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SHORT COMMUNICATIONS

Modulation of mustard toxicity by tacrine

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Abstract—Compounds containing the chloroethyl group are potent inhibitors of DNA synthesis and cell growth. Tacrine, a choline carrier inhibitor, was found to protect both HeLa cells and rat thymocytes against the effects of nitrogen mustard. DNA synthesis was restored from 13 to 71% of the control value and cell viability restored from 27 to 57% of the control value by exposure of the cells to an equimolar concentration of tacrine immediately prior to nitrogen mustard. In contrast, tacrine was unable to significantly protect rat thymocytes against the toxic effects of sulphur mustard. These results have implications for the clinical use of nitrogen mustard.

Key words: nitrogen mustard; sulphur mustard; DNA synthesis; thymocytes; tacrine

HN2* and sulphur mustard and a number of their derivatives are potent alkylating agents and several of the nitrogen mustards are valuable therapeutic agents for the treatment of cancer [1]. There is a substantial body of evidence which implicates DNA in the mechanism of action of these compounds: the first visible signs of damage appear in the nucleus [2, 3]; cells which are in the process of growth and division are more sensitive than differentiated cells; cells are most sensitive during the DNA synthetic phase of the cell cycle; DNA synthesis is one of the first cellular processes to be affected and DNA repair enzymes influence the toxicity of these compounds [4–6]. Metabolic changes resulting from DNA damage, such as depletion of NAD^+, are observed *in vivo* [7]. It is also well known that the mustards alkylate nucleic acids. Alkylation occurs in both DNA and RNA at adenine and guanine residues, the preferred sites being guanine N7 and adenine N1 and N3 [8–10]. Inhibition of DNA synthesis provides a sensitive marker of the activity of these compounds.

Cellular uptake of the HN2 is an active, carrier-mediated process which utilizes the choline carrier [11]. This process reflects the close structural similarity between choline and HN2. Tacrine (1,2,3,4-tetrahydro-9-aminoacridine) is a compound of broad pharmacological activity. The properties of tacrine have been reviewed recently, and the drug has been used as an antibacterial agent, a decurarizing agent and in the treatment of Altzheimers disease and intoxication with psychotropic drugs [12]. Among its broad range of activities, tacrine also inhibits the choline carrier in synaptosomes [13]. Tacrine therefore represents an established clinically useful compound which may moderate the toxic effects of HN2 by means of its ability to inhibit the uptake of HN2 by the choline carrier. The aim of this study was to examine this possibility in isolated cells by using both cell viability, measured by dye exclusion, and DNA synthesis as indicators of the uptake and subsequent damage inflicted by HN2.

Materials and Methods

Tacrine was synthesized at the Institute of Drug Technology (Parkville, Victoria, Australia). HN2 was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.). Sulphur mustard (bis(2-chloroethyl)sulphide, HD) was synthesized at Materials Research Laboratory (DSTO, Australia), and was greater than 98% pure as assessed by ^1H + NMR. [^3H]Thymidine was obtained from Amersham International (Amersham, U.K.). Basal Eagle's medium (HEPES modification) and Folin and Ciocalteu's reagent were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were of analytical grade and all solutions were prepared using distilled, deionized and filtered water from a "Milli-Q" 4-stage water purification system (Millipore, Bedford, MA, U.S.A.).

Thymocyte preparation. Sprague–Dawley rats (male or female) between 4 and 7 weeks old were killed and the thymus removed. The tissue was gently teased apart in Eagle's–HEPES medium, composed of basal Eagle's medium buffered with 0.02 M HEPES buffer, pH 7.4. The cell suspension was filtered through cheesecloth to remove

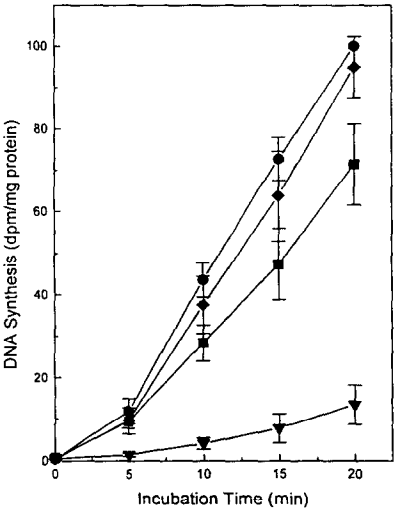

Fig. 1. Effect of HN2 and tacrine on DNA synthesis in rat thymocytes. The results are the means and SD of results from the treatment of five separate animals. (●) Control, (◆) 50 μM tacrine, (■) 50 μM HN2 plus 50 μM tacrine, (▼) 50 μM HN2.

* Abbreviations: HN2, nitrogen mustard, bis(2-chloroethyl)methylamine; TCA, trichloroacetic acid; DMEM, Dulbecco's modified Eagle's medium.

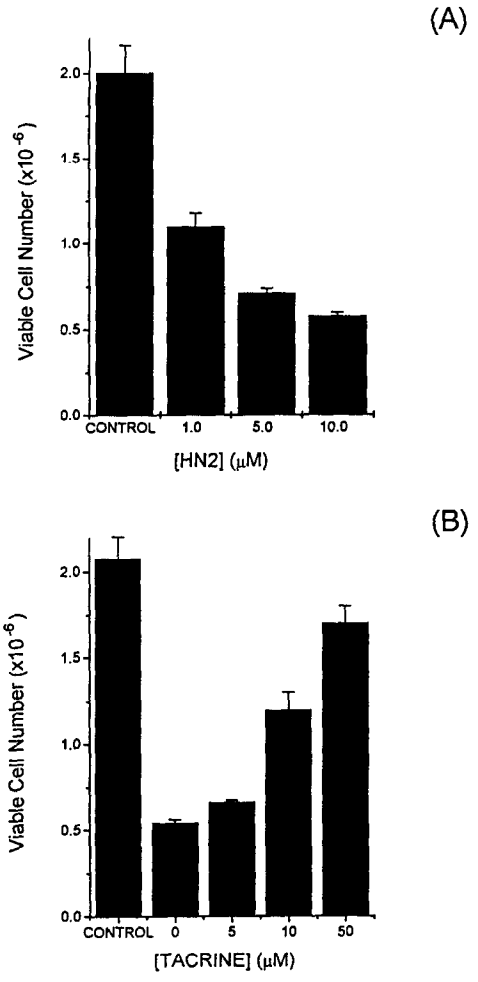

Fig. 2. Effect of HN2 (10 μM) and tacrine (10 μM) on the viability of HeLa cells. The results are the means of three separate cultures and the error bars indicate the maximum error associated with these values. HeLa cells were exposed to 0–10 μM HN2 for 24 hr (A) or to 10 μM mustard for 24 hr in the presence of 0–50 μM tacrine (B).

the tissue debris and the cells concentrated by centrifugation at 2500 rpm in a Clements GS200 centrifuge at 4°. The cells were washed twice with 10 mL Eagle's medium, resuspended in 3 mL medium and counted with a haemocytometer. After counting, the cell concentration was adjusted to 2×10^8 cells/mL.

To examine the effect of mustard and tacrine on DNA synthesis, 1.0 mL of cell suspension, Eagle's medium, tacrine and HN2 (in 100 μL) were added together and the volume adjusted to 5.0 mL with Eagle's medium. The cells were then incubated at 37° for 60 min, centrifuged, washed twice with 5 mL of Eagle's medium and resuspended in 5.5 mL medium. Eagle's medium (50 μL) containing [^{3}H]-thymidine (59 μCi/ml, 82 Ci/mmol) was added and the incubation continued at 37°. Aliquots (500 μL) were removed at 0, 5, 10, 15 and 20 min and added to 2 mL of 6% TCA. The acid insoluble material was precipitated by centrifugation, washed twice with 2 mL of 6% TCA and dissolved in 1.0 mL of 0.1 M NaOH. A 0.5 mL aliquot of the redissolved material was neutralized with 0.1 M HCl and radioactivity determined by liquid scintillation counting. The remaining material was used for protein determination using the method of Hartree [14]. DNA synthesis was determined as dpm/mg protein.

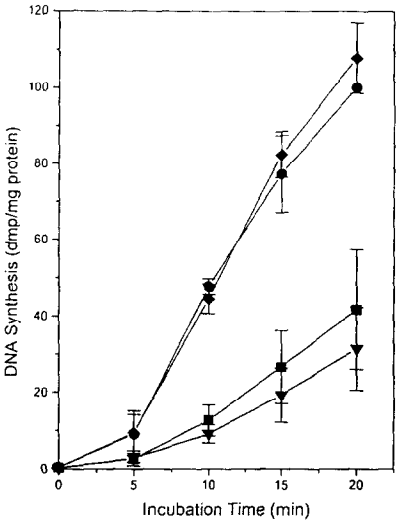

Fig. 3. Effect of sulphur mustard and tacrine on DNA synthesis in rat thymocytes. The results are the means and SD of results from the treatment of five separate animals. (●) Control, (◆) 50 μM tacrine, (■) 50 μM sulphur mustard plus 50 μM tacrine, (▼) 50 μM sulphur mustard.

HeLa cell culture. HeLa cells were grown in DMEM supplemented with 10% foetal calf serum to a confluency of 25% in 5% carbon dioxide at 37°. To determine the effect of HN2 on cell viability in this system, cells were exposed to a range of HN2 concentrations and incubated for 24 hr at 37°. Cells were then harvested, washed twice with PBS and resuspended in PBS. Equal volumes of the resuspended cells and Trypan blue (0.4%) viability stain were mixed and after 5 min the number of viable cells counted using a haemocytometer. Cells showing uptake of the blue dye were scored as non-viable.

To determine the effect of tacrine on cell viability, cells were exposed to 10 μM HN2 and a range of tacrine concentrations and incubated for 24 hr. Treatment of the cells and measurement of viability were as described above.

Results and Discussion

The effects of HN2 and tacrine on DNA synthesis in isolated rat thymocytes are shown in Fig. 1. Cells were exposed to equal concentrations of mustard and tacrine (50 μM) for 60 min, followed by removal of the tacrine and mustard by washing and measurement of DNA synthesis by incorporation of [^{3}H]thymidine. Removal of the tacrine prior to determination of [^{3}H]thymidine incorporation into DNA was necessary since preliminary studies have shown that tacrine inhibits the cellular uptake of [^{3}H]thymidine (results not shown). Exposure of the thymocytes to 50 μM HN2 for 60 min reduced DNA synthesis over 20 min to 13% of the control value. Addition of an equimolar concentration of tacrine to the cells immediately before addition of the mustard protected the ability of the cells to synthesize DNA. Tacrine restored the DNA synthesized over the 20 min incubation period to 71% of the control value.

In addition to re-establishing the DNA synthetic ability of cells, tacrine also increased the viability of HeLa cells exposed to HN2. Exposure to HN2 for 24 hr significantly reduced the viability of HeLa cells. After exposure to 10 μM mustard the number of viable cells was reduced to 31% of the control value (Fig. 2A). Tacrine restored the viability of the cells, with 50 μM tacrine raising the viability of cells treated with 10 μM mustard to 83% of the control value (Fig. 2B).

In contrast to the results achieved with HN2, tacrine was

far less effective in reducing the effects of sulphur mustard. Exposure of the thymocytes to 50 μM sulphur mustard for 60 min reduced DNA synthesis over 20 min to 31% of the control value. Treatment with 50 μM tacrine immediately prior to exposure to the alkylating agent resulted in a small protective effect, with DNA synthesis rising by 11% to 42% of the control value (Fig. 3).

These results, which show the protection afforded by tacrine against HN2 in two completely different cell systems, indicate the apparent importance of the choline carrier in the mechanism of action of HN2. However, the significance of this carrier is based on the assumption that tacrine acts in these cell systems solely as an inhibitor of the choline carrier. This assumption is given some support by the sulphur mustard results. The observation that sulphur mustard, which contains the same bifunctional chloroethyl structure as HN2, inhibits DNA synthesis in the presence of tacrine suggests that tacrine does not simply scavenge the HN2. It should be noted, however, that the role of tacrine in these systems could, in principle, also be due to its effect on other receptor sites or other mechanisms of action. The smaller effect of sulphur mustard on DNA synthesis when compared to equimolar concentrations of HN2, reduction to 31% versus reduction to 13% of the control value, may reflect factors such as membrane solubility and compartmentation within the cell.

The effect of sulphur mustard on unprotected humans was demonstrated graphically during the war between Iran and Iraq [15]. Nitrogen mustards have been proposed as potential chemical warfare agents [16] and would be expected to have similar drastic effects on unprotected individuals. Tacrine or other suitable uptake inhibitors may constitute an effective protection against this misuse. A second situation in which the use of tacrine may be beneficial relates to tissue damage which results from extravasation of therapeutic agents is a common occurrence in cancer patients undergoing intravenous chemotherapy [17]. For HN2, local injection of thiosulphate to scavenge the alkylating agent is a recommended treatment for this problem [17]. Tacrine may represent a suitable alternative or adjunct to this approach. A combined approach of scavenging with thiosulphate and inhibiting uptake with tacrine may prove effective in preventing the ulceration damage which can result from extravasation. Goldenberg *et al.* [18] suggested using choline as a protective agent against the systemic toxicity of HN2 during local application of this agent by regional perfusion or intracavity instillation. Again, tacrine may prove a useful adjunct in this type of therapeutic approach.

In summary, these results show a pronounced effect of tacrine in modulating the cytotoxic effect of HN2 in both HeLa cells and also in rat thymocytes, and serve to indicate the potential use of this drug as a protective agent against exposure to nitrogen mustards in both clinical and non-clinical situations.

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