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THE CYTOTOXICITY, DNA CROSSLINKING ABILITY AND DNA SEQUENCE SELECTIVITY OF THE ANILINE MUSTARDS MELPHALAN, CHLORAMBUCIL AND 4-[BIS(2-CHLOROETHYL)AMINO] BENZOIC ACID

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Abstract—Three aniline derivatives melphalan (L-PAM), chlorambucil (CHL) and 4-[bis(2-chloroethyl)amino] benzoic acid (BAM) have been compared on the basis of their *in vitro* cytotoxicities, DNA interstrand crosslinking ability and DNA sequence selectivity. Cytotoxicity was assessed in the human colonic adenocarcinoma LS174T and leukaemic K562 cell lines using the sulphorhodamine B and tetrazolium dye reduction assays. The order of cytotoxicities was L-PAM > CHL > BAM in both cell lines with K562 being less sensitive than LS174T. This was different from the order CHL > L-PAM > BAM which would be predicted from simple chemical reactivity or rate of hydrolysis, parameters which have been used previously as indicators of biological potency for aromatic nitrogen mustards. DNA interstrand crosslinking in cells as determined by alkaline elution showed a correlation with IC_{50} values. The ranking order of activity was further predicted by the ability of the agents to produce interstrand crosslinks in isolated DNA. The extent of guanine N-7 alkylation, assessed using a modified DNA sequencing technique, mirrored cytotoxicity and crosslinking ability, but at equivalent levels of alkylation there was no significant difference in DNA sequence selectivity. These data demonstrates that simple chemical reactivity or hydrolysis rate is not a good indicator of DNA reactivity or cytotoxicity for a number of aniline mustards, whereas DNA interstrand crosslinking ability either measured directly in cells or in isolated DNA, gives a good indication of biological activity.

Bifunctional alkylating agents have been used to treat cancer for many years. Melphalan (L-PAM)‖ and chlorambucil (CHL), two commonly used nitrogen mustards, are believed to exert their toxic effect by chemically crosslinking two complementary strands of DNA [1, 2]. One approach to increase the selective killing of tumour cells uses a tumour-directed antibody enzyme conjugate [3]. In this Antibody Directed Enzyme Prodrug Therapy (ADEPT) system, a tumour-specific monoclonal antibody is chemically conjugated to an enzyme (e.g. carboxypeptidase G2). The antibody–enzyme complex then localizes to the tumour site. This enzyme then converts a subsequently administered inactive prodrug to a more active drug. One of the prodrugs is 4-[bis(2-chloroethyl)amino]benzoyl-L-glutamate which is activated by the removal of the glutamic acid residue by carboxypeptidase G2 to form the active species 4-[bis(chloroethyl)amino] benzoic acid (BAM) (Fig. 1) [4]. Other prodrug/enzyme systems have also been evaluated [5].

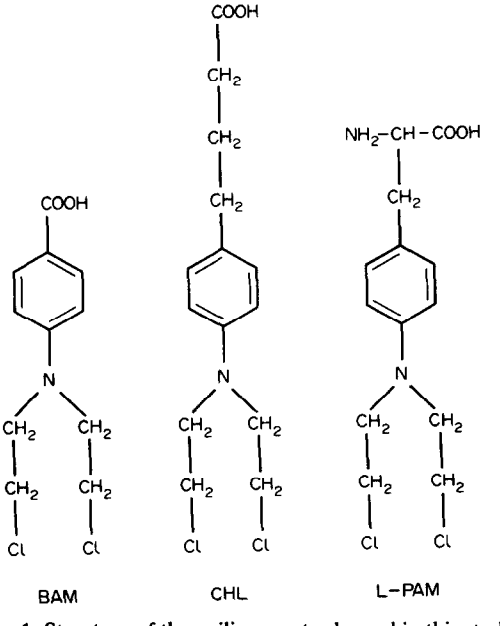

Fig. 1. Structure of the aniline mustards used in this study.

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‖ Abbreviations: L-PAM, melphalan; CHL, chlorambucil; BAM, 4-[bis(2-chloroethyl)amino] benzoic acid; MTT assay, tetrazolium dye reduction assay; SRB, sulphorhodamine B; NBP, 4-(*p*-nitrobenzyl)pyridine.

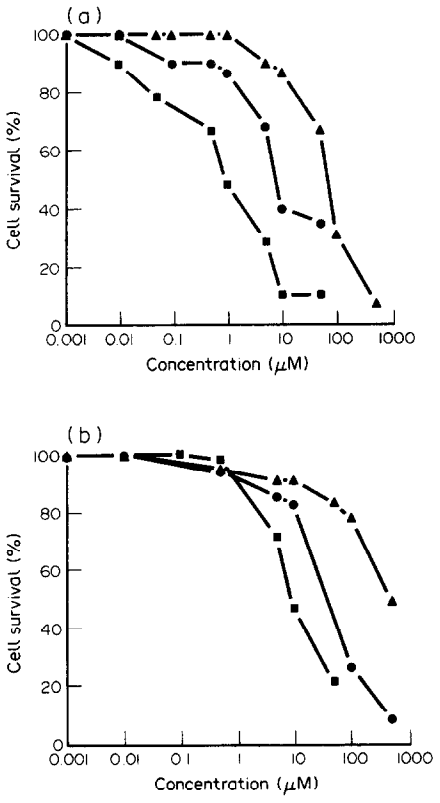

Fig. 2. *In vitro* cytotoxicity of L-PAM (■), CHL (●) and BAM (▲) on (a) L5154T cells (as determined by the SRB assay) and (b) K562 (as determined by the MTT assay).

Table 1. Cytotoxicity of the aniline mustards in LS174T and K562 cell lines

Drug	LS174T IC_{50}* (μM)	LS174T Ratio	K562 IC_{50}† (μM)	K562 Ratio
L-PAM	1.0	1.0	8.5	1.0
CHL	9.0	9.0	45	5.3
BAM	85	85	500	59

* Determined by the sulphorhodamine B assay.
† Determined by the MTT assay.

In the course of studies to develop drugs for use in the ADEPT programme it was necessary to establish assay systems to assess the effectiveness of novel agents. It was generally assumed that the rate of hydrolysis of aromatic nitrogen mustards is an indicator of biological potency [6]. The present study demonstrates that this is not always accurate and that formation of DNA interstrand crosslinks may be a better predictor of biological effect.

MATERIALS AND METHODS

Materials were of analytical grade and purchased from the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. BAM was synthesized as previously published [4].

Cell culture

The human colonic adenocarcinoma cell line LS174T was cultured in DMEM containing 10% foetal calf serum and 25 mM Hepes at 37°. Cells were passaged following trypsinization. The human leukaemia cell line K562 was cultured in RPMI containing 10% foetal calf serum at 37°.

Cytotoxicity assays

Sulphorhodamine B. LS174T cells were plated out at 3000 cells/well in 100 μL DMEM in 96-well plates and left to adhere overnight. Cells were treated with drugs and then incubated at 37° for a further 7 days, after which the plates were fixed with 50 μL of ice-cold trichloroacetic acid for 20 min. Cells were then washed five times in tap water and incubated for 15 min in 0.4% sulphorhodamine B (SRB) solution in 1% acetic acid. The plates were washed five times in 1% acetic acid before being air dried overnight. SRB was dissolved in 100 μL of 10 mM Tris, and the plates read at 540 nm using a plate reader [7].

MTT assays. The tetrazolium dye reduction (MTT) assay was performed as described [8] with K562 cells, as these cells performed poorly in the SRB assay.

DNA crosslinking

The rate of DNA crosslink formation in isolated pBR322 DNA (Northumbria Biologicals Ltd) was assayed by a recently developed agarose gel renaturation technique [9]. pBR322 was 5′ end labelled with ^{32}P, diluted in 25 mM triethanolamine and 1 mM EDTA (pH 7.2), and incubated with the drug for the times indicated. Reactions were terminated by mixing with an equal volume of 0.6 M sodium acetate, 20 mM EDTA and 100 μg/mL tRNA. The reacted DNA was ethanol precipitated, washed and dried by lyophilization. DNA was resuspended in denaturing buffer (30% dimethyl sulphoxide, 1 mM EDTA, 0.04% Bromophenol blue, 0.04% xylene cylanol) and denatured by heating at 90° for 2 min, followed by immersion in an ice/water bath. Control (i.e. non-denatured DNA) was resuspended in 6% sucrose, 0.04% Bromophenol blue buffer. All samples were electrophoresed in 0.8% agarose (Ultra pure grade BRL) in 40 mM Tris, 2 mM EDTA and 20 mM sodium acetate buffer, pH 8.1. Gels were dried onto Whatman DE81 and 3MM paper and autoradiographed at −70°. The percentage DNA crosslinking was determined by quantifying the amount of double stranded (crosslinked) DNA from scanning densitometry on a LKB Ultrascan XL densitometer.

DNA sequence specificity

The sequence selectivity of guanine N-7 alkylation of the drugs used was compared using a modified Maxam–Gilbert method [10]. pBR322 DNA was 5′ end labelled with ^{32}P, digested with Eco R1 (BRL), and reacted for 60 min at 25° with the drug under

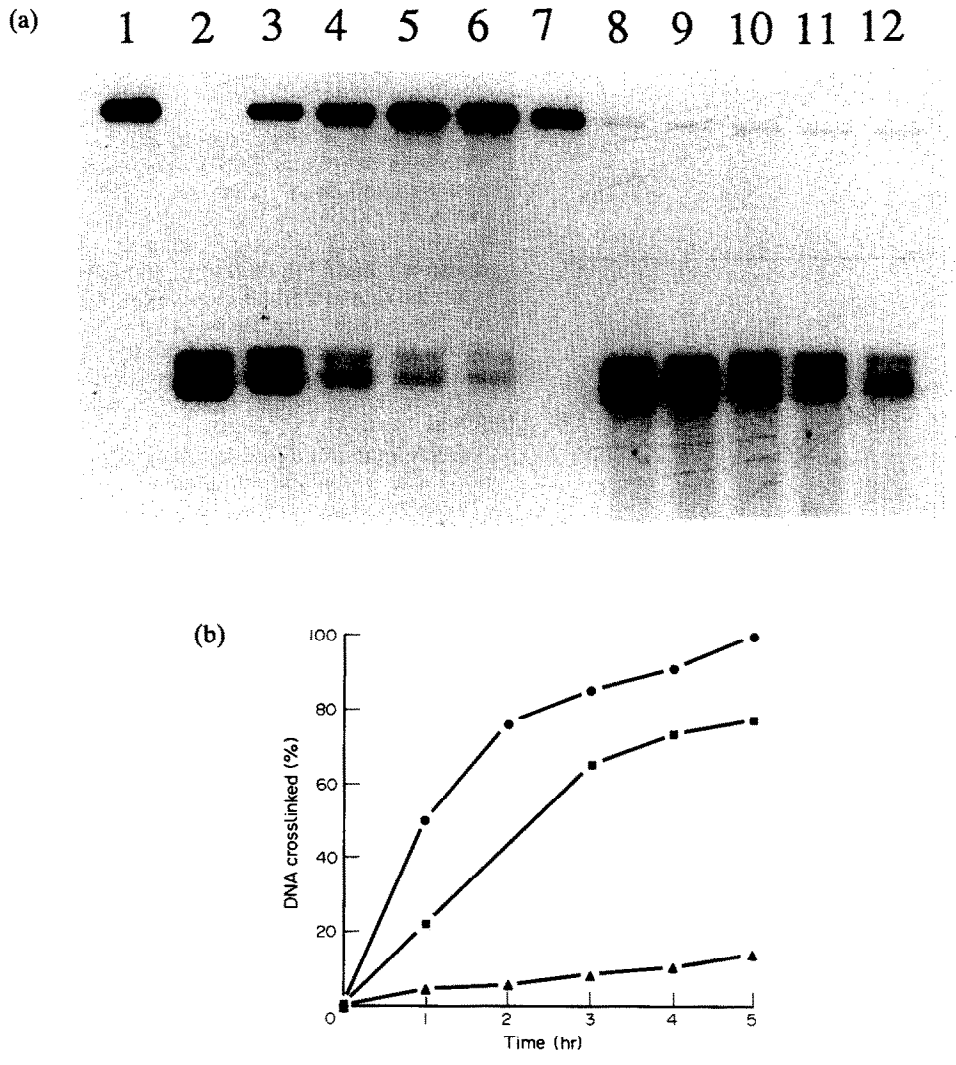

Fig. 3. (a) Autoradiograph showing time course of isolated DNA by CHL 100 μM (lanes 3–7) and BAM 1 mM (lanes 8–12). Times are 1 hr (lanes 3 and 8), 2 hr (4 and 9), 3 hr (5 and 10), 4 hr (6 and 11) and 5 hr (7 and 12). Lane 1 is undenatured, double-stranded DNA and lane 2 denatured single-stranded DNA. (b) Crosslink formation in isolated DNA by L-PAM (10 μM, ■), CHL (100 μM, ●) and BAM (1 mM, ▲).

investigation. The reaction was terminated by the addition of an equal volume of 0.6 M sodium acetate, 20 mM EDTA and 100 μg tRNA. The DNA was then ethanol precipitated, dried and reacted with 1 M piperidine at 90° for 15 min, before being lyophilized overnight. Excess piperidine was removed by twice dissolving the DNA in 10 μL of water, followed by lyophilization. DNA was dissolved in formamide dye and heated to 90° for 2 min, chilled on ice, and loaded onto a 6% acrylamide urea gel. Electrophoresis was at 3000 V and 55°. The gels were dried and autoradiographed at −70°. Autoradiograms were scanned using an LKB Ultrascan XL laser scanning densitometer.

Alkaline elution

Alkaline elution was performed according to Kohn *et al.* [11] using K562 cells in which high molecular DNA had been labelled for 16 hr with [^{14}C]thymidine (final concentration 0.0165 mCi/mM). Drug treatments were for 1 hr at 37°, followed by 4 hr post incubation in drug free medium. Cells were X-irradiated on ice with 450 rad. Alkaline elution was performed for 15 hr at pH 12.1 with the inclusion of proteinase K. A crosslink index was calculated as described previously [11].

RESULTS

The cytotoxicity of three aniline mustards L-PAM, CHL and BAM (Fig. 1) was compared in LS174T and K562 cell lines. The survival curves are shown in Fig. 2 and IC_{50} values presented in Table 1. In the colonic line, L-PAM was approximately 9-fold

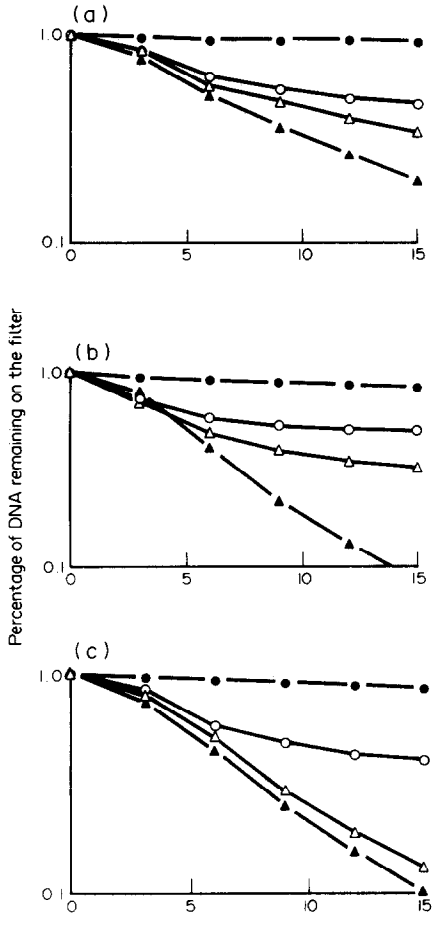

Fig. 4. Alkaline elution profiles from K562 cells treated with (a) L-PAM, (b) CHL and (c) BAM. In each case (●) is unirradiated control DNA and (▲) irradiated control. Doses of drug are L-PAM 5 μM (△) and 10 μM (○), CHL 100 μM (△) and 250 μM (○) and BAM 500 μM (△) and 1 mM (○).

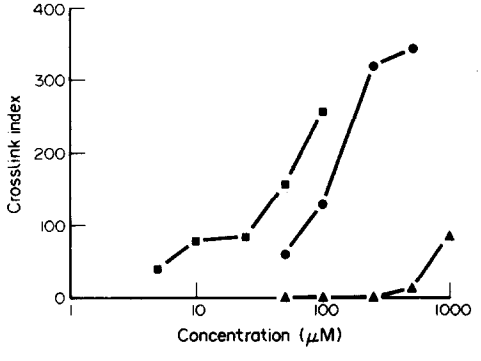

Fig. 5. Crosslink indices derived from alkaline elution profiles from K562 cells treated with L-PAM (■), CHL (●) and BAM (▲).

more active than CHL and 85-fold more active than BAM. The K562 was much more resistant to the nitrogen mustards but showed the same trend in IC_{50} values.

The ability of the drugs to produce interstrand crosslinks in isolated DNA was assessed using an agarose gel-based technique. The crosslinking profiles are shown in Fig. 3 and clearly show that L-PAM (10 μM) is more efficient at producing crosslinks than CHL (100 μM), and BAM (1000 μM) is a relatively inefficient crosslinker, with only a low level of crosslink formation even at 1 mM drug. With BAM, the rate of crosslink formation appears slow and still increasing at 5 hr, whereas with L-PAM and CHL the crosslinking profile is reaching a plateau at 5 hr.

DNA interstrand crosslinking was assessed in cells using the technique of alkaline elution. Elution profiles for K562-treated cells are shown in Fig. 4. Crosslinking is clearly evident at 5 and 10 μM with L-PAM, whereas approximately a 10-fold higher drug concentration is required to give equivalent levels of crosslinking with CHL. Again, BAM is seen to be a poor crosslinker with significant crosslinking at 1 mM, and only low levels at 500 μM. A comparison of crosslink indices for the three drugs is shown in Fig. 5. There is a good correlation between agents of the level of crosslinking at equitoxic doses.

The alkylation by the three agents at guanine N-7 positions in a DNA sequence was compared using a modified sequencing technique (Fig. 6). The order of reactivity of the drugs was the same as in the other assays described. At levels of drug which gave equivalent levels of alkylation the pattern of reactivity was found to be similar, and showed the preference of these agents for runs of guanines as described for several nitrogen mustards previously [10].

DISCUSSION

A comparison of the cytotoxicity of three aniline mustards, L-PAM, CHL and BAM, on two human tumour cell lines ranks them in order of potency to be L-PAM > CHL > BAM. It has generally been assumed that the chemical reactivity of the nitrogen mustard class of alkylating agents can strongly influence their pharmacological properties and biological activity. A positive correlation between chemical reactivity, as measured by reactivity with 4-(*p*-nitrobenzyl)pyridine (NBP), and antitumour activity has been demonstrated by Bardos *et al.* [6] for some members of the aromatic mustard series [6], although L-PAM was not included in this study. Workman *et al.* [12] have shown a good correlation between half-lives derived on the basis of decreased extent of alkylation of NBP and those derived by titrimetric determinations in a series of conjugates of *p*-hydroxyaniline mustards. In the study by Panthananickal *et al.* [13] it was also evident that antitumour activity and toxicity of aniline mustards paralleled hydrolysis although there were important exceptions, most notably CHL. Data shown here suggest that a more effective indicator of biological activity is the efficiency of DNA interstrand crosslink formation. This is illustrated by the behavior of L-

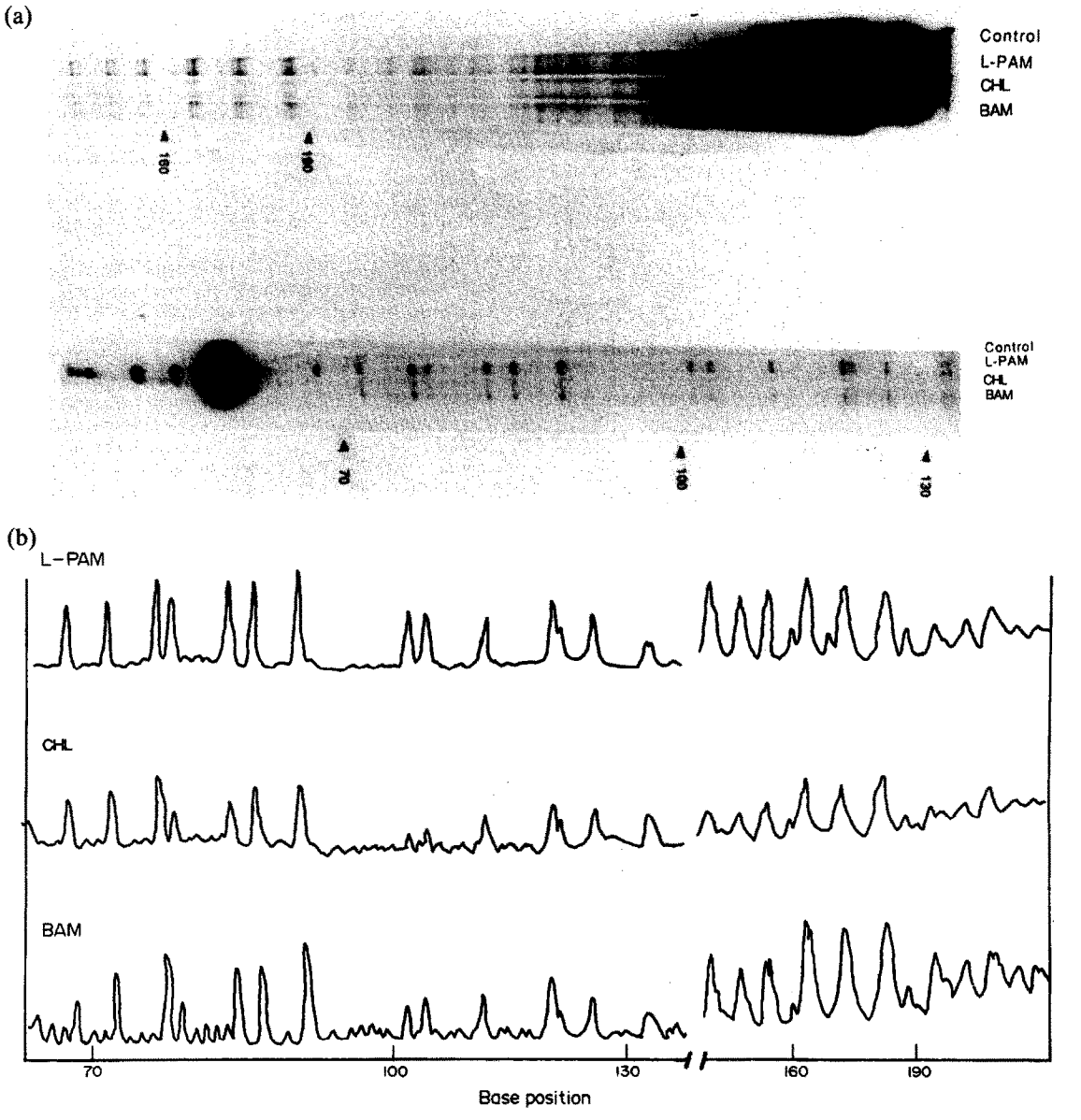

Fig. 6. (a) Autoradiograph of a DNA sequencing gel to assess guanine N-7 alkylation by L-PAM (lane 2, 25 μM), CHL (lane 3, 200 μM) and BAM (lane 4, 1 mM). Lane 1 is control, unalkylated DNA. (b) Densitometric traces from the gel in (a).

PAM and CHL. L-PAM has a longer half-life and lower first order rate coefficient than CHL [12], which would predict L-PAM to be less effective biologically than CHL, however L-PAM has a higher rate of crosslink formation, and a lower IC_{50} than CHL. In the study by Bardos *et al.* [6], CHL has been shown to have a 10-fold increase in alkylating activity and toxicity over BAM which is in agreement with the data presented here.

In contrast, the ability of the agents to produce interstrand crosslinks in cells correlated well with cytotoxicity. Such crosslinking is generally considered to be the cytotoxic lesions for agents of this type [14]. The fact that L-PAM is more effective than CHL could be explained on the basis of different cellular uptake mechanisms rather than chemical reactivity alone. L-PAM has been shown to be transported primarily by a carrier-mediated mechanism [15], whereas CHL accumulates in cells primarily by passive diffusion [16]. Chemical reactivity could also play a role however, since a more unstable and chemically reactive compound may be more likely to react with nucleophilic sites within the cell before the agent reaches the target DNA.

However, the present study shows that the order of activity is also predicted by the ability of the agents to produce crosslinks in isolated DNA as determined by a simple agarose gel technique. This would suggest that cellular factors such as transport

may not be a major determinant of reactivity with cellular DNA. A possible explanation may reside in the nature of the alkylation produced. It is now generally accepted that these agents produce positively charged aziridinium intermediates [17, 18]. The formation of these intermediates would be highly dependent on the electron density of the nitrogen and would be largely influenced by the aniline *para*-substituent. A positively charged species of this type would be expected to react with DNA at sites of highest electronegativity, such as the N-7 position of guanine [19]. The major site of crosslinking in DNA is generally assumed to be between two guanine N-7 sites, and the reactivity of different guanine N-7 sites has been shown to vary according to the influence of neighbouring base pairs on the molecular electrostatic potential [10, 20]. Such studies also indicated that L-PAM was more effective at alkylating guanine N-7 positions than CHL in isolated DNA, and suggested that this was related to the overall charge on the aziridinium intermediate [20]. CHL (and BAM) would be zwitterionic with no net charge at neutral pH, whereas L-PAM has an overall positive charge. An important role for the electrostatic component of the reactivity of these compounds with DNA was supported by the fact that alkylation by L-PAM was reduced much more effectively by the presence of Na or Mg than CHL [20]. In the present study, the extent of alkylation of guanine N-7 positions at low ionic strength mirrored the cytotoxicity and crosslinking ability of the three agents, but at equivalent levels of alkylation the patterns of reactivity were similar. This suggests that although the *para*-substituent on the aniline mustard can markedly influence the reactivity of the mustard, it does not influence the sequence selectivity of the ultimate reaction.

The present study demonstrates that simple chemical reactivity or hydrolysis rate is not a good indicator of DNA reactivity or cytotoxicity for a number of aniline mustards. In contrast, DNA interstrand crosslinking, either measured directly in cells or in isolated DNA by a simple agarose gel technique, gives a good indication of biological activity. BAM was found to be a poor crosslinking agent and relatively inactive compared to the clinically used L-PAM and CHL, and is therefore a poor candidate for the ADEPT programme. As a result, several other prodrug/active crosslinking drug combinations are being investigated.

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