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Mitoxantrone-induced DNA damage in leukemia cells is enhanced by treatment with high-dose arabinosylcytosine*

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Summary. In a phase II study, patients with chronic myelogenous leukemia in blast crisis (CML-BC) were treated with intravenous (IV) mitoxantrone (5 mg/m^2 per day given over 30 min $\times$ 5 days and high-dose arabinosylcytosine (ara-C) (3 g/m^2 IV q 12 h $\times$ 6). The effect of this treatment on DNA damage was studied in the leukemia cells of four patients using the alkaline elution technique modified to measure DNA in unlabeled human cells. A fluorescence assay using Hoechst 33258 dye was applied for the determination of eluted DNA. After a single infusion of mitoxantrone, neither frank nor protein-associated single-strand breaks (SSB) were observed. Even repeated treatment with mitoxantrone on 3 consecutive days did not induce significant SSB. However, after the combined sequential infusion of ara-C and mitoxantrone the DNA elution pattern changed, showing significant DNA damage. SSB remained apparent after 24 h and increased with subsequent doses of ara-C and mitoxantrone. Studies of other patients treated with ara-C alone did not reveal significant SSB (n = 5). Following mitoxantrone infusion the median peak concentrations of intracellular ara-CTP (the triphosphate of ara-C) exceeded 900 μM, a value greater than that observed in CML-BC patients receiving ara-C alone (230 μM, n = 15, $P < 0.02$). The present study shows the applicability of the alkaline elution method for the assay of DNA damage in vivo. The enhanced DNA damage after combined treatment with mitoxantrone and high-dose ara-C suggests a synergistic drug effect.

Introduction

Mitoxantrone [1, 9, 34] and ara-C [3, 7, 16, 23] used as single agents are effective drugs in the treatment of refractory acute leukemia. These drugs show different biochemical mechanisms of action. The anthracenedione mitoxantrone acts as an intercalating agent [24, 29, 37] and has a base specificity for the G-C base pair [10]. Mitoxantrone binds to both DNA and RNA in the nuclear chromatin with preference for the nucleolus and cytoplasmic RNA [22, 24]. The drug induces frank and protein-associated DNA strand breaks [5, 20, 28]. Frank DNA strand breaks presumably occur via the production of free radicals due to the quinone structure of mitoxantrone [2]. Protein-associated strand breaks are assumed to result from covalent binding of the drug to DNA-topoisomerase II and formation of cleavable complexes [20, 28]. Under the lysis conditions of the alkaline elution procedure and in the presence of proteinase K (PK), the cleavable complexes become evident as DNA strand breaks.

Ara-C phosphorylated to the active triphosphate ara-CTP inhibits DNA polymerase activity [6, 13], but also serves as a substrate in replicative [14, 26] and repair [19, 27] DNA synthesis. The action of ara-C as a chain terminator and as an inhibitor of DNA polymerase induces dose-dependent single-strand breaks (SSB) in replicating DNA [11, 12] and in DNA undergoing repair synthesis [19, 38].

Recent clinical studies investigating the combined use of both drugs have shown encouraging results for the treatment of acute leukemia [17, 18]. The interaction of mitoxantrone and ara-C at the molecular level and its relation to either the induction or the repair of DNA damage is not yet understood. Based on in vitro investigations it is reasonable to hypothesize that DNA damage observed during treatment is related to the cytotoxic potential of the combination therapy. The present study was performed to analyze the DNA damage introduced in vivo into unlabeled leukemia cells from patients with CML-BC during treatment with a combination of high-dose ara-C and mitoxantrone.

Materials and methods

Patients and therapy. Four CML-BC patients, two male and two female, median age 42.5 years, were analyzed for the development of DNA damage during the course of therapy. All patients refractory to a variety of previous treatment protocols were entered into the study after informed consent had been given. The detailed patient characteristics are shown in Table 1.

The chemotherapy protocol consisted of mitoxantrone 5 mg/m^2 IV given over 30 min daily for 5 days, and ara-C

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Abbreviations. ara-C, 1-β-D-arabinosylcytosine; ara-CTP, the triphosphate of ara-C; SSB, single-strand breaks; SSF, strand scission factor; PK, proteinase K; CML, chronic myelogenous leukemia; BC, blast crisis; AML, acute myelogenous leukemia

Table 1. Patient characteristics

Patient	1	2	3	4
Age/sex	34/female	37/male	51/female	42/male
Diagnosis	CML-BC, undiff.	CML-BC, lymphoid	CML-BC, undiff.	CML-BC, myeloid
Cytogenetics	Philadelphia pos.	Philadelphia pos.	Philadelphia pos. [46xx, t(9:22)] [45xx, −7 t(9:22)] [47xx, +8, t(9:22)]	Philadelphia pos.
Pretreatment failure with	VAD	VAD, L-asparaginase, + methotrexate	alpha interferon, gamma interferon, VAD	alpha interferon, gamma interferon, high-dose hydroxyurea, mithramycin/hydroxyurea
Start of therapy WBC	6.54×10^3/l	83.9×10^3/l	54.3×10^3/l	25.2×10^3/l
Blasts	10%	88%	81%	72%

VAD, vincristine, ara-C, daunorubicin

3 g/m^2 given IV over 2 h at 12-h intervals for 6 doses. In patients 1, 2, and 3 the treatment began with an infusion of mitoxantrone on day 1. The first ara-C dose was given 24 h later, followed immediately by the mitoxantrone infusion (schedule I). For patient 4 the treatment protocol was changed so that on day 1 and day 2 mitoxantrone was given as a single agent. Ara-C was given to patient 4 for the first time on day 3 following the third mitoxantrone infusion (schedule II).

Cellular pharmacology studies. Peripheral blood samples were obtained from patients at the indicated time points before and during the course of therapy. The blood samples were placed in an ice slurry immediately after drawing and processed expeditiously. All further processing of the samples was carried out at freezing point in order to inhibit DNA repair processes. After separation of plasma and cells by centrifugation, mononuclear cells were isolated by step gradient centrifugation on Ficoll-Hypaque [35, 36]. Cell number and volume were determined using a model ZM Coulter Counter equipped with a model C-1000 Coulter Channelyzer (Coulter Inc., Hialeah, Fla). For determination of intracellular ara-CTP, leukemia cells were extrated with 0.4 *N* $HClO_4$ as previously described. The intracellular ara-CTP concentration in the neutralized acid-soluble cell extract was analyzed by high-pressure liquid chromatography (HPLC) using an ALC-204 HPLC system (Waters Associates, Milford, Mass) with two model 6000A pumps, a model 660 gradient programmer, and a Partisil-10 SAX anion-exchange column. Ara-CTP was separated from natural cellular nucleosides triphosphates by a concave gradient (curve 9) run over 30 min at a flow rate of 3 ml/min. The initial buffer composition was 65% buffer A (0.005 *M* $NH_4H_2PO_4$, pH 2.8) and 35% buffer B (0.75 *M* $NH_4H_2PO_4$, pH 3.5) and the final composition was 100% buffer B.

Alkaline elution. The alkaline elution technique as described by Kohn et al. [25] was adapted for the measurement of DNA damage in unlabeled cells in vivo [30, 32, 33]. Human leukemia cells ($6-8 \times 10^6$) were impinged onto polycarbonate filters 47 mm in diameter and with 2-μm pore size (Nucleopore, Pleasanton, Calif) and were washed twice with 5 ml ice-cold phosphate-buffered saline (PBS), 5 *mM* EDTA. The cells were subsequently lysed for 30 min with 10 ml of 2 *M* NaCl, 0.04 *M* EDTA, 0.2% Sarkosyl (pH 10.0) either with or without proteinase K (PK) (0.5 mg/ml, lysis solution). The lysis solution was allowed to flow through the filter by gravity. After the lysate had been washed twice with 5 ml 0.02 *M* EDTA (pH 10.3), the DNA was eluted in the dark with 0.1 *M* tetrapropylammonium hydroxide containing 0.02 *M* EDTA (free acid), pH 12.1. The elution was carried out at a constant flow rate of 0.035–0.04 ml/min. Fractions were collected at 90-min intervals over 15 h. The DNA retained on the filter was brought into solution by extensive vortexing in 5 ml 0.02 *M* EDTA (pH 10.3) after a 30-min incubation at 50° C [32].

Microfluorimetric assay for DNA. A microfluorimetric assay using the Hoechst 33258 dye to quantitate unlabeled DNA in the elution fractions was performed according to the method of Cesarone et al. [4, 32, 39]. Aliquots of each elution fraction (1 ml), as well as of the solutions containing the DNA retained on the filter and the wash of the filter holder were neutralized with 0.4 ml 0.2 *M* KH_2PO_4. The sample volume was brought up to 2.0 ml with 0.6 ml H_2O, and 1 ml 1.5×10^{-6} *M* Hoechst 33258 dye in standard saline citrate was added. Fluorescence due to binding of the Hoechst dye to DNA was determined using an Aminco SPF-125 spectrofluorometer with the excitation wavelength set at 350 nm and the emission at 460 nm. Background fluorescence was determined by analysis of a blank (no cells added).

Calculation of the strand scission factor. SSB were quantitated from alkaline elution profiles by calculating a relative strand scission factor (SSF) = $-\log(f_d/f_C)$; f_D and f_O are respectively equivalent to the percentage of DNA retained on the filter after a volume of 17.5 ml had eluted for the drug treated and the control sample. The elution volume of 17.5 ml, which is 50% of the total elution volume, was chosen to avoid higher statistical variability due to lower DNA concentrations in the later elution fractions. An SSF value of zero indicated no induced SSB. Gamma irradiation of human CCRF-CEM leukemia cells with

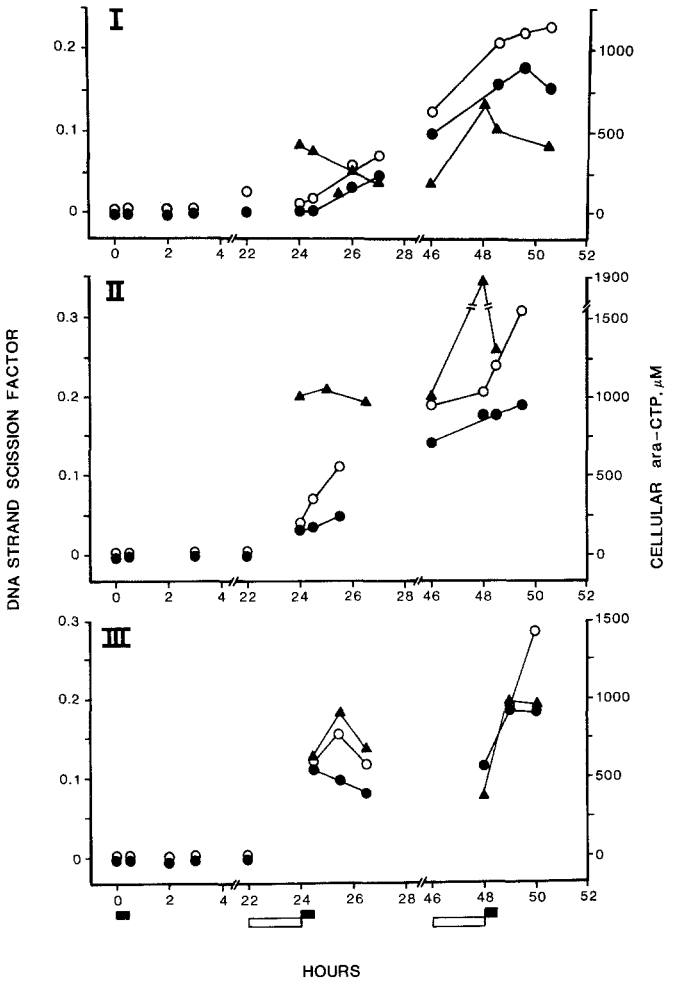

Fig. 1. Patients 1, 2, and 3 were treated with mitoxantrone 5 mg/m^2 IV over 30 min at 24-h intervals (*filled bars*). Ara-C was given IV over 2 h at 12-h intervals for six doses starting on day 2 at 24 h after treatment begin (*open bars*). During the course of treatment DNA damage was monitored by measurement of the DNA strand scission factor (SSF). ○, SSF measurements after treatment of the cell lysate with proteinase K; ●, determinations of SSF which did not include PK; ▲, intracellular ara-CTP concentrations

4 Gy at freezing point in vitro induced SSB equivalent to an SSF of 0.367 ± 0.047.

Results

The interaction of mitoxantrone and ara-C on the induction of DNA damage was analyzed in the leukemia cells of four patients treated with combination protocols. Measurements of DNA damage undertaken at 0, 0.5, 2, and 3 h after the start of treatment with mitoxantrone alone did not show significant SSB in any of the three patients treated according to schedule I (Fig. 1). Repeated treatment with mitoxantrone as a single drug given three times consecutively at 24-h intervals did not induce measurable amounts of SSB in patient 4 (Fig. 2). Protein-associated SSB, evident after treatment of the cell lysate with PK, were not observed in any of the patients during their treatment with mitoxantrone alone (Figs. 1 and 2). These data indicate that mitoxantrone infusions of 5 mg/m^2 per 30 min in vivo induced neither frank SSB nor protein-associated SSB at the sensitivity level of the alkaline elution procedures used.

Consecutive infusion of ara-C followed by mitoxantrone in patients 1, 2, and 3 resulted in a significant increase of measured SSB (Fig. 1). A linear increase of DNA damage was observed in patients 1 and 2 subsequent to the first combination treatment. The SSB remained apparent up to 24 h after the first combined drug treatment, indicating that rapid DNA repair was not taking place.

The second combination treatment with mitoxantrone/high-dose ara-C on day 3 further increased the SSB levels. In the cells of patients 1, 2, and 3, levels of frank SSB saturated 1–3 h after the end of the second combination treatment whereas total SSB, frank plus protein-associated, did not. At the end of measurements, SSF values as high as 0.3 were reached for total SSB corresponding to nearly 3 Gy of ionizing radiation.

The pattern of DNA damage with its onset at the time of combined mitoxantrone/ara-C treatment was uniform in all patients. In all patients SSB increased with the progression of the treatment course. Once induced, protein-associated SSB constituted 30%–40% of total SSB.

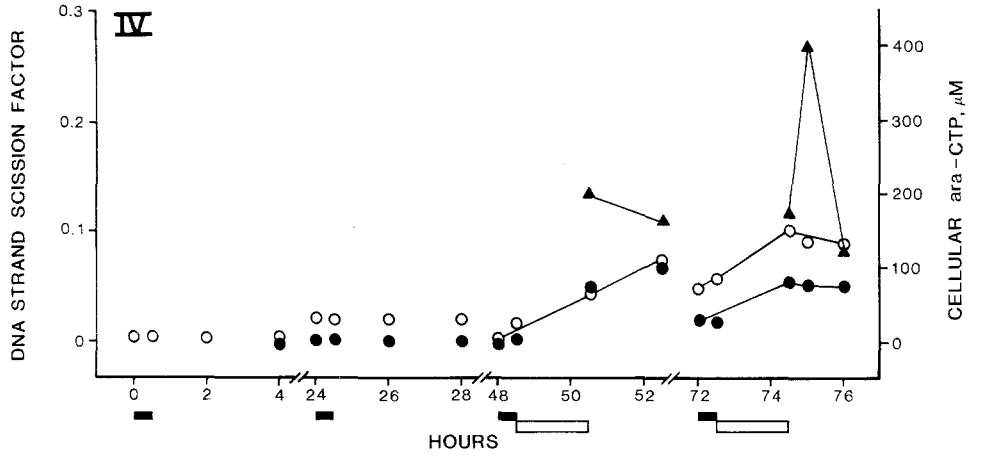

Fig. 2. Patient 4 was treated with mitoxantrone 5 mg/m^2 IV over 30 min at 24-h intervals (*filled bars*). Ara-C (*open bars*) given IV over 2 h at 12-h intervals for six doses was started on day 3. During the combination treatment ara-C was applied subsequent to mitoxantrone. ○, SSF measured after exposure of the cell lysate to proteinase K; ●, SSF determinations which did not include the use of PK; ▲, intracellular ara-CTP concentrations

Schedule II provided the opportunity to evaluate the effect of multiple doses of mitoxantrone in the absence of ara-C treatment (Fig. 2). No significant DNA damage was observed after two consecutive doses of mitoxantrone. In fact, a determination made immediately after the third dose of mitoxantrone, prior to ara-C infusion, failed to demonstrate an increase in SSB. Significant DNA damage became apparent only after the first combination therapy with mitoxantrone and ara-C was started on day 3. SSB then increased linearly for at least 2 h after the first ara-C infusion. Patient 4 showed essentially no protein-associated SSB after the first combination treatment. Two hours after the second combination treatment on day 4 both frank SSB and protein-associated SSB were comparatively lower than in schedule I and reached SSF values of 0.050 and 0.040 respectively. In contrast to the patients treated under schedule I, total SSB in patient 4 reached a plateau at the end of the second combination treatment on day 2.

Ara-C is known to introduce SSB into DNA of cells in culture [11, 12]. During the treatment course with mitoxantrone/high-dose ara-C the median peak concentrations of intracellular ara-CTP exceeded 900 μM, a significantly greater value than that previously observed in CML-BC patients on a different protocol who received ara-C as a single drug (230 M, $n = 15$, $P < 0.02$) [21]. Separate measurements of DNA damage were conducted for 5 patients treated with infusions of ara-C alone at 24-h intervals. These patients achieved median peak ara-CTP concentrations of 402 μM (range 327–661 μM). Following the first infusion of ara-C a median SSF of 0.009 was measured; 22 h later the median SSF was 0.003. Subsequent to the second ara-C infusion a median SSF of 0.013 was calculated, which decreased to SSF = 0.008 at 48 h. The results indicate that repeated exposure of leukemia cells to peak intracellular concentrations in the range of 400 μM ara-CTP did not induce significant DNA damage during the 48-h observation period.

Discussion

The present study was undertaken to elucidate the in vivo kinetics of DNA damage induced in leukemia cells of CML-BC patients undergoing treatment with mitoxantrone and high-dose ara-C. The pattern of SSB induction during the combination treatment was similar in the four patients examined. Mitoxantrone given as a single agent did not cause significant SSB. In vitro experiments with mitoxantrone-treated mammalian cell lines showed a fast development of SSB reaching a plateau after 60 min of exposure (unpublished data). The repair of SSB after washout of mitoxantrone was slow relative to the cell-cycle time [15]. It is therefore unlikely that the lack of SSB observed in CML-BC patients treated with mitoxantrone alone can be attributed to delayed development of DNA damage or of rapidly progressing DNA repair.

Treatment of cells with ara-C in vitro is known to induce SSB in mature and nascent DNA [11]. To evaluate whether a similar effect could be observed in vivo, the induction of SSB by treatment with high-dose ara-C alone was studied in the leukemia cells of five additional patients. No significant SSB were observed suggesting that at the sensitivity level of our techniques, ara-C, administered at high doses as a single agent, does not cause measurable DNA damage.

On the other hand, the consecutive infusion of mitoxantrone and ara-C independent of the infusion sequence resulted in a significant rise in SSB. Subsequent treatment with the drug combination induced further increases in DNA damage. Significant SSB remained apparent even 24 h after the first manifestation of DNA damage. It appears that in the presence of ara-C, repair of mitoxantrone-induced DNA damage is a rather slow phenomenon in vivo. Protein-associated and frank SSB increased essentially in parallel. It is of interest that the greater proportion of the observed total SSB were frank or non-protein-associated SSB. This finding contrasts with the present notion of other intercalating agents, such as m-AMSA, which predominantly induce protein-associated SSB [31]. However, it must be considered that the use of polycarbonate filters may lead to an underestimation of protein-associated DNA cleavage [25, 33].

To exclude the possibility that the rise of SSB observed for patients 1–3 on day 2 of the treatment was merely a consequence of cell sensitization caused by the previous exposure to mitoxantrone on day 1, the treatment regimen for patient 4 was changed. Although this patient was treated on 3 consecutive days with mitoxantrone, no DNA damage was evident until the start of the combination therapy. It therefore may be concluded that even repeated exposure to mitoxantrone in vivo does not result in DNA damage that can be observed by our techniques. Whether the comparatively lower level of DNA damage observed overall in patient 4 was due to the drug scheduling remains to be clarified. In vitro data obtained for Chinese hamster ovary cells [15] indicate that cytotoxic synergism is best achieved by ara-C pretreatment and subsequent exposure to mitoxantrone, while the inverse drug sequence does not lead to synergistic cell kill. Further analyses of the interaction of mitoxantrone and high-dose ara-C in vitro suggest a strong schedule dependence of DNA damage and cytotoxic drug effect. Thus an 18- to 36-h interval between ara-C pretreatment and exposure to mitoxantrone yielded the greatest enhancement of DNA strand-break induction and cytotoxicity in CHO cells (unpublished results). These observations correspond with the schedule dependence described for synergistic interaction of ara-C with daunorubicin [8] or m-AMSA [31]. For L5178Y cells, however, a significant, synergistic increase of SSB and cytotoxicity was reported when the cells were simultaneously exposed to mitoxantrone and ara-C [40].

The mechanistic background for the synergistic DNA cleavage by mitoxantrone and ara-C is unclear. Ara-C-induced alterations in cell-cycle distribution, DNA conformation, DNA methylation or DNA-histone interaction [31] may play a role. Also, an effect of ara-C on the repair of mitoxantrone-induced DNA damage cannot be ruled out. Cell death and concomitant DNA fragmentation may contribute to the amount of total SSB measured. However, repeated treatment with toxic doses of mitoxantrone alone did not cause a significant increase in frank SSB (Fig. 2). Moreover, increases of frank SSB were paralleled by protein-associated SSB which are assumed to result from drug interaction with topoisomerase II and thus do not reflect enzymatic degradation of DNA induced by cell death.

In conclusion, the modified alkaline elution method proved to be a suitable technique for the investigation of DNA damage in leukemia cells of patients undergoing chemotherapy. The analysis of DNA damage in vivo may

help to understand the mechanism and schedule dependence of drug interaction. The amount of SSB observed during the combination treatment showed a substantial interpatient variability and thus should be analyzed as a factor of prognostic significance. We propose therefore that the determination of DNA damage in vivo may provide a parameter which reflects drug activity at the intracellular target level, and also allows a correlation to treatment response.

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