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Protection of Ehrlich Ascites Tumor Cells against the Antiproliferative Effect of Mechlorethamine (Nitrogen Mustard) by 5-*N,N*-Dimethylamiloride[1]

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ABSTRACT

5-*N,N*-Dimethylamiloride protects Ehrlich ascites tumor cells against the antiproliferative effect of nitrogen mustard. The drug reduces the frequency of DNA interstrand cross-links introduced by nitrogen mustard. Cells with a defective choline carrier are not protected against nitrogen mustard by dimethylamiloride. As nitrogen mustard is taken up by the choline carrier, it is concluded that the recently reported inhibition of the choline transport system by amiloride and its dimethyl derivatives (W. Doppler *et al.*, Biochem. Pharmacol., *36:* 1645–1649, 1987) is responsible for the protection against the alkylating agent.

INTRODUCTION

Amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylene) pyrazinecarboxamide] is used clinically as a potassium-sparing diuretic drug. Its diuretic properties have been attributed to the blockade of Na^+ channels (1, 2). Amiloride and some if its derivatives have gained special attention as inhibitors of the Na^+/H^+-antiporter which plays a major role in the regulation of internal pH and represents an important element in signal transduction of growth factors, hormones, and other agents (3–10).

In a recent publication we have demonstrated that amiloride, as well as dimethylamiloride, interferes with the choline transport system (11). As the uptake of nitrogen mustard has been shown to occur via the choline carrier (12) it seemed possible that amiloride and dimethylamiloride might block the uptake of nitrogen mustard and thereby protect cells against the effects of the alkylating agent. It is shown here that this is indeed the case for dimethylamiloride.

MATERIALS AND METHODS

DMA[3] was kindly donated by the Austrian branch of Merck, Sharp & Dohme; nitrogen mustard [mechlorethamine; *N*-methylbis(2-chloroethyl)amine HCl; HN2) was from Aldrich Chemie, Steinheim, FRG; chlorambucil and choline chloride were obtained from Sigma Chemicals, Munich, FRG; ASTA Z7557 (4-sulfonatoethylthiocyclophosphamide) was a gift from ASTA-Werke A. G., Bielefeld, FRG; sodium dodecyl sulfate was from Serva, Heidelberg, FRG; tetrapropylammonium hydroxide was from Fluka, Neu Ulm, FRG; proteinase K was from Merck, Darmstadt, West Germany; AR 20 and AR200 silicon oils were from Wacker Chemie, Vienna, Austria; and [*methyl*-^{14}C] choline (50 mCi/mmol), ^3H_2O, and [^{3}H]inulin were obtained from the Radiochemical Centre, Amersham, England.

Cell Culture. Ehrlich ascites tumor cells were cultured in Eagle's minimal essential medium as described elsewhere (8), and cells resistant to nitrogen mustard were obtained as described previously (13).

Measurement of Cell Proliferation. For the determination of cell proliferation (p), the numbers of cells at time 0 (n_0) and after 48 h (n_{48}) were measured with a Coulter Counter Model ZM (Coulter Electronics, Luton, England), and p was calculated by the equation $p = (n_{48} - n_0)n_0$. The mean ± SE (number of experiments) of p was, for the untreated parental Ehrlich ascites tumor cell line, 3.4 ± 0.37 (9), and for the HN2-resistant cell line, 2.3 ± 0.43 (3), which corresponds to mean cell doubling times of 22.4 h for sensitive and 27.7 h for resistant cells. The ratio of p in the presence of drugs compared to untreated controls (expressed as a percentage) was taken as a measure for inhibition of cell proliferation. For calculating the IC_{50} values for inhibition of cell proliferation, the data of dose-response curves were linearized according to the method of Chou and Talalay (14). Stock solutions of DMA in DMSO, HN2 in 1 mM HCl, chlorambucil in ethanol, and ASTA Z7557 in water were prepared, and the appropriate amount was added to the culture medium. The maximal final DMSO and ethanol concentrations in the medium were 0.1% and had no significant effect on cell proliferation.

DNA Interstrand Cross-Linking. Alkaline elution assays for DNA-DNA interstrand cross-linking were performed as described by Kohn (15). [^{14}C]Thymidine-labeled cells (3.5×10^5), treated for 1 h with nitrogen mustard and DMA as indicated, were mixed with 4×10^5 [^{3}H]thymidine-labeled internal standard cells and subjected to 300 rads of X-rays at ice temperature. The cells were deposited on polycarbonate filters, 0.8-μm pore size (Nucleopore Corp., Pleasanton, CA), and lysed with 2% sodium dodecyl sulfate and 0.05 M Tris/HCl, pH 9.7. Proteolytic digestion of the lysate was accomplished by the addition of 0.5 mg/ml of proteinase K. The eluting solution contained 0.1 M tetrapropylammonium hydroxide, 0.02 M EDTA, and 0.1% sodium sulfate, pH 12.1, and was pumped at a rate of 1.5 ml/h. Elution were performed for 14 h, and fractions were collected at 2-h intervals for scintillation counting. Drugs were added to the culture medium as described previously in "Materials and Methods."

Choline Uptake Measurement. The amount of [^{14}C]choline/liter of cell water was determined by the silicon oil layer technique as described (11). One hundred to 200 μl of cells containing [^{14}C]choline and tritiated water were layered on the top in 400-μl tubes containing a lower phase of 20 μl of detergent solution (2 M NaCl/40 mM EDTA/0.2% sodium *N*-lauryl sarcosine, pH 10) and an upper phase of 100 μl of silicone oil (10 parts of AR 200/3 parts of AR 20) and centrifuged in a Beckman Microfuge B for 15 s at 10,000 × *g*. The amount of ^{3}H and ^{14}C in the cell pellet and the supernatant were measured by liquid scintillation counting, and the nmol of choline/liter of water volume (11) in the pellet were calculated from these data. The amount of extracellular water was determined as 15% of total water volume by measuring the distribution of [^{3}H]inulin in a separate experiment. Extracellular water was subtracted from the measured water volume to obtain the cell volume. No significant differences in cell volumes between HN2-sensitive and -resistant cells could be observed. All experiments were performed at 37°C.

RESULTS

Effect of DMA on the Inhibition of Cell Proliferation by HN2. Cell proliferation of EATC in culture is affected by nanomolar concentrations of HN2. The dose-response curve for this drug is shown by *closed circles* in Fig. 1. DMA exhibits an antiproliferative effect on Ehrlich cells by itself as depicted by the *open circles*. Half-maximal inhibition was calculated from these data by median effect plots (14) to occur at 69 ± 6 (4) μM (mean ±

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[3] The abbreviations used are: DMA, 5-*N,N*-dimethylamiloride; HN2, nitrogen mustard; DMSO, dimethyl sulfoxide; EATC, Ehrlich ascites tumor cells; NBP, *p*-nitrobenzylpyridine; FRG, Federal Republic of Germany; IC_{50}, 50% inhibitory concentration.

SE). A similar activity of DMA has been demonstrated in other cell lines (16, 17). The effect of a combined treatment by DMA and HN2 on cell proliferation as a function of the total concentration of both drugs is shown in Fig. 1. The molar ratio of DMA to HN2 in the mixture was kept constant at 200:1 in this experiment. The antiproliferative activity of the mixture is almost identical with the antiproliferative activity of the DMA component. The results indicate a complete suppression of the activity of the HN2 component in the mixture by DMA. Since the mixture of HN2 and DMA is less effective than HN2, a suppression of DMA activity by HN2 alone cannot account for the observed effects.

To test whether the protective effect of DMA shown in Fig. 1 is due to an increase in the rate of inactivation of the chloroethyl groups of HN2 in the presence of DMA, the alkylating activity of HN2 in the presence of DMA was measured. A 200-fold molar excess of DMA does not reduce the capacity of HN2 to alkylate NBP under the conditions of the NBP assay as described by Friedmann and Boger (18) (data not shown), ruling out the possibility of a direct effect of DMA on HN2.

DNA-DNA Cross-Link Formation by HN2 in the Presence of DMA and Choline. We investigated the effect of DMA on the capability of HN2 to form DNA-DNA interstrand cross-links. Cross-link formation was determined by the alkaline elution technique (15). Fig. 2 demonstrates that 50 μM DMA reduced the number of DNA cross-links formed in the presence of 1 μM HN2 in the culture medium. In the absence of DMA, cells treated with 0.1 μM HN2 exhibit higher levels of DNA-interstrand cross-linking than cells exposed to 1 μM HN2 in the presence of 50 μM DNA. The frequency of DNA-DNA cross-links at a given time point is dependent on the rates of formation and repair. The pronounced effect of DMA on DNA-DNA cross-link frequency by HN2 was measured 1 h after addition of HN2 and DMA to the culture medium. At this early time point the effect of repair processes on the amount of cross-links by HN2 is low (19). Thus, an interference with cross-link formation rather than with repair most likely accounts for the observed effect of DMA.

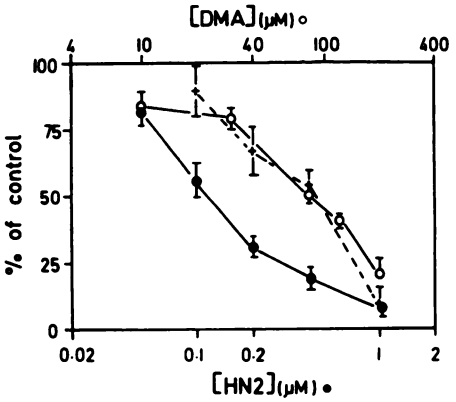

Fig. 1. Effects of HN2 and DMA on cell proliferation of sensitive EATC. Cell proliferation in the presence of inhibitors after cultivation for 48 h was determined after cultivation for 48 h as described in "Materials and Methods" and is shown as the percentage of untreated controls. *Bars*, SEM of at least 4 experiments. ●, HN2; ○, DMA; + – +, HN2 plus DMA, molar ratio (1:200).

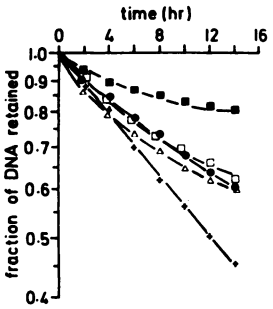

Fig. 2. DNA-DNA interstrand cross-link formation by HN2 in sensitive EATC. DNA-DNA interstrand cross-link formation of sensitive EATC was measured as described in "Materials and Methods." *Points*, mean of two separate experiments. Untreated controls (+ – +); cells treated in culture for 1 h with 1 μM HN2 (■); 0.1 μM HN2 (□); 1 μM HN2 plus 50 μM DMA (△); 1 μM HN2 plus 10 mM choline (●).

A major factor in cross-link formation is the intracellular concentration of HN2. In contrast to *Vinca* alkaloids and anthracycline antibiotics, no extrusion system which affects intracellular concentrations of HN2 has been demonstrated (20). Uptake of HN2 has been shown to be mediated by the choline carrier (12). Therefore, uptake can be reduced by addition of choline to the culture medium which competes with HN2 for the same carrier. As depicted in Fig. 2, 10 mM choline caused a similar reduction in the DNA-DNA cross-links produced by 1 μM HN2 as did 50 μM DMA (Fig. 2, *triangles*). The addition of choline also protects the EATC against the cytostatic activity of HN2. Cell proliferation over a 48-h period was measured. Addition of 1 μM HN2 for 1 h to the culture medium inhibits cell proliferation to 33 ± 14.4% (mean ± SE) of control cells. When 10 mM choline and 1 μM HN2 are added to the culture medium, cell proliferation is 93 ± 5.3% of untreated control cells.

Impairment of Carrier-mediated Drug Uptake as a Common Mechanism for Protection against the Cytostatic Activity of HN2. Ehrlich ascites tumor cells with an acquired resistance to HN2 were obtained by cultivating the cells in the presence of increasing concentrations of HN2 for a period of 6 mo (13). Table 1 shows that the sensitivity of these cells to the cytostatic activity of HN2 is reduced 40-fold, whereas their sensitivity to the cyclophosphamide ASTA Z7557 and chlorambucil is not reduced. Fig. 3 demonstrates that the frequency of DNA-interstrand cross-links observed after treatment with HN2 is much lower in resistant cells compared to the sensitive parental line. Resistant cells require a 10 times higher HN2 concentration in order to obtain a similar cross-linking frequency than do HN2-treated sensitive cells.

Fig. 4 depicts the uptake kinetics for choline by the HN2-resistant cell line compared to the parental cell line. There is no significant difference in the apparent V_{max} values for choline uptake between the two cell lines. However, the affinity of the carrier for choline is reduced: the K_m value for choline by the resistant cell line was determined as 124 μM which is 8-fold higher than the K_m value observed with sensitive cells (15.2 μM). We conclude that reduced uptake of HN2 by the defective choline carrier is responsible for at least a major part of the resistance to HN2 in our cell line. Other HN2-resistant cell

Table 1 *Effect of HN2, ASTA Z7557, and chlorambucil on cell proliferation of HN2-sensitive and -resistant EATC*

IC_{50} values for inhibition of cell proliferation were obtained from dose-response curves as described in "Materials and Methods." ASTA Z7557 was given for 4 h, chlorambucil was given for 6 h, and HN2 was continuously present during the 48-h interval.

Inhibitor	IC_{50} (μM)	
	Sensitive EATC	Resistant EATC
HN2	0.12 ± 0.017[a] (6)[b]	4.9 ± 0.50 (6)
ASTA Z7557	1.8 ± 0.44 (4)	1.6 ± 0.38 (4)
Chlorambucil	1.3 ± 0.13 (3)	1.2 ± 0.20 (3)

[a] Mean ± SE.
[b] Numbers in parentheses, number of experiments.

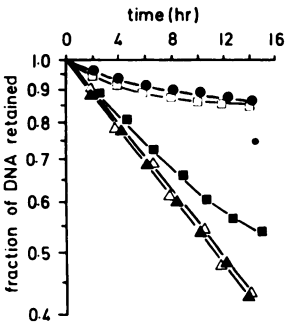

Fig. 3. DNA-DNA interstrand cross-link formation in HN2-resistant EATC compared to sensitive EATC. Cross-link formation was measured as described in Fig. 2. *Open symbols*, sensitive cells; *closed symbols*, resistant cells. △ and ▲, untreated; □ and ■, treated with 1 μM HN2; ●, treated with 10 μM HN2. Incubation with HN2 was 1 h at 37°C. Cross-link formation was measured immediately after the incubation period. *Points*, means of two separate experiments.

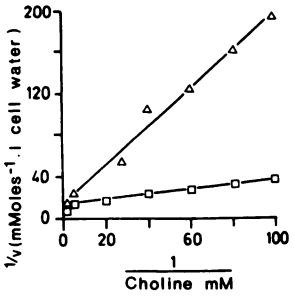

Fig. 4. Choline uptake kinetics of nitrogen mustard-sensitive (□) and -resistant (△) Ehrlich ascites tumor cells. Exponentially growing cells from culture were concentrated to a density of 2×10^6 cells/ml by centrifugation (5 min at 600 × *g*) and resuspension in Eagle's minimal essential medium with Eagle's salts buffered with 20 mM 3-(*N*-morpholino)propane sulfonic acid at pH 7.35. Tritiated water (10 μCi/ml) was added to the suspension. The concentration of choline in this medium was taken as 7.1 μM. After a preincubation period of 10 to 40 min at 37°C, 135-μl aliquots of the cellular suspension were transferred to the test tubes with 150 μl of the same medium, but with different amounts of [^{14}C]choline, and mixed rapidly. Ten min later the cells were separated from the medium by the silicone oil layer method, and the amount of choline/liter of cell water was calculated as described in "Materials and Methods." Each *point* represents the mean of two determinations. Data were plotted by the method of Lineweaver and Burk.

lines which exhibit defects of the choline carrier have been described (21, 22).

The data shown so far demonstrate that a reduced sensitivity to the antiproliferative activity of HN2 can be produced in sensitive cells by addition of DMA or choline. A reduced sensitivity to HN2 is also seen with choline carrier-defective cells (Fig. 1; Table 1). Thus, in all cases the depression of the antiproliferative activity of HN2 can be attributed to a common mechanism, namely, a depression of the choline carrier-mediated uptake of HN2. The HN2-resistant cells are characterized by a defective choline carrier leading to a reduced uptake of HN2. It is not surprising, therefore, that the DMA concentrations used in the experiment described in Fig. 1 with sensitive cells do not protect against the antiproliferative activity of HN2 in the resistant cell line (data not shown). However, for the resistant cells, the molar ratio of DMA to HN2 can only be adjusted to 20:1 (in contrast to 200:1 in the case of the HN2-sensitive line) which makes an interpretation of the data obtained with resistant cells more difficult. The reason why the DMA/HN2 ratio cannot be further increased in resistant cells is due to the fact that they require 10-fold higher HN2 concentrations for equitoxic effects. A corresponding 10-fold increase in DMA, however, would not be tolerated by the cells and would exhibit DMA toxicities only.

DISCUSSION

This paper demonstrates that dimethylamiloride is able to protect Ehrlich ascites tumor cells in culture against the cytotoxic activity of nitrogen mustard. Basically the same but less pronounced effects are obtained if amiloride instead of its dimethyl derivative is used (data not shown). The conclusion that this effect is caused by an interference with nitrogen mustard uptake is based on the following arguments. (*a*) Nitrogen mustard has been shown to be transported into the cells by the choline carrier (12). (*b*) Amiloride and dimethylamiloride inhibit the uptake of choline in Ehrlich ascites tumor cells (11). (*c*) Half-maximal inhibition of choline transport by amiloride and dimethylamiloride occurs at 60 and 20 μM, respectively (11). Protection against nitrogen mustard is seen within the same dose range. (*d*) The frequency of DNA-interstrand cross-links introduced by nitrogen mustard can be reduced by both choline and dimethylamiloride. Neither choline nor dimethylamiloride is alkylated by nitrogen mustard. Thus, under the conditions used cross-linking frequency is proportional to the transport capacity for nitrogen mustard. (*e*) Cells with a defective choline carrier exhibit a reduced sensitivity to nitrogen mustard and show less cross-linking of DNA compared to cells with a normal choline carrier activity. Amiloride and dimethylamiloride inhibit the Na^+/H^+-antiporter of Ehrlich ascites tumor cells at 1 mM external sodium with IC_{50} values of 25 and 0.6 μM, respectively. The IC_{50} values for the inhibition of the choline transport by amiloride and dimethylamiloride have been determined as 60 and 20 μM, respectively (11). Thus, the affinity of amiloride to the choline carrier is lower than to the Na^+/H^+-exchange system, but seems to be higher when compared to the affinity of amiloride to Na^+/K^+-ATPase (23) or the Na^+/Ca^{2+}-antiporter (24). The data indicate that the interference of amiloride and its dimethylcongener with nitrogen mustard uptake occurs within the same dose range as many of the pharmacological effects of these drugs. Furthermore, in contrast to the effect of amiloride on the Na^+/H^+-antiporter, the inhibition of the choline carrier by amiloride is not antagonized by Na^+ ions (11), which favors the interaction with the choline carrier at physiological Na^+ concentrations.

Amiloride exerts its diuretic effects at concentrations which are about one-tenth of those discussed here (2). It seems unlikely, therefore, that amiloride, if administered at a diuretic dose, interferes with nitrogen mustard. It should be emphasized, however, that cells have been shown to accumulate amiloride (25, 26). It remains to be checked, therefore, whether amiloride at diuretic concentrations antagonizes the antitumor activity of nitrogen mustard during chemotherapy. It is conceivable that the observations presented here could be used for the design of a protective scheme against unwanted, toxic side effects of nitrogen mustard. Derivatization of the amiloride molecule has shown that the affinity to some transport systems can be dramatically increased with little effect on other functions (3, 5). Thus, systematic investigation on the available amiloride derivatives and/or synthesis of new compounds of this series may lead to even more powerful antagonists of nitrogen mustard.

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