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SYNERGISTIC CYTOTOXICITY OF CARBOPLATIN AND DIVICINE ON MURINE ERYTHROLEUKEMIC CELLS

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Summary. Carboplatin (CBDCA) and the pyrimidine aglycone divicine displayed cytotoxic effects on murine erythroleukemic cells (MELC), with ID_{50} values of 158 and 37 μM, respectively. Combination of CBDCA and divicine, at a 2:1 ratio, increased cytotoxicity considerably. Under specific conditions of time schedule of administration, the association of CBDCA and divicine resulted in a clear synergistic activity. Alkaline elution studies on both unirradiated and γ-irradiated MELC demonstrated opposite patterns of DNA damage with the two molecules. Thus, CBDCA elicited DNA interstrand crosslinks (ISC), while divicine resulted in DNA single strand breaks (SSB). Association of both molecules led in the unirradiated cells to a higher SSB frequency than recorded with divicine alone. Accordingly, intracellular activation of CDBCA by redox cycling of divicine seems not to be involved. Rather, intracellular platinum appears to enhance cytotoxicity of divicine.

Carboplatin (CBDCA) is a platinum coordination complex, which exhibits a potent antitumor activity (1). It is increasingly used in clinical trials due to an improved therapeutic index over Cisplatin (CDDP), the first of the several platinum complexes introduced for therapy (2). The cytotoxic effects of these drugs are thought to be due to the formation of DNA adducts, i.e. intra- and inter-strand crosslinks and DNA-protein crosslinks, which result in the inhibition of DNA duplication and transcription (3-6). Growing evidence suggests that the displacement of the leaving ligands (chlorides for CDDP and cyclobutanedicarboxylic acid for CBDCA) by water molecules to form the aquated species represents the limiting step for reaction with DNA (7). The presence of a bidentate ligand in the CBDCA molecule makes it more stable and resistant to aquation, compared to CDDP, where two independent leaving ligands are present. As consequence, the rate of reaction of CBDCA with isolated DNA *in vitro* is significantly

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Abbreviations used: CBDCA, Carboplatin (*cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II)); CDDP, Cisplatin (*cis*-diamminedichloroplatinum(II)); MELC, murine erythroleukemic cells; PBS, 20 mM Na phosphate, pH 7.4, containing 146 mM NaCl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulphoxide; ID_{50}, dose producing 50% growth inhibition; CI, combination index; F_a, fraction affected; SSB, DNA single strand breaks; ISC, DNA interstrand crosslinks.

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reduced (8). However, *in vivo*, platinum complexes containing bidentate ligands are only slightly less effective than CDDP (9), thus suggesting that their activation can occur inside the cells (10). Identification of these still undefined mechanisms of activation is therefore expected to shed light on the cytotoxicity of CBDCA, with practical outcomes in antitumor therapies (11-13).

We have previously demonstrated that *in vitro* oxygen free radicals decrease the CBDCA stability in aqueous solutions, enhancing both the rate and the extent of its conversion to the aquated forms and consequently the rate of platinum reaction with isolated DNA (11). Additional evidences suggest that reactive oxygen species, produced by oxyhemoglobin, can play a role also in the activation of CBDCA inside erythrocytes (12) and hemoglobin-producing erythroleukemic cells (13).

The studies described here were designed to investigate the effects of oxygen free radicals on the CBDCA toxicity on tumor cells and, in particular, to determine if oxygen radical species could enhance the intracellular activation of the drug. We used as free radical generating system the quinoid molecule divicine, which undergoes redox cycling, thereby generating $O_2^{\cdot}$ and H_2O_2 (14-17). It can be easily obtained from its corresponding β-glucoside vicine, particularly abundant in fava beans, by chemical or enzymatic cleavage. The two species obtained through the different treatments are both able to produce oxygen free radicals, even though the deaminated form produces them at a faster rate (17).

MATERIALS AND METHODS

Chemicals. CBDCA was from Bristol-Myers (Evansville, IN, USA). Vicine was purchased from Serva (Heidelberg, FRG). All other reagents were from Sigma Chemicals (St. Louis, MO, USA).

Cell cultures and cytotoxicity assays. Murine erythroleukemic cells (MELC, kindly provided by Prof. E. Melloni) were grown in suspension in α-MEM, supplemented with 10% FCS. Cytotoxicity was extimated by the MTT assay, performed as described (18). Briefly, exponentially growing cells (2.5×10^5/ml) were treated for two hours with different concentrations of drugs, either alone or in combination. Divicine was used before, together or at different times after exposure to CBDCA. After each treatment the cells were washed twice with ice-cold PBS and resuspended in fresh medium. They were then plated in 96-wells microtiter plates, at 5000 cells/well in 200 μl. After 2 days, 50 μl of MTT (2 mg/ml in PBS) were added to each well and incubated for 4 hours. Then, the medium was aspirated and replaced with 200 μl of DMSO. Absorbance was determined with a microplate reader at 540 nm.

Divicine preparation. Divicine was freshly prepared immediately prior to use, by chemical hydrolysis of its β-glucoside vicine (15, 16). Vicine (20 mg/ml in 1 N HCl) was put under N_2 to prevent divicine oxidation by O_2 and boiled for 10 min. The solution was then neutralized with 1 N NaOH, sterile filtered and immediately added to the cells.

Data analysis. The ID_{50}s of the drugs were calculated by plotting $\text{Log}[f_a/(1-f_a)]$ versus LogD (median effect plot) (19), where f_a is the fraction affected and D is the drug dose. The interaction between CBDCA and divicine was analyzed by calculating the combination index (CI), a parameter that derives from the comparison of isoeffective doses of the drugs when applied alone or in combination (19).

$$CI = D_1/(D_x)_1 + D_2/(D_x)_2 + \alpha(D_1)(D_2)/(D_x)_1(D_x)_2,$$

where $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that elicit x fractional effect and D1 and D_2 are the doses that elicit the same effect when the two drugs are combined. $\alpha = 0$ is used

for mutually exclusive effects (drugs act on the same site or with the same mechanism) and $\alpha = 1$ for mutually non exclusive drugs (different sites or mechanisms). CI < 1, CI = 1 and CI > 1 indicate synergistic, additive and antagonistic effects, respectively (19).

Alkaline elution. Alkaline elution studies were performed, following the Kohn procedure (20), as described (13). Cells were treated with CBDCA and divicine, alone or in combination for two hours. Divicine was added 5 hours after the beginning of CBDCA incubation. Elution was performed 1 hour after the end of divicine incubation. For the determination of DNA interstrand crosslinks (ISC), cells were irradiated at 4 °C with γ-rays (300 rad) immediately prior to cell lysis.

RESULTS AND DISCUSSION

Cytotoxicity assays

Both CBDCA and divicine, individually tested, exhibited cytotoxic effects on MELC, with ID_{50} values of 158 μM and 37 μM, respectively (Fig.1A). The antiproliferative activity of divicine is remarkable, this confirming a previous report on different tumor cells (21). The two drugs used in combination, at a CBDCA:divicine ratio of 2:1, induced a sharp increase in cytotoxicity, as shown in Fig. 1A (where the abscissa indicates the sum of doses of the two molecules). To obtain a more precise characterization of the antiproliferative effects elicited by exposure of the cells to both drugs, the combination index (CI) was calculated from the profiles shown in Fig. 1A for different effect levels (Fig. 1B). The CI proposed by Chou et al. (19) is based on the median effect principle applied for two or more drugs and allows to determine, a) the degrees of synergism (CI < 1), additivity (CI = 1) or antagonism (CI > 1) at different doses and effect levels, b) the schedule dependency and, c) the optimal combination ratio to achieve maximal effect. Fig. 1B clearly shows that in these experimental conditions, the association of CBDCA and divicine resulted in a synergistic action, assuming either mutually exclusive or non exclusive mechanisms.

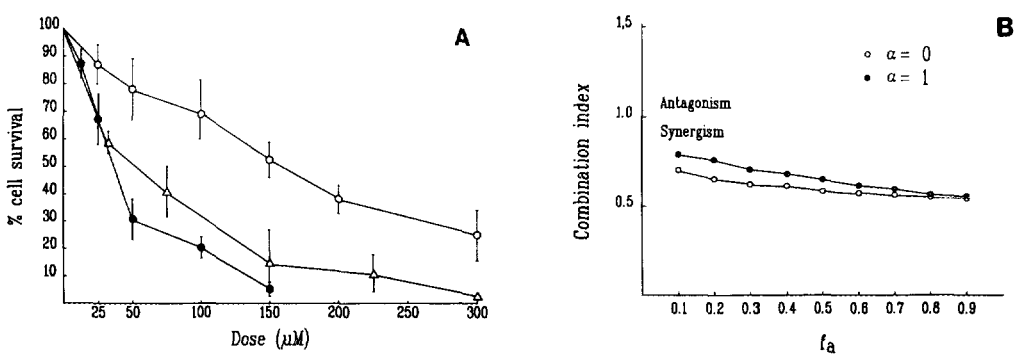

Figure 1. Cytotoxic effects on MEL cells of CBDCA and divicine either alone or used in combination. (A) Dose-response plot of CBDCA (○), divicine (●) and CBDCA + Divicine (△). Cells were exposed to both drugs for two hours. In the combination experiments, divicine was administered 6 hours after starting the exposure to CBDCA. The CBDCA:divicine ratio was 2:1. In this case, the abscissa represents the sum of doses of the two drugs, e.g.,150 μM means 100 μM CBDCA + 50 μM divicine. Data derive from at least three independent experiments. (B) f_a-CI plot derived from the curves of (A). f_a represents the different effect levels (for explanation of $\alpha = 0$ and $\alpha = 1$, see Materials and Methods). CI < 1, = 1 and > 1 indicate synergism, additive effect and antagonism, respectively.

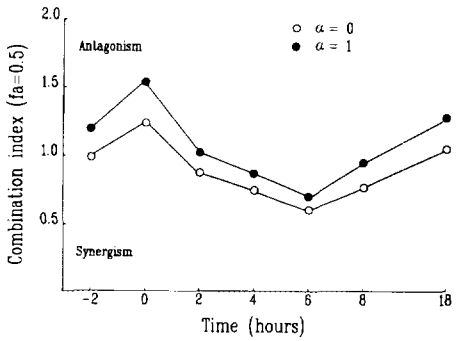

Figure 2. CI values obtained for $f_a = 0.5$, as function of the schedule of divicine administration. Time 0 indicates the beginning of CBDCA treatment. Divicine was added 2 hours before (time -2), together (time 0) or at different times after CBDCA.

The time schedule of exposure to divicine, with respect to treatment with CBDCA, was critical for the type of cytotoxic effects. The best sequence, allowing a synergistic action to be achieved, was administration of divicine within few hours after the end of CBDCA treatment. Fig. 2 shows CI values, determined for $f_a = 0.5$, i.e. the median effect, as related to the timing of divicine exposure. When divicine was used before or at 18 hours after CBDCA, a simple additive mechanism was apparent, assuming mutually exclusive effects. Conversely, the simultaneous administration of both drugs resulted in an unequivocally antagonistic effect, possibly due to direct interactions between the two molecules (due to the pyrimidine structure of divicine) (22) in the culture media (Fig. 2).

The CBDCA:divicine concentration ratio eliciting the maximal effect was 2:1. However, a lower synergistic action was observed also for other combination ratios (4:1, 10:1) (data not shown).

Alkaline elution

Alkaline elution studies were performed to elucidate the effects of CBDCA and divicine, either alone or in combination, on cellular DNA. The alkaline elution technique allows to discriminate DNA single strand sizes by their rate of elution from the filter (20). Single strand breaks (SSB) are measured on the basis of an increase in the DNA elution rate, while interstrand crosslinks (ISC) induce a delay in the elution rate. Since ISC reduce the effects inherent to the presence of SSB, their determination is usually performed after irradiation of the cells to obtain a known, standard frequency of SSB (Fig. 3B). Divicine alone, because of its ability to generate oxygen free radicals, produced *per se* a significant amount of SSB, monitored as an increased elution rate compared to the untreated cells (Fig. 3). In agreement to previous data (13), exposure to CBDCA resulted in the formation of ISC, even in unirradiated cells (Fig. 3A).

Analysis of DNA lesions produced by the combination of CBDCA and divicine was complicated by the simultaneous presence of both ISC and SSB. Specifically, the effect of either type of damage can lead to an underestimate of the other one (20). The combination of both DNA alterations can be sought by comparing the elution profiles obtained from irradiated and from unirradiated cells as well. Irradiation, by causing a large DNA fragmentation, minimizes the

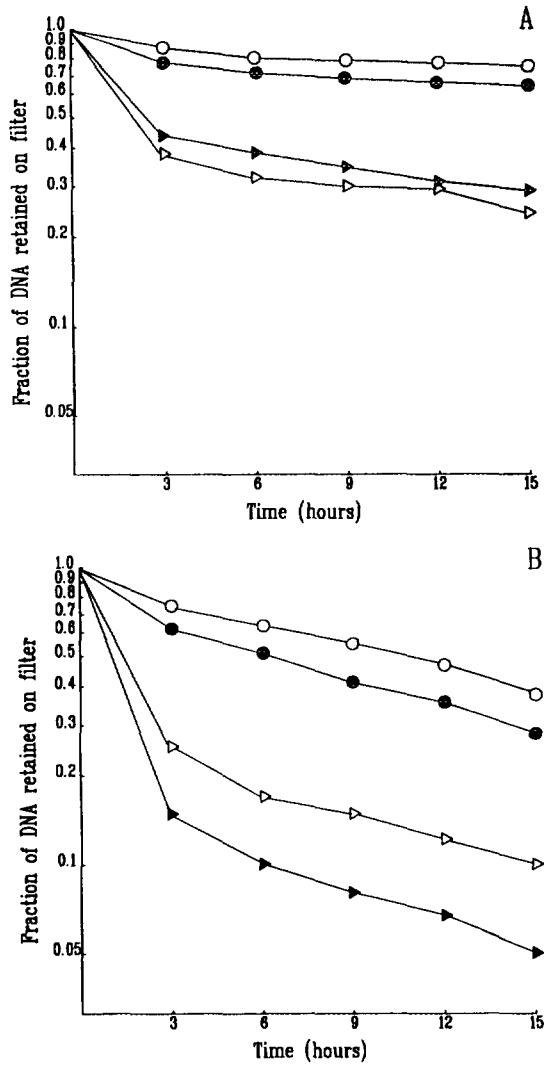

Figure 3. Profiles of alkaline elution of DNA derived from cells treated with CBDCA and divicine alone or with a combination of the two drugs. Untreated (●), CBDCA (○), divicine (▲), CBDCA + divicine (△). (A) Unirradiated cells. (B) Cell irradiated with 300 rad before elution.

effects of divicine and allows to determine ISC presence due to platinum crosslinking (Fig. 3B). The presence of ISC in cellular DNA should decrease the elution rate determined by fragmentation by divicine. However, as shown by Fig. 3A, the elution rate in unirradiated cells treated with both drugs is slightly greater than that obtained after treatment with divicine alone. Accordingly, an increased frequency of SSB appears to be present in the cells treated with both drugs. Whether and how this type of DNA damage compares with the synergistic cytotoxicity of the two drugs (Fig. 1), is still unknown.

Rather unexpectedly, no evidence whatsoever emerged for intracellular activation of CBDCA by the oxygen free radicals produced by divicine, which would result in an enhanced ISC formation. The presence of platinum seems, on the contrary, to increase the ability of

divicine to produce SSB (Fig. 3A). This effect may be due to inefficient DNA repair mechanisms, impaired by platinum itself. However, a direct effect of platinum on radical generation cannot be excluded. This possibility is suggested by an *in vitro* enhanced $OH^{\cdot}$ production with other oxygen free radical generating systems (FeEDTA/ascorbate and Fe/H_2O_2) in the presence of either CBDCA or CDDP (Tonetti et al., unpublished results).

Preliminary data, obtained in our laboratory, indicate that, similarly to CBDCA (11), oxygen free radicals are able to potentiate the *in vitro* cytotoxicity of CDDP too, which quickly undergoes a spontaneous conversion to the aquated forms. Recently, the association of CDDP and acute oxidant stress was demonstrated to induce a schedule-dependent synergistic cytotoxicity on murine and human cell lines (23). Oxidative stress has also been postulated to be responsible for the *in vivo* CDDP potentiation induced by interleukin-1α (24). However, the mechanisms responsible for such synergism have not been elucidated so far.

The data obtained in this study represent a further step toward elucidation of the mechanisms of platinum coordination complexes. In addition, they can also offer new clues to possible combination schemes to be used for antitumor therapy, for example association with the glucoside vicine, which can be activated to yield the redox cycling divicine by the β-glucosidase presents inside the cells.

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