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Activity of a chartreusin analog, elsamicin A, on breast cancer cells

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The *in vitro* activity of elsamicin A (ELS) was investigated compared with that of doxorubicin (DX) on two sensitive breast cancer cell lines: one estrogen receptor-positive (ER^+, MCF7) and one estrogen receptor-negative (ER^-, MDA-MB-231) line, and on a DX-resistant subline (MCF7DX). The activity of the two drugs was also investigated on 19 clinical breast cancer specimens from untreated patients. The drugs were tested at pharmacologically relevant concentrations, as calculated from the area under the curve for a 3 h exposure to the lethal dose producing 10% mortality (LD_{10}) in mice, and at 10- and 100-fold concentrations. In DX-sensitive lines, a greater inhibition of RNA and DNA precursor incorporation, as well as of cell proliferation, was caused by ELS than by DX. Moreover, the antiproliferative effect was 10-fold higher in the ER^+ MCF7 than in the ER^- MDA-MB-231 cell line (IC_{50}: 0.025 versus 0.21 μg/ml). ELS was cross-resistant to DX in the MCF7DX subline. In clinical specimens, effects on DNA precursor incorporation were more often observed for ELS than for DX at the same drug concentrations. The *in vitro* sensitivity to ELS was more pronounced for ER^+ than for ER^- tumors: minimal inhibiting concentrations of the drug were 0.1 and 3.5 μg/ml, respectively, in the two groups. If confirmed in a larger series of human breast tumors, these *in vitro* results would indicate a promising role for ELS in clinical treatment, mainly of ER^+ breast cancer patients.

Key words: Doxorubicin, elsamicin A, human breast cancer.

Introduction

Elsamicins are aminoglycosides, isolated from the fermentation broth of an unidentified actinomycete strain.[1] In particular, elsamicin A (elsamitrucin or Y-28090; ELS) has in common with chartreusin the aglycone, chartarin, and differs for the sugar moiety, which consists of neutral sugar and amino sugar, and which increases water solubility and confers different kinetics. The amino sugar moiety is responsible for the *in vitro* and *in vivo* antitumor activity, which has not been observed for the analog elsamicin B, and for the higher activity of ELS than chartreusin.[2]

A relevant antitumor activity of ELS has been detected in murine P388 leukemia and B16 melanoma cell lines *in vitro* by a microtiter cytotoxicity assay, and in MX-1 human breast cancer xenografts implanted in the subrenal capsule in mice.[3] In the P388 leukemia cell line, antitumor activity was related to the induction of DNA single- and double-strand breaks.[4] A cross-resistance with doxorubicin (DX) and other antitumor antibiotics, consequent to a reduction of drug retention, has been described in DX-resistant P388 leukemia cell line.[4]

In view of the interesting activity of ELS on the MX-1 human breast cancer line, and in order to further explore the clinical therapeutic potential of ELS, we investigated its antimetabolic and antiproliferative activity in cell lines and clinical specimens of breast cancer with different estrogen receptor (ER) profiles. The activity of ELS was also analyzed compared with that of DX in cell lines and in tumor surgical specimens sensitive or resistant to the anthracycline.

Materials and methods

Cell lines and clinical specimens

MCF7 is an ER-positive (ER^+) cell line obtained from pleural effusion of a human adenocarcinoma of the breast.[5] Its DX-resistant subline (MCF7DX) was developed by selection with excalating doses of DX.[6] MDA-MB-231 is an ER-negative (ER^-) cell

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line also obtained from pleural effusion of a human adenocarcinoma of the breast.[7] MCF7 and MDA-MB-231 cell lines were maintained as monolayer cultures in Dulbecco's F12 minimal essential medium enriched with 10% fetal calf serum. MCF7DX cells were grown in medium containing 10 μM DX and passaged for at least 3 weeks in drug-free medium before each experiment. Clinical specimens were obtained from 19 previously untreated breast cancer patients during mastectomy and immediately processed. Tumor sample adequacy was verified by the pathologist.

Drugs and concentrations

ELS (succinate salt) was kindly provided by Dr A M Casazza from the Experimental Chemotherapy Laboratory, Bristol-Myers Co. (Willingford, CT). DX was purchased from Farmitalia-Carlo Erba (Nerviano, Italy). The drugs were reconstituted in saline. ELS was stored as stock solutions at $-80°C$ and DX was stored as a powder. Basic concentrations for ELS and DX were calculated from the area under curve (AUC) obtained after a 3 h drug exposure to the LD_{10} in mouse,[8] corresponding to the AUC at the maximal tolerated dose in man according to Collins *et al.*[9] These concentrations corresponded to 0.1 and 0.8 μg/ml for ELS and DX, respectively. A second pharmacologically relevant concentration was calculated by the formula proposed by Tisman *et al.*,[10] according to the criteria previously used in our studies,[11] and corresponded to 1 and 3.5 μg/ml for the two drugs, respectively. In addition 10- and 100-fold concentrations were tested for each drug.

In vitro chemosensitivity assays

In cell lines, drug activity was analyzed as the antiproliferative effect after prolonged exposure, and as the antimetabolic effect on DNA and RNA precursor incorporations, after short-term drug exposure. In clinical specimens, only the short-term antimetabolic effect was evaluated, owing to the difficulty of adequately growing cells from clinical breast tumors *in vitro*.[12]

For the evaluation of the antiproliferative effect, exponentially growing cells in 24-well microtiter plates were treated for 72 h with the drugs and during the last 24 h [^{3}H]thymidine ([^{3}H]dT, 5 μCi, specific activity 5 Ci/mmol) was added to the culture medium. The radioactivity incorporated in DNA was evaluated in trichloroacetic acid (TCA)-precipitable material after overnight solubilization in 0.3 M KOH at room temperature by liquid scintillation counting, as previously described.[13]

The drug effects on DNA or RNA synthesis was evaluated according to the previously described methods for cell suspensions[14] and tissue fragments.[15] Briefly, exponentially growing cell lines or small fragments from clinical specimens were incubated for 3 h with the drugs. During the last hour of treatment, [^{3}H]dT and [^{3}H]uridine (5 μCi, specific activity 5 Ci/mmol and 5 μCi, specific activity 28 Ci/mmol, respectively) were added to the culture medium. Nucleic acids were extracted from TCA-precipitable material and separated by alkaline/acid extraction. Precursor incorporation was evaluated by liquid scintillation counting. In solid samples, the incorporation was expressed as fractional incorporation.[15] Samples incubated with drug solvent or drugs were run in triplicate.

For both the antiproliferative and antimetabolic assays, drug effects were expressed as the ratio between the incorporation values in drug-treated versus control samples. In human specimens, any inhibition exceeding the median coefficient of variation of incorporation values in control samples (20%) was considered significant, according to our previously reported criteria.[11,15]

Determination of proliferative activity

Cell lines and tumor fragments were incubated with [^{3}H]dT for 1 h at 37°C. Proliferative activity was evaluated by autoradiography, as previously described,[16] and expressed as the [^{3}H]dT labeling index ([^{3}H]dT LI), i.e. the ratio between the number of labeled cells and the total number of cells.[16] [^{3}H]dT LI was around 40% for all cell lines growing exponentially and ranged from 0.1 to 16.2% in human tumors.

Evaluation of cytoplasmatic ER content

The ER content was assessed by using the dextran-coated-charcoal technique according to the EORTC method[17] and evaluated according to Scatchard.[18] The cutoff value used to define ER^+ and ER^- tumors was 10 fmol/mg cytosol protein.

Data analysis

The mean IC_{50} values for the two drugs were compared by Student's *t*-test.

Results

The antiproliferative activity of ELS and DX in the different breast cancer cell lines is reported in Table 1. MCF7DX was cross-resistant to ELS. In DX-sensitive cell lines, ELS was about 10-fold more active in ER^+ than in ER^- cells and the IC_{50} was 3 times lower and only slightly lower, respectively, than the DX IC_{50}.

The effect on DNA and RNA precursor incorporation after 3 h of treatment is shown in Table 2. An antimetabolic effect was caused by ELS on the MCF7DX cell line. In particular, the IC_{50} was of the same order as that observed for DX-sensitive cell lines for RNA precursor incorporation and 10 times higher for DNA precursor incorporation. Moreover, in both DX-sensitive cell lines, DNA and RNA syntheses were similarly affected by DX, whereas at least in the MCF7 ER^+ line, the ELS IC_{50} was 5 times lower for RNA than for DNA precursor incorporation.

The activity of ELS in relation to that of DX was studied in 19 clinical specimens from breast cancer patients. Cell kinetics were evaluated as [^{3}H]dT LI in 14 of these 19 tumors (Table 3). LI values ranged from 3.5 to 16.2% for ER^- tumors and from 0.1 to 5.5% for ER^+ tumors. The median LI value was higher for ER^- than ER^+ tumors. Drug activities were analyzed as interference in nucleic acid synthesis on solid tumor samples immediately after surgery. In particular, DNA synthesis was chosen as the more representative indicator of any antiproliferative effect. As shown in Table 4, 0.1 μg/ml represented the minimal concentration at which an inhibition by ELS could be observed (one of 19 sensitive tumors). In contrast, no inhibition by DX could be observed in this case series up to 3.5 μg/ml. Moreover, a marked increase in the frequency of sensitive tumors could be observed at 10 μg/ml for ELS and only at 35 μg/ml for DX.

Analysis as a function of ER content showed a trend in favor of a higher sensitivity to ELS in ER^+ than in ER^- tumors (Table 4).

Table 1. Antiproliferative effect of ELS and DX in breast cancer cell lines

	IC_{50} (μg/ml)[a]	
	ELS	DX
MCF7 ER^+	0.025[b]	0.09
MDA-MB-231 ER^-	0.21[b]	0.35
MCF7DX	>80	47

[a] Mean value from four independent experiments.
[b] $p < 0.05$; Student's *t*-test.

Table 2. Antimetabolic activity of ELS and DX in breast cancer cell lines

	IC_{50} (μg/ml)[a]			
	ELS		DX	
	RNA	DNA	RNA	DNA
MCF7 ER^+	0.07[b]	0.35[b]	0.50	0.18
MDA-MB-231 ER^-	0.20	0.55	1.00	0.80
MCF7DX	0.07[c]	4.00[c]	50.0	40.0

[a] Mean value from four independent experiments.
[b] $p < 0.05$; Student's *t*-test.
[c] $p < 0.05$; Student's *t*-test.

Table 3. Cell kinetics of 14 human breast tumors ([^{3}H]dT LI %) in relation to ER status

ER^+	ER^-
0.1	3.5
1.5	3.8
2.0	5.1
2.4	6.6
4.1	13.2
4.1	16.2
4.5	
5.5	
3.25[a]	5.85
3.03 ± 1.81[b]	8.07 ± 5.34

[a] Median.
[b] Mean ± SD.

Discussion

Breast cancer is one of the most important causes of death in women. The search for drugs with specificity against tumor cells or more active and non-cross-resistant with those of the conventional armamentarium therefore remains an important goal. The activity of ELS on breast cancer cell lines transplanted in the subrenal capsule in mice indicated a potential therapeutic importance for this neoplasm. In the present study the antimetabolic and antiproliferative activities of ELS were investigated and analyzed in comparison with those of DX, also a product of biosynthesis, on established cell lines and on clinical breast cancers.

Table 4. Activity of ELS and DX on DNA synthesis in clinical specimens

Drug dose (μg/ml)	No. of sensitive tumors/no. of tested tumors							
	ELS				DX			
	overall	ER^+	ER^-	unknown	overall	ER^+	ER^-	unknown
0.1	1/19	1/10	0/6	0/3	ND[a]	ND	ND	ND
0.8	1/19	1/10	0/6	0/3	0/19	0/10	0/6	0/3
1.0	2/19	2/10	0/6	0/3	0/19	0/10	0/6	0/3
3.5	2/19	2/10	0/6	0/3	1/19	0/10	1/6	0/3
10.0	8/19	5/10	1/6	2/3	3/19	2/10	0/6	1/3
35.0	6/9	3/4	2/4	1/1	7/15	4/9	1/3	2/3

[a] ND, not done.

Drugs were tested at pharmacologically relevant concentrations chosen on the basis of the AUC obtained with a 3 h exposure to the LD_{10} in mice. For ELS, phase I clinical studies confirmed the reliability of this criterion.[8] Moreover, additional concentrations defined according to the formula proposed by Tisman[10] were also tested. ELS concentrations derived in such a way are in the range of peak plasma levels obtained with this drug in clinical pharmacokinetic studies.[8]

ELS caused a greater antiproliferative effect in ER^+ than in ER^- established breast cancer cell lines. Such preferential activity was consistently observed in a preliminary series of clinical breast tumors. The finding is of utmost importance considering the high frequency (80%) of ER^+ human breast cancers and needs to be confirmed in a larger series of clinical tumors. The higher activity cannot be explained on the basis of a higher proliferative rate, since ER^+ and ER^- cell lines have a similar proliferative rate, and ER^+ tumors in the present series, as in larger series,[16] showed a lower proliferative activity than ER^- tumors. Therefore, other mechanisms are probably responsible for the differential activity of ELS as a function of ER status.

ELS showed a greater activity than DX both in DX-sensitive cell lines and clinical tumors, and it was cross-resistant with the anthracycline. Conversely to DX, which inhibited DNA and RNA synthesis to a similar extent, ELS inhibited RNA more than DNA synthesis in both sensitive and resistant cell lines. This greater effect on RNA synthesis could be related to the early inhibition of topoisomerase I activity, as already reported in human and mouse tumor cell lines.[4] The lack of activity on DNA synthesis in the MCF7DX cells could be explained by the repair of DNA single-strand breaks and/or by the lack of inhibition of topoisomerase II activity and induction of double-strand breaks, as already reported for P388 cells resistant to DX.[4]

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