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SHORT COMMUNICATION

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In vitro antitumor activity of 3′-desamino-3′(2-methoxy-4-morpholinyl) doxorubicin on human melanoma cells sensitive or resistant to triazene compounds

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Abstract A new methoxymorpholinyl derivative of Adriamycin (ADR), FCE 23762 (MRD), has recently been selected for phase I clinical trials for its reduced cardiotoxicity and for its cytotoxic activity against a broad spectrum of solid tumors and leukemias that are sensitive or resistant to ADR. The purpose of the present study was to compare the in vitro antitumor activity of MRD and ADR on human melanoma lines with different chemosensitivity to triazene compounds, among which dacarbazine remains a reference drug in the treatment of melanoma. Both MRD and ADR were tested in vitro on three melanoma lines, MI13443-MEL, SK-MEL-28, and M14, previously screened for their chemosensitivity to the triazene compound *p*-(3-methyl-1-triazeno) benzoic acid, potassium salt (MTBA). The three lines were also analyzed for P-170 expression, total glutathione (GSH) content, and GSH-related enzyme activity. All melanomas, whether sensitive or resistant to MTBA, were susceptible to anthracycline treatment. The cytotoxic activity of MRD was comparable with that of ADR, and no substantial difference was found in cell growth inhibition between the two drugs. When the relative chemosensitivity of the three lines was considered, SK-MEL-28 was found to be slightly less sensitive to MRD treatment than the other tumors. This finding seems to correlate with the higher GSH-peroxidase activity of this melanoma relative to that of the MI13443 and M14 lines. These results show a homogeneous response of melanoma lines to MRD treatment in vitro, suggesting that phase I clinical trials concerning this drug, which in vivo appears to be activated to a more cytotoxic metabolite, could be extended to metastatic melanomas, including those completely resistant to triazene compounds.

Key words Melanoma · Anthracyclines · Triazenes · Chemotherapy

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Introduction

The management of disseminated malignant melanoma represents one of the most challenging problems in clinical oncology [22]. In monochemotherapy regimens the triazene compound (TZC) dacarbazine (DITC) remains the most effective agent, although total responses do not exceed 15–20% [23]. A significant improvement in the management of melanoma has been achieved with selected multidrug regimens [1, 6, 22, 24]. However, a pressing need remains for the identification of new chemotherapeutic agents effective against this tumor.

Anthracyclines are a class of antineoplastic agents with a wide spectrum of activity against leukemias and solid tumors. Among them, Adriamycin (ADR) is the most widely used, in spite of its high cardiotoxicity. Tumor-cell resistance to anthracyclines is mainly associated with overexpression of the P-170 membrane glycoprotein (P-170), which extrudes the drugs before cytotoxic intracellular concentrations can be reached [29]. However, other mechanisms of resistance to an-

thracyclines, in particular, high levels of glutathione (GSH) and GSH-related enzymes [29, 33, 39] and reduced activity of topoisomerase II [9, 30, 38], have been described.

Recently, many anthracycline derivatives have been developed in attempts to improve the antitumor activity and to overcome the cardiotoxicity that restricts the use of these drugs in cancer treatment. Among these derivatives, the ADR analogue FCE 23762 [3′-desamino-3′(2-metoxy-4-morpholinyl)-doxorubicin, MRD], is one of the most promising compounds. It is endowed with low-level cardiotoxicity and potent antitumor activity both in vitro and in vivo against ADR-resistant tumors and leukemias [12, 31, 36]. This compound has recently completed a phase I clinical study, the dose-limiting toxicity being reversible myelosuppression, producing little, if any, cardiotoxicity [37].

Relative to ADR, MRD is more lipophilic, and this allows rapid influx of the drug into cells, resulting in a high intracellular concentration, even in tumors expressing P-170 [13, 31]. Moreover, it has been shown that MRD can be activated by microsomal enzymes to more cytotoxic, not yet identified metabolite(s) with DNA interstrand cross-linking activity [16–18, 20]. The finding that the molecular mechanisms underlying MRD activity are at least in part different from those of ADR opens up the possibility that this drug could be effective on human tumors that are not highly sensitive to anthracyclines, including melanoma. No experimental data are presently available concerning the antitumor activity of MRD on melanomas. Therefore, the aim of the present study was to compare the in vitro suppressive activity of ADR and MRD on three human melanoma lines with different sensitivity to *p*-(3-methyl-1-triazeno) benzoic acid, potassium salt (MTBA) an in-vitro-active TZC [27].

Materials and methods

Cell lines

Three human melanoma cell lines, MI13443-MEL (hereafter referred to as MI13443), SK-MEL-28, and M14, were used in the present study. MI13443 [35] and M14 [10] were kindly supplied by Dr. G. Parmiani (National Cancer Institute, Milan, Italy) and Dr. G. Zupi (Regina Elena Institute, Rome, Italy), respectively. SK-MEL-28 was obtained from American Type Culture Collection (Bethesda, Md, USA).

All cells lines were maintained in RPMI-1640 culture medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 50 IU penicillin/ml, and 50 μg streptomycin/ml (all from Gibco Laboratories, Paisley, Scotland, UK; hereafter referred to as complete growth medium, CGM).

Drug treatment and cell-growth evaluation

ADR and its methoxymorpholinyl derivative MRD were kindly provided by Farmitalia Carlo Erba (Milan, Italy). MTBA was kindly supplied by Dr. G. Lassiani (Institute of Pharmaceutical Chemistry, University of Trieste, Trieste, Italy). Melanoma cells were plated (4 wells/point) in 6-well plates (Falcon, Beckton Dickinson Labware, Oxnard, Calif., USA) at 2×10^4 cells/well in 2 ml of CGM and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 18 h. ADR or MRD was then added in 1 ml of RPMI-1640 to give final concentrations ranging from 12.5 to 1600 n*M*, and cellular monolayers were incubated at 37 °C for 4 h. MTBA treatment was performed at concentrations ranging from 31.25 to 500 μ*M* for 1 h. At the end of the incubation period, melanoma cells were washed twice with phosphate-buffered saline (PBS) and reincubated in CGM at 37 °C in a humidified atmosphere containing 5% CO_2.

After 72 h of culture [25], viable cells (according to the trypan-blue dye-exclusion test) were counted in a hemocytometer. Cell-line chemosensitivity was expressed in terms of the 50% inhibitory drug concentration (IC_{50}, i.e., the concentration of drug capable of inhibiting cell growth by 50%) calculated on the regression line in which viable cell numbers were plotted against the logarithm of drug concentration.

Detection of P-170

For determination of P-170 expression, cells were permeabilized with 70% methanol at −20 °C, washed in PBS, and stained with fluorescein isothiocyanate (FITC)-labeled C219 monoclonal antibody (P-glycoCHEK C219, Centocor, Inc., Malvern, Pa.). Melanoma cells were then analyzed by the FACScan flow cytometer (Beckton Dickinson, Mountain View, Calif., USA) using Lysis II software.

O^6-Alkylguanine-DNA alkyltransferase activity assay

Cells extracts were assayed for O^6-alkylguanine-DNA alkyltransferase (OGAT) activity by measurement of the transfer of tritium-labeled methyl groups from a substrate DNA to the OGAT protein according to the method of Morten and Margison [26]. Labeled methylated substrate DNA for use in the OGAT assay was prepared using *N*-[^{3}H]-methyl-*N*-nitrosourea (Amersham, specific activity 23 Ci/mmol). OGAT activity was expressed in fentomoles of [^{3}H]-methyl group removed/per milligram of protein in the cell extract. The protein content of the cell extract was evaluated according to the method of Bradford [2].

Evaluation of GSH levels and of GSH-peroxidase and GSH-transferase activity in cell extracts

Total GSH was determined using the method of Griffith [14]. GSH-transferase (GSHT) activity was measured according to the method of Habig and Jakoby [15]. Selenium-dependent and selenium-independent GSH-peroxidase (GSHPx) activity was assayed as described by Lawrence and Burk [19] using H_2O_2 or cumenehydroperoxide as substrates. Protein concentration was determined following the method of Lowry et al. [21].

Statistical analysis

P values were calculated according to Student's *t*-test analysis.

Results

The antitumor effects of ADR and MRD were evaluated on MI13443, SK-MEL-28, and M14 cell lines selected for differential sensitivity to MTBA. The main biochemical lesion responsible for the cytotoxic activity of TZC appears to be the methylation of DNA at the O^6 position of guanine [5]. Methyl adducts at O^6-guanine

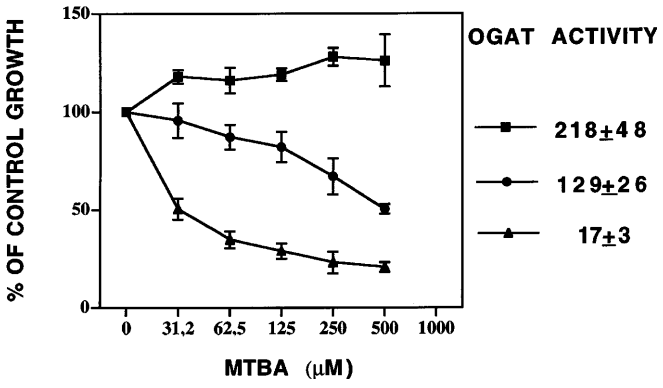

Fig. 1 Effect of MTBA on cell growth of melanoma lines expressing different OGAT levels. MI13443 (■), SK-MEL-28 (●), and M14 (▲) cells were treated with increasing concentrations of MTBA for 1 h as described in Materials and methods. Cell numbers were evaluated on quadruplicate samples at 72 h after drug removal. Data are expressed as mean values ± SD for the percentage of cell growth in drug-treated cultures relative to untreated cells. For each cell line the OGAT activity calculated in 3 different determinations and expressed as the mean value ± SD in fmol of [^{3}H]-methyl groups removed/mg protein is also shown

are removed by the DNA-repair enzyme OGAT [28] and, therefore, high levels of this enzyme are expected to be associated with cell resistance to TZC. The results illustrated in Fig. 1 confirm the existence of an inverse correlation between OGAT levels and cell sensitivity to MTBA. Indeed, the MI13443 line (OGAT activity 218 fmol/mg) was completely resistant to concentrations of MTBA ranging up to 500 μ*M*. On the other hand, SK-MEL-28 cells (OGAT activity 129 fmol/mg) showed an intermediate degree of sensitivity to MTBA, and the growth of the M14 line (OGAT activity 17 fmol/mg) was markedly inhibited by all drug concentrations tested.

Table 1 summarizes data concerning the sensitivity of melanoma lines to ADR and MRD as well as their P-170 expression, total GSH content, and GSHPx and GSHT activity. Chemosensitivity of melanomas is expressed in terms of IC_{50} values calculated on the concentration-response curves. The results show that both anthracyclines were equally suppressive for the three lines. Moreover, SK-MEL-28 appeared to be moderately but significantly ($P < 0.05$) less sensitive than the other two melanoma lines to MRD.

In terms of P-170 expression, total GSH content, and GSHPx and GSHT activity, the results illustrated in Table 1 show that (a) the three melanoma lines did not express the transmembrane protein P-170, the number of positive cells not exceeding 5%; (b) slight but significant differences ($P < 0.05$) in the intracellular level of GSH were found between MI13443 and the other two melanoma lines; (c) the GSHPx activity of SK-MEL-28 cells was significantly higher ($P < 0.01$) than that of M14 and MI13443 cells – essentially all the GSHPx activity detected in the three lines was due to selenium-dependent peroxidase, since the activity levels were almost the same when cumene hydroperoxide (Table 1) or hydroxiperoxide was used as the substrate (data not shown); and (d) the total GSHT activity found in MI13443 cells was significantly lower ($P < 0.01$) than that detected in the SK-MEL-28 or M14 line.

Discussion

Human melanomas are considered to be rather refractory to anthracycline treatment [1]. However, Chawla and co-workers [3] found that detrorubicine, a semi-synthetic anthracycline, was capable of inducing a high response rate in previously untreated patients with metastatic melanoma. In the present study the suppressive effects of the new anthracycline derivative MRD and of ADR on melanoma cells in vitro were comparatively analyzed on three cell lines previously selected on the basis of their sensitivity to TZC.

The results indicate that all three melanoma lines can be considered susceptible to ADR, whereas MI13443 cells were entirely resistant to the triazene MTBA. Indeed, the IC_{50} values recorded for ADR (Table 1) are in the range of those described for human tumor cell lines considered sensitive to ADR treatment [16, 32] and well under the peak plasma levels (5–10 μ*M*) obtainable after ADR administration to patients [34]. The inhibitory effect of MRD on melanoma cell growth does not sub-

Table 1 P-170 expression, GSH levels, GSH-related enzyme activity, and chemosensitivity of melanoma cells to ADR and MRD

Cell line	P-170[a]	GSH[b]	GSHPx[c]	GSHT[d]	IC_{50}	
					ADR[e]	MRD[e]
SK-MEL-28	2.0	1.35 ± 0.36	75 ± 1.10*	490 ± 61.5	305 ± 66.2	409 ± 62.2**
M14	1.0	1.45 ± 0.29	40 ± 4.95	570 ± 44.3	308 ± 16.3	247 ± 77.9
MI13443	4.5	2.16 ± 0.09**	30 ± 6.49	220 ± 67.5*	268 ± 10.7	248 ± 16.8

*$P < 0.01$, significantly different from the other two values in the same column
**$P < 0.05$, significantly different from the other two values in the same column
[a]Percentage of cells expressing the P-170 glycoprotein. Data refer to a representative experiment
[b]Total cellular GSH, expressed in nmol/10^6 cells
[c]Selenium-dependent GSH-peroxidase activity, expressed in mU/mg protein in the cell extract
[d]GSH-transferase activity, expressed in mU/mg protein in the cell extract
[e]Data are expressed as mean values ± SD for 3–5 experiments. GSH, GSHPx, and GSHT data are expressed as mean values ± SD for 3 different determinations

stantially differ from that of ADR. Moreover, analysis of cell-cycle perturbations in cells treated with MRD or ADR indicates that both drugs cause an accumulation of cells in the G_2/M phase, with no induction of apoptosis occurring in the range of concentrations tested (data not shown).

In several tumor models, both in vitro and in vivo, MRD has been described to be more active than ADR [12, 31, 36]. The greater efficacy of MRD has been correlated with its ability to reach high intracellular concentrations even in the presence of P-170 overexpression; to inhibit both topoisomerase I and topoisomerase II [7, 18]; and to generate DNA cross-linking metabolite(s) [18].

The finding that MRD and ADR are equally effective in inhibiting the melanoma lines used in this study can be explained by taking into account that (a) the three cell lines do not express P-170; (b) inhibition of topoisomerase I probably does not play a role in the cell-growth impairment induced by MRD – indeed, the IC_{50} values noted for MRD were lower than 500 n*M* for all melanoma lines, and Lau and co-workers [18] have shown that in a cell-free assay, topoisomerase I inhibition occurs at MRD concentrations higher than 500 n*M*; moreover, at this concentration, ADR has also been described to inhibit topoisomerase I [8]; and (c) activation of MRD to the more cytotoxic metabolite(s) requires the presence of cytochrome P450 mixed-function oxidase [11, 17, 20]. Cell lines generally show poor expression of most P450 isoforms, including CYP 3A4, which is reported to activate MRD [20].

As far as the relative cell-line chemosensitivity is concerned, the data illustrated in Table 1 show that no marked difference exists among the lines with regard to ADR treatment, whereas, SK-MEL-28 cells appear to be significantly less susceptible than the M14 or MI13443 line to MRD treatment. The data listed in Table 1 suggest that the minor susceptibility of SK-MEL-28 to MRD could be related to GSHPx activity. All three lines are indeed negative for P-170, and no correlation exists between GSH content or GSHT level and cell sensitivity to MRD.

The finding that MRD is as effective as ADR in inhibiting melanoma cell growth in vitro suggests the possibility that MRD could be significantly more active than ADR in vivo. However, in this regard, one must consider the dose of drug that can be clinically given. For ADR the standard clinical doses range between 60 and 90 mg/m^2 [1]. For MRD, according to the recent phase I clinical study [37], the maximum tolerated dose is 1.5 mg/m^2 given by i.v. bolus injection. Since it has been shown that metabolic activation of MRD in vivo results in at least an 80-fold increase in its potency [12], it can be speculated that MRD could actually be more effective than ADR on tumor cells showing the same in vitro chemosensitivity to ADR and to MRD. Moreover, it should be stressed that on the basis of equiactive doses in vivo, MRD shows substantially lower level cardiotoxicity than ADR [4, 37].

In conclusion, the results illustrated in the present report indicate that MRD has a cytotoxic effect against melanoma cell lines that are sensitive or even entirely resistant to TZC. Moreover, this agent was found to possess antimelanoma activity similar in vitro to that of ADR on an equimolar basis. Under these conditions it would be expected that MRD would be more active than ADR in vivo, although no direct proof is presently available to support this hypothesis in melanomas. In any case, the higher level of cytotoxic activity expressed by MRD after in vivo activation, its capability to overcome ADR resistance, and its decreased cardiotoxicity suggest that MRD could be a new interesting candidate for the treatment of advanced melanoma.

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