Cancer Chemother Pharmacol (1986) 18: 198–202

Cancer Chemotherapy and Pharmacology

Original articles

Enhancement of etoposide-induced cytotoxicity by cyclosporin A

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Summary. Following the clinical observation of enhanced antineoplastic action of etoposide in the presence of cyclosporin A (CyA), we investigated this drug interaction in several in vitro and in vivo tumor systems. Macromolecular DNA damage induced by etoposide at drug levels comparable to plasma AUC values achieved in patients was increased not only in leukemic peripheral blood cells from patients but also in mononuclear peripheral blood cells from a healthy donor. Intracellular retention of radioactivity from ^{3}H-etoposide was increased by a factor of 1.5 at the most in the presence of CyA. The cytotoxicity of etoposide and adriamycin to L 1210 leukemic cells was clearly enhanced, whereas CyA had no effect on the action of cisplatin or ionizing irradiation. At CyA blood levels not exceeding 1.44 μg/ml, increased tumor inhibition of etoposide was observed in a human embryonal cancer xenograft, but there was also higher lethality in normal mice. We conclude from our own data and from other recent findings that with respect to chemosensitization the effects of CyA resemble those of calcium channel blockers or anticalmodulin agents. In contrast to calcium channel blockers, however, adequate plasma levels of CyA can well be achieved in patients.

Introduction

Cyclosporin A (CyA) is a cyclic polypeptide with unique pharmacodynamic properties. It interferes with T cell growth and differentiation by inhibiting the production of interleukin 2 at the transcriptional level [6]. Thus, the drug may be selectively cytotoxic to human T cells in Sézary's syndrome [13]. On the basis of these findings, a patient with T cell leukemia in relapse after multiple drug treatment was given CyA and etoposide. The patient achieved complete clearance of leukemic cells in his bone marrow but succumbed to severe systemic infection 21 days after initiation of treatment [8].

This clinical observation prompted us to investigate the mechnisms of drug interaction. Three lines of investigation were followed. At the subcellular level, macromolecular DNA damage was monitored after exposure to etoposide in the presence or absence of CyA in normal and leukemic peripheral human blood cells.

At the cellular level, survival was determined by soft agar cloning after treatment with antineoplastic agents or ionizing irradiation. Drug retention with or without simultaneous CyA was determined using triatied etoposide. Finally, in vivo treatments were given to nude mice bearing human embryonal cancer xenograft Ma, and tumor growth curves were monitored after treatment with etoposide in the presence or absence of CyA. Changes in lethality as a function of cytotoxic drug exposure with or without CyA were also investigated.

This approach at various levels of investigation was chosen to gain rapid information as to the mode of interaction and its potential clinical relevance.

Materials and methods

Etoposide-induced macromolecular DNA damage was determined by the alkaline filter elution technique. Peripheral blood cells from a healthy donor and from patients with a predominance of leukemic cells in their peripheral blood were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. Neither of the two patients with leukemic peripheral blood cells had been pretreated with etoposide. One was suffering from refractory myeloblastic leukemia, whereas the other had a leukemic variant of non-Hodgkin's lymphoma. The final cell concentrations for in vitro treatments were always set to 10^6 cells/ml, and incubations were for 1 h unless explicitly stated otherwise. For each dose level alkaline elutions were performed in triplicate. Means were plotted without SEM values for the sake of clarity. Elutions were performed in a similar way to that previously described, i.e., internal radioactive standards were omitted and DNA was determined by microfluorometry [7]. Values for fractional DNA retention were converted to radiation damage (Gy) equivalents by means of a linear regression function obtained from untreated cells irradiated with 0–6 Gy in every elution experiment. The influence of CyA on the uptake of tritium-labeled etoposide was measured in L1210 cells as previously described [11]. The duration of incubation was 30 min. ^{3}H-Etoposide with a specific activity of 88 μCi/mg and pure etoposide were a gift from W. Achterrath of Bristol-Myers Co. We are indebted to Dr A. A. Miller at our own insti-

* Supported by BDMFT (PTB 8315)
** Supported by SFB 102 (Deutsche Forschungsgemeinschaft)
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tute for confirming the radiochemical purity of ^{3}H-etoposide by HPLC at the time of the experiments. Pure CyA was given to us by Dr J. F. Borel of Sandoz Co., Basle, Switzerland, and was dissolved in pure ethanol. Further dilutions were made with appropriate culture medium.

Clonogenic survival of L1210 leukemia cells was determined according to established methods [4]. While etoposide was used as the pure chemical dissolved in DMSO, with a final concentration of the solvent not exceeding 0.05%, adriamycin (Adriblastin) and cisplatin (Platinex) from commercial sources were used. No effect on clonogenicity of L 1210 cells was recorded if DMSO, ethanol, and CyA were present in the incubation in any one combination.

For oral and intraperitoneal administration etoposide from commercial sources (Vepesid) was diluted appropriately and mice received the drug in a volume of 0.01 ml/g body weight.

Ionizing irradation was given with a 300-kV roentgen source (Siemens Stabiliplan II) at a dose rate of 1.25 Gy/min in a normal atmosphere.

The details of maintaining and treating the human embryonal carcinoma line Ma as a xenograft in nude mice have already been published [10]. Treatment groups consisted of six mice each and tumor volumes (V) were calculated from caliper measurements of tumor length (L) and width (W) using the formula:

$$V = 0.5\ L \times W^2.$$

Data were plotted as mean relative tumor volumes and set at 1 at the time of initiation of treatment. Lethality studies were performed in groups of nine male NMRI mice per dose level; the mice were 4–6 weeks old and weighed 25 g × each. CyA (the oral preparation of Sandimmun obtained from Sandoz AG, Basle, contains 100 mg CyA per ml and is an oily suspension prepared with 12.6% ethanol) was administered 25 h and 1 h before cytotoxic treatment with etoposide, both being given via the oral route. The nine doses tested were spaced logarithmically by a factor of 1.25, and the survival data were subjected to probit analysis by the SAS statistics program. The probability of survival was then plotted on a linear scale from the computer printout.

CyA levels in EDTA-treated whole blood were determined 25 h after the first administration of CyA (i.e., the time of etoposide administration) by radioimmune assay (Ciclosporin Ria Kit, Sandoz Ltd, Basle, Switzerland).

Results

The DNA from human peripheral blood cells elutes with a rate proportional to the dose of etoposide. The elution rate is clearly increased at all dose levels if cells are treated simultaneously with CyA 2 μg/ml.

Quantitation of elution rates in terms of radiation damage equivalents yields interesting information, in that enhancement is most prominent in cells with low damage in the absence of CyA. Thus, in the very sensitive lymphoblastic cells DNA damage induced by treatment with 10 μmol etoposide for 1 h increased only from 4.25 to 4.89 Gy equivalents in the presence of CyA (Fig. 1b). In the mononuclear cells from the healthy donor there was an increase from 1.66 to 3.21 (Fig. 1c), and in the resistant myeloblastic cells the values rose from 1.03 to 5.13 Gy equivalents (Fig. 1a). While these data are compatible with a preferential enhancement in drug-resistant cells, more data are needed before firm conclusions on this effect are possible.

The net accumulation of ^{3}H-etoposide into L 1210 cells increased as a function of incubation time (data not shown). If retention after 30 min of incubation was determined as a function of the dose of etoposide, intracellular radioactivity was increased by a factor of 1.5 at most. This was more evident at high doses of etoposide (Fig. 2).

Cloning efficiency of L 1210 was 73% in the presence or absence of CyA 2 μg/ml. Neither ethanol nor DMSO up to the final concentration of 0.05% interacted with CyA in such a way as to cause cytotoxicity.

The slope of curves for cellular survival of L1210 cells was changed to a greater extent by CyA if cells were exposed to etoposide rather than to adriamycin. The dose-response curves for cisplatin and ionizing irradiation were not changed at all, indicating some specificity in the chemosensitizing action of CyA (Fig. 3). No enhancement was observed when cells were pretreated with etoposide, washed twice, and then incubated for an additional hour with or without CyA (data not shown).

The enhancement of cytotoxicity from a fixed dose of etoposide (3 μg/ml) was monitored as a function of the dose of CyA in L1210 cells (Fig. 4). The degree of enhancement by CyA shows a linear dose dependency. The linear regression (with y denoting percentage survival and x concentration of CyA in μg/ml) is described by:

$$y = 97.7 - 32.1\,x;\ \text{with}\ r^2 = 0.953$$

We determined blood levels in normal mice after oral ad-

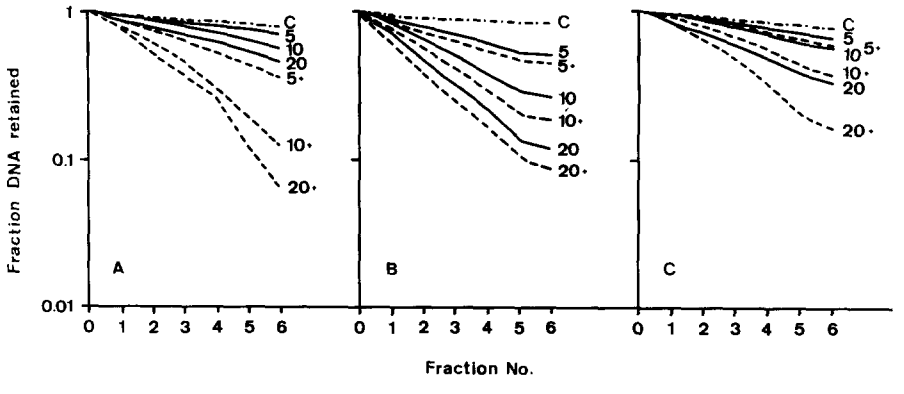

Fig. 1 A–C. Alkaline elution patterns of DNA extracted from peripheral leukemic mononuclear blood cells exposed to etoposide 5, 10, 20 μ*M* for 60 min with *(dashed lines)* or without *(solid lines)* CyA 2 μg/ml. **A** Patient suffering from acute myeloblastic leukemia refractory to any treatment; **B** patient suffering from a leukemic variant of NHL; **C** mononuclear cells from a healthy donor

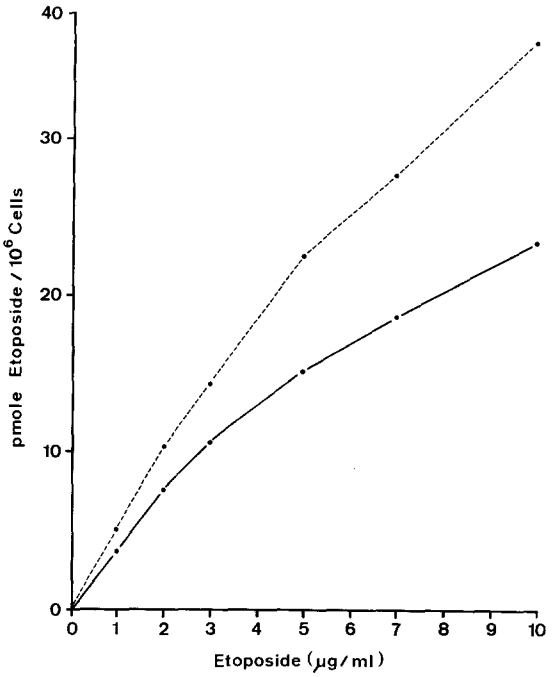

Fig. 2. Retention of radioactive ^{3}H-etoposide in L1210 cells in the presence *(dashed lines)* or absence *(solid lines)* of CyA 2 μg/ml. SEMs were too small to show

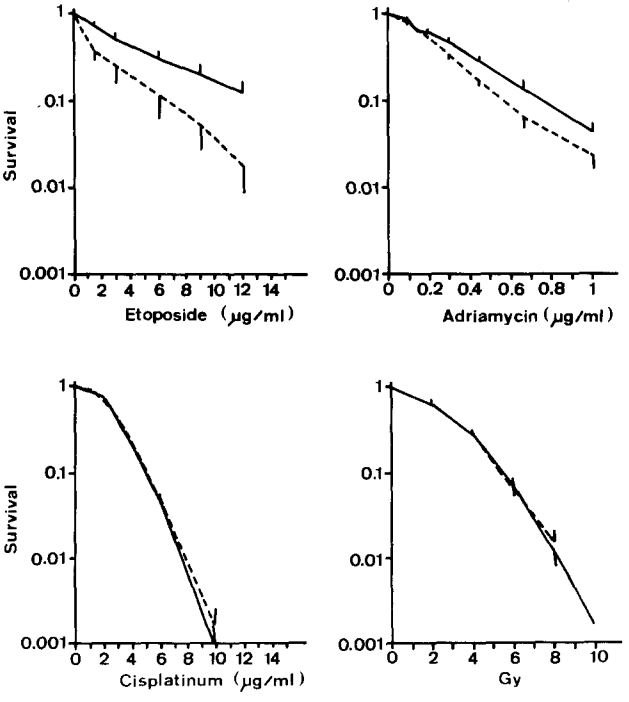

Fig. 3. Clonogenic survival of L1210 cells treated with etoposide, adriamycin, or cisplatinum for 1 h and then plated in soft agar. Ionizing irradiation was given in normal atmosphere. *Solid lines* without CyA, *broken lines* with CyA 2 μg/m. Points are means from five culture dishes in each case. *Bars* SEM

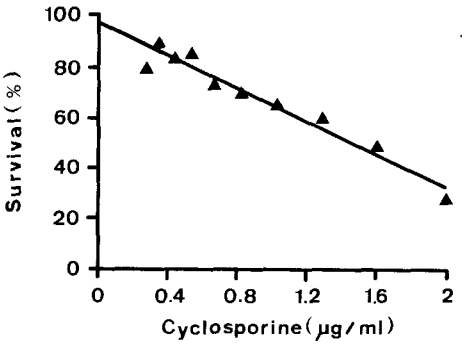

Fig. 4. Survival of L1210 cells after treatment with etoposide 3 μg/ml for 1 h in the presence of increasing doses of CyA. Survival of 43% is set to 100% for treatment with etoposide alone. The linear regression curve was computed from two separate experiments each involving five dishes per dose of CyA. The values for this function are given in *Results*

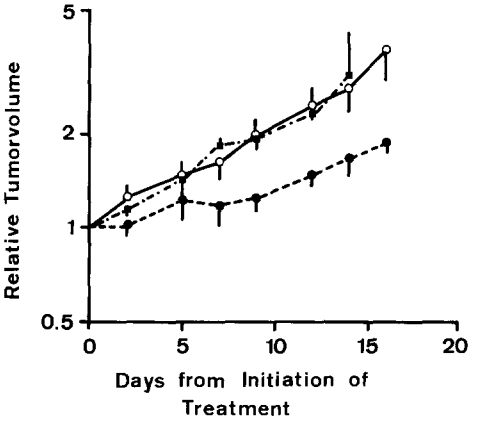

Fig. 5. Tumor volume growth curves of human embryonal cancer xenograft Ma. There were six mice per treatment group. *Dashed line (closed rectangles)*, untreated controls; *dashed line (closed circles)*, etoposide 25 mg/kg i.p., concurrently with two doses of CyA 50 mg/kg given 25 h and 1 h before cytotoxic treatment. *Solid line (open circles)*, etoposide 25 mg/kg i.p. *Bars*, SEM

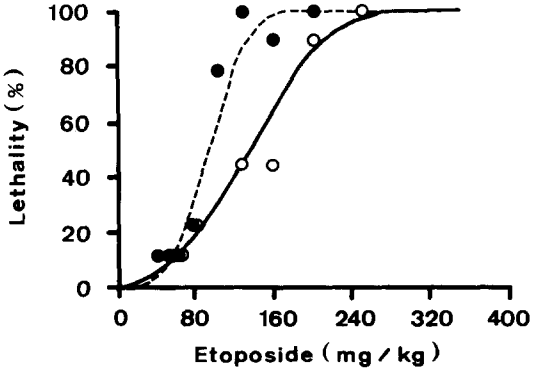

Fig. 6. Lethality of male NMRI mice 4–6 weeks old, determined at 30 days after single oral doses of etoposide with *(dashed line, closed circles)* or without *(solid line; open circles)* pretreatment with CyA 50 mg/kg given 25 h and 1 h before administration of etoposide (some numerical values are given in Table 1)

ministration of CyA. A total CyA dose of 100 mg/kg given in two fractions over 24 h yielded a blood level of 1.44 μg/ml. This was not the peak blood level achieved, but the actual level of CyA at the time of etoposide administration.

The growth of human embryonal cancer xenograft Ma was clearly retarded by the concurrent administration of etoposide and CyA, but not by either drug alone (Fig. 5). CyA alone has no effect on tumor growth (data not shown here).

The lethality of single oral doses of etoposide at 30

Table 1. Lethality data[a] for etoposide in the absence (−) or presence (+) of CyA

	−	+
LD10:	54.4 (7.9–79.2)	52.8 (28.5–66)
LD50:	132.9 (110.6–159)	93.2 (80.9–108.1)
LD90:	211.5 (179.8–276.4)	134.3 (117.3–166.2)

[a] Values are given in milligrams of etoposide per kilogram, with confidence limits in brackets. There is no overlap of these 95% limits for the LD_{50} or LD_{90} values

days, however, was also increased by CyA. The values for LD_{10}, LD_{50}, and LD_{90} are given in Table 1 together with confidence limits.

Discussion

Unlike the majority of investigations on chemosensitization, the series of experiments described in this paper originated from a clinical observation [8] and was designed to analyze the general relevance of this drug interaction in various tumor systems. By this approach we hoped to minimize the probability that any interference with lymphokine functions was an essential feature of the effects observed. As with other chemosensitizers, there was some specificity concerning the kind of cytotoxic damage that could be enhanced. So far, that caused by etoposide and adriamycin has been shown to be clearly enhanced, whereas that resulting from cisplatin and ionizing irradiation has not. This suggests some parallel with the actions of verapamil or anticalmodulin agents [14]. The specificity of drug interaction is currently being explored with other antineoplastic agents. Postincubation with CyA after treatment with etoposide does not block repair of DNA damage or change cellular survival to a significant degree (R. Osieka 1986, unpublished data).

While these experiments were in progress it was shown that CyA binds to calmodulin [5]. We therefore conclude that our experiments and the data of Colombani et al. [5] have revealed hitherto unsuspected properties of this drug, which had so far been regarded solely as a highly selective immunosuppressive agent. The calmodulin system is, of course, of rather broad significance in human physiology, and any interference could have toxic side-effects. The system may play an important role in signal transmission for growth regulation, and it might be possible to confer some selectivity if the susceptibility of this system differs among normal and neoplastic cells [3]. Yet, the observed changes in lethality in normal mice and the increased DNA damage in normal peripheral blood cells leave no doubt that the selectivity of chemosensitization by CyA is limited.

The data on the increase in macromolecular DNA damage in peripheral human mononuclear cells are compatible with the notion that preferential chemosensitization takes place in drug-resistant cells. DNA damage after exposure to etoposide correlates well with cellular survival [16]. We have been able to extend the experiments reported here, and have found a significant negative correlation between DNA damage inflicted by etoposide alone and the increase in such damage conferred by simultaneous incubation with CyA 2 μg/mg (R. Osieka 1986, manuscript in preparation). There have been several reports of reversal of drug resistance in selected sublines of rodent or human origin [14, 15]. Reversal of acquired resistance to vincristine and adriamycin in a human leukemic cell line was recently reported by Slater et al. [12], confirming our results by different methods. These authors mentioned that the chemosensitization was not mediated by changes in drug retention and specifically ruled out altered drug efflux.

The differential observed between the parental line and the resistant variant cell line may also depend on the inherent sensitivity of the parental line. Thus, we have observed more sensitization in L1210 cells than in HL-60 promyelocytic leukemia, which is much more sensitive to etoposide alone (R. Osieka 1986, unpublished data).

If drug resistance may indeed be reversed by chemosensitization, the identification of sensitizing agents without dose-limiting acute toxicity in humans is an important step in the direction of clinical exploitation of this effect.

In contrast to clinical experiences with calcium channel blockers [1], chemosensitization by CyA occurs at drug levels achieved without undue toxicity to patients in the short time necessary [2]. The doses of etoposide used in our studies on macromolecular DNA damage were also in the range of plasma AUC values observed in patients [9]. To our mind, CyA appears to be a very promising candidate for clinical trials. Studies to determine the optimum timing of administration, the tissue distribution of CyA, and its influence on retention of antineoplastic drugs are in progress. A crucial element in the optimum spacing of CyA and etoposide may be the differential retention of CyA in neaplastic vs normal tissues. This might be another mechanism of preferential sensitization under in vivo conditions.

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Received February 10, 1986/Accepted August 21, 1986