancement by ethacrynic acid of the cytotoxicity of either of the agents evaluated.

Initial studies conducted with ethacrynic acid at 3.3 and 1.0 μM revealed that 3.3 μM was toxic to a substantial number of tumor specimens (33%), in primary culture. For this reason, subsequent evaluations were conducted at 1 μM which resulted in toxicity to only 4% of studied specimens. Tew et al. [30] used ethacrynic acid at 10 μM in the Walker 256 carcinoma cell line, and Smith et al. [25] reported the use of 20 μM ethacrynic acid in 9L rat gliosarcoma cell lines obtaining little direct toxicity. This may reflect the characteristics of the particular cell lines studied or the durations of exposure. Our observation of ethacrynic acid cytotoxicity is of interest and possibly suggests that the drug itself might be tumoricidal if adequately high concentrations could be achieved in vivo.

Ethacrynic acid was found to potentiate significantly the activity of nitrogen mustard and doxorubicin at one of the five concentrations studied for each of these agents (1.7 and 0.13 μM, respectively) as evaluated in normal peripheral blood lymphocytes. In this report, the concentration ranges for cytotoxic drugs were selected for their known activities against drug-resistant tumor cells [33]. The resultant substantial cell kill obtained in normal peripheral blood lymphocytes at these cytotoxic drug concentrations prevented the identification of enhancement by ethacrynic acid in these cells at higher concentration ranges. An additional finding in this study was the degree of variability between individual specimens within a diagnosis. Samples manifested substantial interspecimen variability as illustrated by the survival plots of individual specimens from patients with chronic lymphocytic leukemia (CLL) (Fig. 1).

The actions of many chemotherapeutic agents are multifactorial. Anthracyclines have been shown to be free radical generators [3] and inhibitors of topoisomerase II [31]. Functioning through the release of electrophilic intermediates, alkylating agents cause DNA alkylation as well as lipid peroxidation and the release of free-radical oxygen species. Cellular defenses against many of these processes occur through intracellular thiols, the most abundant nonprotein thiol being glutathione.

Possible mechanisms of action for ethacrynic acid include depletion of intracellular glutathione and inhibition of glutathione-S-transferase activity. Alterations in antioxidant enzymes in CLL cells have been described [9], and the expression of glutathione-S-transferase isozymes in CLL cells has been shown to vary considerably in individual patient specimens [24]. Isozyme expression variability has also been identified in human lung-tumor biopsies [5]. Of course, it is possible that the actions of ethacrynic acid observed in our system may be entirely unrelated to the glutathione redox cycle.

The intent of the current study was not to elucidate the mechanisms of ethacrynic acid's actions but, instead, to determine whether ethacrynic acid has the ability to enhance cytotoxic drug activity in primary cultures of human tissues and to identify the most promising target neoplasms for future clinical trials. Our observations suggest that in some individual tumors, drug resistance appeared to be perturbed by ethacrynic acid. Enhancement of cytotoxic drug activity was confined largely to lymphatic malignancies. The results suggest that among the tumor types evaluated, CLL and non-Hodgkin's lymphomas may be targets for resistance-modifying approaches that use ethacrynic acid. However, the degree of enhancement was lower than that observed in the same tissues at concentrations of verapamil and lidocaine that are completely nontoxic to normal lymphocytes ([33] and unpublished data). Although verapamil-lidocaine [18] and verapamil alone [6, 17] appear to have some efficacy in enhancing the clinical activity of doxorubicin-based chemotherapy, these clinical benefits have been short-lived in the setting of acquired drug resistance in advanced disease. Clinical trials will be required to determine whether the observed in vitro activity for ethacrynic acid is associated with clinical benefit in unselected or assay-selected patients.

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