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Comparative in vitro toxicity of mitoxanatrone and adriamycin in human granulocyte-macrophage progenitor cells

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Summary. Mitoxantrone (MIT) has recently been introduced into cancer therapy as a possible substitute for the structurally related drug, adriamycin (ADR), because it causes less cardiotoxicity and fewer gastrointestinal side effects. However, the dose-limiting toxicity of MIT is pronounced neutropenia. The in vitro hematoxicity of both drugs in granulocyte-macrophage precursor cells (GM-CFCs) was analyzed using drug-exposure schedules analogous to the principles of the in vivo pharmacokinetics of MIT. Bone-marrow and peripheral-blood cells were exposed to 0.075–20 ng/ml MIT or ADR for 5, 20, 60, and 120 min, and for 14 days. The 14-day exposure resulted in D_0 values of 0.95 and 0.68 ng/ml for bone-marrow and peripheral-blood GM-CFCs subjected to MIT. Exposure to ADR resulted in D_0 values of 5.43 and 5.13 ng/ml, respectively. As was the case after 14-day exposure to MIT or ADR, short-term exposure again revealed that peripheral-blood GM-CFCs were more sensitive to both drugs. Moreover, at low concentrations, ADR was less toxic than MIT in both types of GM-CFCs, but was more toxic than MIT when a concentration of 20 ng/ml was used. The intracellular concentration of MIT, as measured by high-performance liquid chromatography, was constantly below 1 ng per 2×10^7 cells, even when it was applied at a concentration of 20 ng/ml for an exposure time of 2 h. The fact that such low concentrations of MIT are toxic for hemopoietic precursor cells may explain the myelotoxicity of this drug. However, the difference between the precursor-cell toxicity of MIT and that of ADR was small when their respective therapeutic doses were taken into consideration. Further analyses of their toxicity in stem cells and/or the microenvironment would appear to be needed.

Introduction

Mitoxantrone (MIT; dihydroxyanthracenedione) is a recently developed chemotherapeutic drug for cancer which belongs to the synthetic class of anthraquinones (for a recent review, see [26]). MIT intercalates to DNA, thus inhibiting nucleic-acid synthesis regardless of the cell-cycle phase [22, 26]. In both animal and in vitro test systems, MIT has been found to have cytotoxic effects [19, 25, 26], and its antitumor activity seems to be equal to or even better than that of structurally related anthracycline drugs [1, 26, 30]. Moreover, there is evidence that it has only weak cardiotoxic and gastrointestinal side effects [1, 3, 22, 26, 27, 31]. In clinical studies, the antitumor activity of MIT has been demonstrated in patients with advanced breast cancer, lymphoma, and leukemia [6, 11, 14, 16, 18, 24, 26, 28, 32]. For these reasons, MIT has been proposed as a possible substitute for the established anthracycline drug, doxorubicin (adriamycin; ADR), thus making it, in a sense, a 'second-generation drug'. However, the dose-limiting toxic effect of MIT is myelosuppression, especially neutropenia. In this context, it is of interest that OKunewick et al. [23] have shown that MIT is more toxic than ADR when applied to murine granulocyte-macrophage progenitor cells (GM-CFCs). To the best of our knowledge, there have been no studies of the effects of MIT on human GM-CFCs.

In order to compare the in vitro toxicity of MIT and ADR in human myeloid precursor cells, we investigated their effects in both permanent and short-term incubation schedules. Our experiments revealed that MIT has pronounced toxic effects on human GM-CFCs even when applied at very low concentrations. Pronounced differences between the in vitro toxicity of MIT and ADR in GM-CFCs were observed. However, in view of the different therapeutic doses applied, these differences with respect to toxicity do not appear to be the only factors responsible for the different degrees of in vivo myelosuppression produced by MIT and ADR.

Materials and methods

Drugs. Mitoxantrone (Novantrone) was supplied by Cyanamid-Lederle (Wolfratshausen, FRG) in its crystalline form. It was diluted in double-distilled water for use in our pilot experiments. All other experiments were performed using the commercially available form of the drug (Cynanamid-Lederle). Doxorubicin (Adriblastin) was purchased from Farmitalia Carlo Erba (Freiburg im Breisgau, FRG) and was diluted in sterile saline.

Preparation of cells. Normal human bone-marrow cells (BMCs) from patients with miscellaneous disorders were aspirated into a syringe containing preservative-free heparin. The patients underwent routine diagnostic bone-

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marrow puncture, and their hematopoiesis appeared to be normal in terms of the blood and bone-marrow findings. Alternatively, BMCs were obtained from patients undergoing thoracotomy for cardiac surgery or hip dissection for hip transplantation.

Normal peripheral blood cells (PBCs) were obtained from hematologically normal adults by being aspirated into a syringe containing preservative-free heparin.

PBCs and aspirated BMCs were diluted (1:2) in phospate-buffered saline (PBS) at human tonicity. The cells were layered over Percoll (1.077 g/ml; Seromed, West Berlin), and buffy-coat cells were obtained by centrifugation at 400 *g* for 30 min at room temperature. Interface cells were collected, washed three times in PBS, and than resuspended in cold PBS. Alternatively, BMCs from intraoperative specimens were flushed from the spongiosal cavities, and a single-cell suspension was prepared by repeated pipetting. Cell counting was performed in Türk's solution (Merck, Darmstadt, FRG) using a hemocytometer. All cells were kept on ice until further use.

Drug exposure. For the permanent drug-incubation schedule, mononuclear cells (MNCs) were added directly to culture medium containing various concentrations of MIT, ADR, or PBS. For the short-term incubation schedule, various concentrations of MIT and ADR were incubated with 10^6 MNCs per milliliter for different periods in a volume of 1 ml. PBS incubation served as a negative control. Cells were washed twice in PBS and kept on ice until used for culture.

GM-CFC assay. All cultures were performed in triplicate or quadruplicate in a volume of 1 ml in 35-mm plastic Petri dishes (Greiner, Nürtingen, FRG) using an equal-parts mixture of doublestrength Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) containing 40% fetal calf serum (FCS; Flow Labs, Meckenheim, FRG) and 0.6% Bacto agar (Difco, Detroit, Mich) to provide a final single-strenght medium containing 20% FCS and 0.3% agar. MNCs were added at a concentration of 10^5 per milliliter for BMC, and 10^6 per milliliter for PBC. The culture technique used has been described elsewhere [21]. The growth of GM-CFCs was stimulated by the addition of 10% giant-cell-tumor-conditioned medium (GCT-CM; Gibco) which provided a source of colony-stimulating factor [8, 9]. The cultures were allowed to gel at room temperature and were subsequently incubated for 14 days at 37° C in a humidified atmosphere consisting of 10% CO_2 in air. Aggregates containing 40 or more cells were scored as being colonies. Optical examination was performed using an Olympus dissecting microscope with indirect illumination at a magnification of $\times 32$.

The D_0 value, i.e., the drug concentration at which 37% of the cells survived exposure, was subsequently calculated for both bone-marrow- and blood-derived GM-CFCs.

High-performance liquid chromatography. After preincubation with MIT, 2×10^7 MNCs from each sample were not subjected to the GM-CFC assay, but instead were suspended in 1 ml PBS and subsequently lysed by repeated freezing and thawing. The intracellular drug concentration was determined by high-performance liquid chromatography (HPLC). The HPLC system, the chromatographic conditions, and the sample cleaning procedure used have been described elsewhere [12].

Table 1. Effects of crystalline MIT and Novantrone on human GM-CFC numbers in vitro

Drug	Survival of GM-CFCs (%)[a]
Crystalline MIT	32.1 ± 5.2
Novantrone	27.0 ± 5.0

BMCs of hematologically normal adults were permanently exposed to 1 ng/ml crystalline MIT or its commercially available therapeutic form (Novantrone). They were analyzed for GM-CFC numbers in soft agar using GCT-conditioned medium as a source of colony-stimulating factor

[a] Mean ± SEM of three experiments as a percentage of the negative control value

Results

The hematotoxic effects of MIT in its commercially available water-soluble form may be mediated by its dissolving agents, i.e., sodium metabisulfite, acetic acid, etc. [26]. In a first set of experiments, we therefore analyzed the effects of MIT in both its crystalline form, in which it is available for laboratory use only, and its dissolved form, in which it is available as a therapeutic drug. As can be seen from Table 1, significant differences with respect to the cytotoxicity of both forms of the drug were not detected. Moreover, it was difficult to keep the concentration of the dissolved crystalline form constant because of absorption by both sterile filters and plastics. Accordingly, the commercially available form of MIT (Novantrone), which is adjusted to a constant concentration of 2 mg/ml and is also water soluble, was used in all further experiments.

Permanent exposure

The pharmacokinetics of MIT in humans seem to be adequately described by a three-compartment model showing short-term peaks of MIT in the serum, a superficial tissue compartment with rapid release of the drug and a 'deep' tissue compartment with slow release of the drug, the last being consistent with the drug's long terminal half-life of 214.8 h [13, 15]. In order to analyze the effects of this last compartment, peripheral-blood and bone-marrow GM-CFCs were assayed in the continuous presence of either MIT or ADR. In all, GM-CFCs exhibited greater sensitivity to MIT than to ADR (Fig. 1). This is shown by D_0 values of 0.95 ng/ml for bone-marrow GM-CFCs and 0.68 ng/ml for peripheral-blood GM-CFCs when incubated with MIT. ADR incubation resulted in D_0 values of 5.43 and 5.13 ng/ml, respectively.

In this context it is of interest to note that the survival of peripheral-blood GM-CFCs was consistently lower than that of bone-marrow GM-CFCs at all concentrations of both drugs (Fig. 1); however, the differences were not significant.

Short-term exposure

The toxic effects of the tested drugs on GM-CFCs are not necessarily mediated by long-lasting concentrations in the deep tissue compartment, but might instead be caused by short-term serum and/or tissue peaks. Hence, PBCs and BMCs were subjected to different periods of in vitro exposure to different concentrations of either MIT or ADR

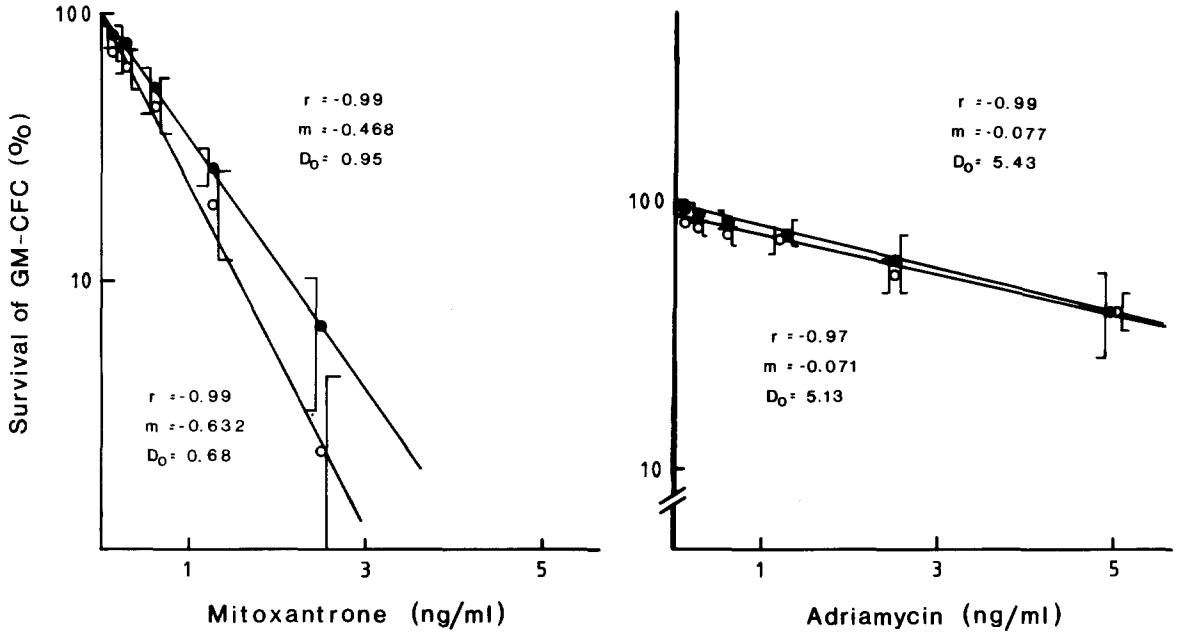

Fig. 1. Effects of various concentrations of MIT or ADR on both human bone-marrow GM-CFCs (●) and human peripheral-blood GM-CFCs (○). Cells were continuously exposed to the drugs in semisolid agar cultures that were stimulated for 14 days by GCT-CM as a source of colony-stimulating factor. Each *point* represents the mean ± SEM of three or four separate experiments. *r*, coefficient of regression; *m*, slope of the curve; D_0, dose which allowed survival of 37% of the GM-CFCs

(Table 2). Bone-marrow-derived GM-CFCs did not exhibit significant differences with respect to their survival when subjected to MIT for 5, 20, 60, or 120 min (Table 2). In further experiments, therefore, only exposure times of 20 or 120 min were used. Especially when higher drug concentrations were applied, peripheral-blood GM-CFCs were more sensitive to both drugs (Fig. 2).

Table 2. Exposure of BMCs and PBCs to MIT or ADR

Drug concentration (ng/ml)	Exposure time (min)	Survival of GM-CFCs (%)[a]			
		MIT		ADR	
		BMC	PBC	BMC	PBC
0.2	0	100± 0	100± 0	100± 0	100± 0
	5	84± 4	nt	nt	nt
	20	81±14	82± 7	96± 3	96± 4
	60	75± 7	nt	nt	nt
	120	67± 6	69±10	90±12	79± 7
2.0	0	100± 0	100± 0	100± 0	100± 0
	5	75±12	nt	nt	nt
	20	72± 3	73± 5	90±10	84±17
	60	74± 5	nt	nt	nt
	120	62± 6	58±17	63±17	73± 6
20.0	0	100± 0	100± 0	100± 0	100± 0
	5	70± 0	nt	nt	nt
	20	68±11	35±16	53± 2	11± 6
	60	60± 8	nt	nt	nt
	120	45± 7	20±17	25± 6	4± 5

Cells of hematologically normal adults were incubated for different periods with various concentrations of the drugs, or with PBS as a negative control. Cells were washed twice and analyzed for GM-CFC survival in soft agar using GCT-conditioned medium as a source of colony-stimulating factor

[a] Mean ± SEM of four experiments as a percentage of the negative control value. nt, not tested

The longer the incubation period with various concentrations, the more toxic was the effect of both MIT and ADR. On the other hand, ADR, which was less toxic than MIT in peripheral-blood and bone-marrow GM-CFCs when used at low concentrations, was the more cytotoxic than MIT at a concentration of 20 ng/ml. This was true for both incubation periods, but was more pronounced in PBCs (Fig. 2).

Intracellular MIT concentration

In order to compare the in vivo levels attained after the therapeutic application of MIT [13], we examined the intracellular concentrations of MIT after preincubation of the drug at various concentrations and after different incubation periods. Interestingly, even at a concentration of 20 ng/ml and an incubation period of 2 h, the intracellular MIT concentration was below 1 ng per 2×10^7 cells, i.e., below the detection limit of the method. This also applied to all other drug concentrations and incubation periods tested.

Discussion

Since its introduction into cancer therapy as a possible substitute for ADR, MIT has been reported to exhibit less cardiotoxicity and less gastrointestinal side effects than ADR [1, 3, 22, 26, 27, 31]. The dose-limiting toxicity of MIT, however, is neutropenia, which is especially long-lasting in leukemic patients. This side effect is the result of bone-marrow aplasia and may reach WHO grade 4 (leukopenia below, 1,000 leukocytes/mm^3). It is therefore surprising that, in this neutropenic state, patients show few septic complications [11, 31]. A comprehensive review of the effects and clinical applications of MIT has been published very recently [26].

To provide an analogy with the in vivo pharmacokinetics of MIT (i.e., short-term peaks and long-lasting tissue

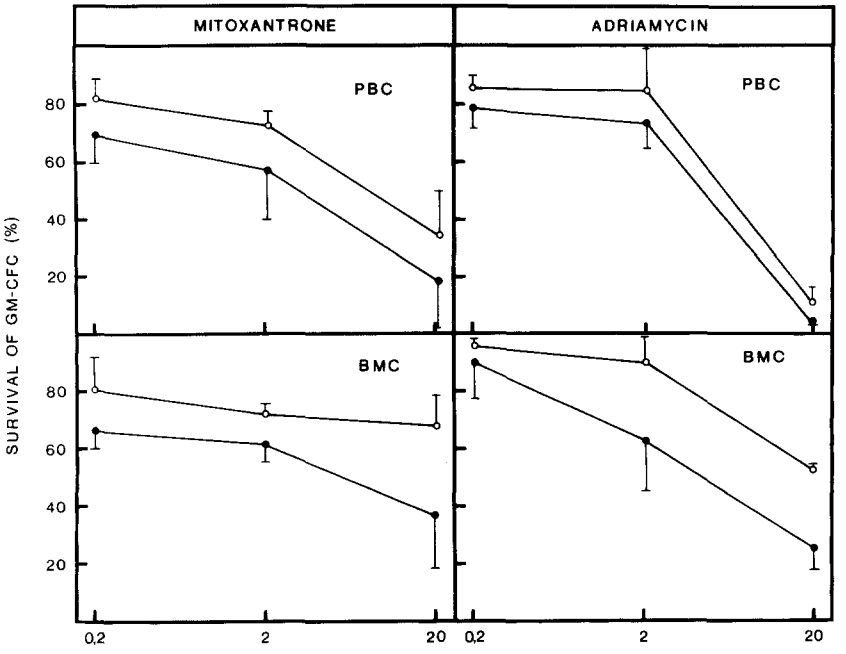

Fig. 2. Effects of various concentrations of MIT or ADR on human bone-marrow and human peripheral-blood GM-CFCs. Cells were preincubated with the drugs for 20 (○) or 120 min (●). The cells were washed twice and assayed in semisolid agar cultures that were stimulated for 14 days by GCT-CM as a source of colony-stimulating factor. Each *point* represents the mean ± SEM of three or four separate experiments

concentrations), MIT and ADR were applied for different incubation periods. Permanent exposure of BMCs and PBCs generally revealed that MIT has more toxic effects on GM-CFCs than ADR. This is shown by the ADR/MIT-ratio D_0 values of 5.72 for BMCs and 7.54 for PBCs. However, when comparing the in vivo myelotoxicity of these two drugs, the different doses used for their therapeutical application need to be considered, i.e., 40 mg/m^2 for ADR versus 12 mg/m^2 for MIT. On this basis, their differences with respect to GM-CFC toxicity are reduced to a factor of 1.7 for BMCs and 2.3 for PBCs. It is of interest that, in the experiments of OKunewick et al. [23] involving in vivo exposure of murine bone-marrow GM-CFCs, MIT was found to be more toxic than ADR by a factor of 1.75. Furthermore, these investigators showed that the nadir of the MIT effect occurred within the first 6 h, while that of the ADR effect lagged 1 day behind. However, when analyzing the effects of ADR and MIT on splenic pluripotent stem cells (CFU-S), the killing effect of these drugs was found to be identical on a milligram per kilogram basis. In view of increasing evidence of the antileukemic effect of MIT [14, 16, 18, 24], analyses of its effects on human pluripotent stem cells and the human microenvironment are needed. These could be performed by applying a recently developed liquid-culture system which apparently allows human stem cells to be maintained on a stromal layer [20]. In vivo myelotoxicity, however, is not necessarily mediated by the pure drug as such, but may also be mediated by its metabolites. Accordingly, the pronounced precursor-cell toxicity of MIT does not seem to be the sole factor responsible for the neutropenia occuring in patients receiving this drug. Appart from possible damage to pluripotent stem and stromal cells, disturbed granulocyte release from the bone marrow may contribute to the leukopenic effect of MIT.

In both exposure schedules tested, blood-derived GM-CFCs appeared to be most sensitive to the two drugs. It would be interesting to know whether this difference in the sensitivity of blood and bone-marrow GM-CFCs is due to the existence of different populations. This must remain a matter of speculation, although it has been demonstrated that different populations of precursor cells do exist in the peripheral blood and bone marrow [5, 17, 29].

The long terminal half-life of MIT, resulting from its slow release from a deep tissue compartment accounting for 70% of the whole drug dose [13, 15], prompted us to analyze the intracellular concentrations of MIT that are toxic for GM-CFCs. To our surprise, concentrations of below 1 ng per 2×10^7 MNCs invariably proved to be toxic. This would seem to emphasize the biological relevance of the hematotoxicity of MIT. In this context, it is interesting to note that high tissue concentrations of MIT were found in the liver, bone marrow, heart, lungs, spleen, and kidneys of a patient autopsied 35 days after applications of the drug [2]. Moreover, the distribution volume of MIT, i.e., 3,792 l, indicates the large amount of tissue incorporation. However, GM-CFCs account for a very small percentage of the whole MNC population tested. If they accumulate higher concentrations of MIT than other MNCs, their small numbers would ensure that the effect on the total accumulation value would be undetectable.

To date, there have been several examples of 'second-generation' drugs replacing established anticancer agents, e.g., piperazindione in lieu of cyclophosphamide for leukemia [4], new platinum compounds substituting for cisplatinum [7, 10], and MIT partially replacing ADR. Most of the research on the toxicity of these new agents has focused on comparison with the specific organ toxicity associated with the first-generation drug for which they have been proposed as a substitute. However, myelosuppression appears to be the principal dose-limiting factor for nearly all of these new drugs. It would therefore seem to be important to investigate not only the specific organ toxicity associated with the first-generation drugs, but also the myelosuppressive effects of the new drugs.

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