Mechanism of Action of Fotemustine, a New Chloroethylnitrosourea Anticancer Agent: Evidence for the Formation of Two DNA-Reactive Intermediates Contributing to Cytotoxicity†

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ABSTRACT: Methyl excision repair deficient human tumor cells (Mer$^-$) were found to be hypersusceptible to killing by the antimelanoma agent fotemustine (FM) implicating alkylation of O^6 guanine as the major contributor to toxicity. Preincubation of the drug in aqueous solution for 5 min resulted in an immediate reduction in cytotoxicity (35−50%), *in vitro* DNA alkylation (31%), and DNA interstrand cross-linking (40%) followed by a second reaction with considerably slower kinetics. Electrospray ionisation mass spectrometry (ESI-MS) showed that in aqueous solution FM rearranged rapidly to form either a metastable tautomer or decomposed to form a highly reactive diazohydroxide ($t_{1/2}$ < 2 min). These results suggest the presence of two DNA-reactive species relevant to biological activity. Coincubation of ellagic acid (an inhibitor of O^6-guanine alkylation) with FM inhibited *in vitro* ISC, suggesting that the O^6-chloroethyl lesion is the predominant cause of the cross-link. On the basis of these findings, we propose that FM breaks down to form a short-lived intermediate, 2-chloroethyldiazohydroxide, which rapidly generates O^6-guanine lesions responsible for the drug's initial activity and a long lived iminol tautomer responsible for the remaining O^6 guanine alkylation and cytotoxicity.

Fotemustine (FM), adds to the existing series of antitumor 2-chloroethyl-1-nitrosoureas (CENUs):[1] carmustine (BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea), lomustine (CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea), semustine (methyl-CCNU, 1-(2-chloroethyl-3-(trans-4-methylcyclohexyl)-1-nitrosourea) and chlorozotocin (1-(2-chloroethyl)-3-(D-glucopyranos-3-yl)-1-nitrosourea). With moderate levels of side effects, FM gave encouraging responses in phase I and II drug trials for a number of tumor types, particularly metastatic melanoma including brain metastases (Schallreuter *et al.*, 1991a,b; Oliver *et al.*, 1993; Frenay *et al.*, 1991; Lasset *et al.*, 1993; Khayat *et al.*, 1992, 1994; Calabresi *et al.*, 1991. The formation of O^6-chloroethylguanine adducts, which subsequently lead to covalent interstrand cross-links, is thought to be central to the cell killing capacity of these agents (Avril *et al.*, 1992; Lemoine *et al.*, 1991; Ludlum, 1986; Gibson, 1989; Robins, 1983). Being lipophilic, chloroethylnitrosoureas cross the blood-brain barrier (Calabresi & Chabner, 1991) and thus are of special interest for use against meningeal leukemia and brain tumors.

Tapiero *et al.* (1989) found that methyl excision repair deficient cell lines (Mer$^-$) were more susceptible to FM than were Mer$^+$ cell lines. The drug has shown a similar efficacy to dacarbazine in clinical trials against disseminated metastatic melanoma and its action is enhanced by pretreatment with dacarbazine, an alkylating agent which presumably reduces the capacity of the O^6-methyl guanine methyl transferase to cope with the lesions formed by FM (Lee *et al.*, 1991, 1993, 1994; Avril *et al.*, 1990; Binder *et al.*, 1992). The base sequence specificity and alkylation patterns of CENUs have been studied by a number of workers (Briscoe *et al.*, 1988, 1990; Prakash & Gibson, 1992; Hartley *et al.*, 1986). In general they alkylate both O^6 and N^7 positions of

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[1] Abbreviations: FM, fotemustine; MGMT, O^6 methyl guanine methyl transferase; CENU, chloroethylnitrosourea; CNU, 2-chloroethyl-1-nitrosourea; Mer$^-$, methyl excision repair deficient; Mer$^+$, methyl excision repair sufficient; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; SM, semustine.; methyl CCNU, 1-(2-chloroethyl-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea); CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; chlorozotocin, 1-(2-chloroethyl)-3-(D-glucopyranos-3-yl)-1-nitrosourea; MMS, methyl methane sulphonate; ESI-MS, electrospray ionization mass spectrometer; ISC, DNA interstrand cross-links: HODEP, diethyl{1-[2-(hydroxyethyl)ureido]ethyl}phosphonate; DAP, diethyl(1-aminoethyl)-phosphonate; DEPISO, diethyl(1-isocyanoethyl)phosphonate.

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guanine in runs of guanine, to varying degrees which depend upon chemical and steric factors.

This study examined the sequence selectivity and cross-linking properties of FM on a plasmid DNA *in vitro* and survival of human cell lines. HeLa and MM96L were selected as representative Mer^- and Mer^+ cell lines, respectively, for length of exposure studies because they provided the sharpest contrast in response to FM. Semustine (SM) was chosen as a representative lipophilic CENU, for comparison with FM. Here we propose a mechanism involving the highly reactive 2-chloroethyldiazohydroxide and a relatively stable tautomer of the parent drug as intermediates with differing cytotoxicity properties, responsible for the production of sequence-selective DNA reaction products.

MATERIALS AND METHODS

FM [muphoran, S10036, diethyl-1-(3-(2-chloroethyl)-3-nitrosoureido) ethylphosphonate)] was kindly provided by Servier Laboratories (Melbourne, Australia). Ellagic acid, SM, and MNNG were obtained from Sigma Aldrich. The plasmid pBR322 and restriction enzymes *Bam*HI (Boehringer Mannheim), *Eco*R I (Promega), and the Klenow fragment (Boehringer Mannheim) were obtained commercially. The [α-^{32}P]dATP used for end labeling was obtained from Bresatec (Adelaide, Australia).

Cell Biology. The origin and properties of the human melanoma cell lines MM96L and MM253cl and the cervical cancer cell line HeLa have been described elsewhere (Hayward & Parsons, 1984; Maynard *et al.*, 1989). Cells were cultured in Roswell Park Memorial Institute Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 5% fetal calf serum, benzyl penicillin (100 IU/mL), streptomycin (100 μg/mL), and fungizone (250 μg/mL). Assays for *Mycoplasma* were routinely conducted and found to be negative (Chen, 1977). For determination of drug toxicity, cells were seeded into 96-well microtitre plates (Nunc, Denmark), 24 h prior to drug treatment, at 10^3 cells/well for HeLa and 2×10^3 for MM96L. Treatments were conducted in quadruplicate for 1 h, then the medium was replaced. For the preincubation experiments, the drug was preincubated in medium for increasing periods up to 1 h, then placed on the cells. Plates were incubated at 36.5 °C in an 5% CO_2 atmosphere until control wells were confluent (5 days), when the medium was replaced with medium containing [*methyl*-^{3}H] thymidine (2 μCi/mL, 40 Ci/mmol; Radiochemical Center, Amersham, UK). After incubation for 4 h, the cells were rinsed with phosphate-buffered saline, then detached with trypsin solution (0.2 mg/mL, 7.5 mM EDTA in Dulbecco's solution). The detached cells were harvested in a Pharmacia Betaplate 96-well plate harvester onto fiberglass mats, dried, placed into plastic envelopes with scintillation fluid and radioactivity counted on a Wallac 1205 Betaplate liquid scintillation counter.

Labeling and Isolation of a Plasmid DNA Fragment. A 375 base pair *Eco*RI to *Bam*HI fragment of plasmid pBR322 DNA was 3′ end labeled at the *Eco*RI site using Klenow fragment and [α-^{32}P]dATP (3000 Ci/mmol) according to a published procedure (Prakash *et al*., 1990). The fragment was isolated on a 4% nondenaturing polyacrylamide gel. Below is a partial sequence of the pBR322 DNA used in this work:

^{31} ^{5}′GCTTTAATGC CGTAGTTTAT CACAGTTAAA
TTGCTAACGC AGTCAGGCAC

^{3}′CGAAATTACG GCATCAAATA GTGTCAATTT
AACGATTGCG TCAGTCCGTG

^{81}CGTGTATGAA ATCTAACAAT GCGCTCATCG
TCATCCTCGG CACCGTCACC

GCACATACTT TAGATTGTTA CGCGAGTAGC
AGTAGGAGCC GTGGCAGTGG

^{131}CTGGATGCTG^{-3}′

GACCTACGAC^{-5}′

N^7-Guanine Sequence Specificity. FM and SM were prepared fresh as a 100 mM ethanol solution from lyophylized powder and added as a 1:100 dilution to the labeled DNA (10 000 cpm) in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.2). Reactions were incubated at 37 °C unless otherwise stated and were stopped by precipitation of the DNA with ethanol. The treated DNA pellets were dissolved in 0.1 mL of 1 M piperidine (Prakash *et al*., 1990) and heated at 90 °C for 10 min prior to lyophylization. Samples were then dissolved in formamide dye (98% formamide, 1 mM EDTA, 0.1% w/v xylene cyanol, and bromophenol blue, pH 7.5) and denatured at 90 °C for 2 min before loading onto a 6% denaturing polyacrylamide gel. Gels were run at 50 °C until the xylene cyanol dye front had travelled 26 cm. Autoradiographs were taken using Kodak XAR5 film. The relative intensities of the alkylation peaks were determined using Molecular Dynamics Imagequant software to analyze the phosphorimages and densitometer scans of the gels.

DNA Cross-linking. Cross-linking can be detected using the difference in migration rate between denatured and native DNA in agarose. Cross-linked DNA renatures rapidly and comigrates with native, control DNA while uncross-linked DNA remains single stranded and comigrates with denatured, control DNA. Linearized end labeled pBR322 plasmid DNA was obtained by cutting the plasmid with *Eco*RI, then end labeling with Klenow and [α-^{32}P]dATP. In preliminary experiments (data not shown) duplicate samples of DNA were exposed to drug for 5 min, precipitated to remove excess drug, then one sample was further incubated in TE buffer for 3 h to allow cross-linking to proceed. Only the postincubated sample formed detectable levels of DNA interstrand cross-links (ISC), indicating that the second arm reaction is a slow process. In order to study the kinetics of cross-linking, all DNA samples were exposed to FM or SM for 1 h or less, followed by precipitation then incubation in 10 mM TE buffer (pH 7.2) for 3 h to allow for completion of the second arm reaction. The DNA was then precipitated, washed with ethanol, and resuspended in 10 μL denaturing buffer (30% DMSO, 1 mM EDTA, and 0.04% xylene cyanol), heated for 2 min at 90 °C then chilled on ice. Control DNA was resuspended in non-denaturing loading buffer (6% sucrose, 0.04% xylene cyanol and 0.04% bromophenol blue) without heating. Samples adjusted to equal levels of radioactivity were loaded on a 1% agarose gel, run for 16 h at 40 V, dried, and autoradiographed using Kodak XAR5 film (Prakash & Gibson, 1992).

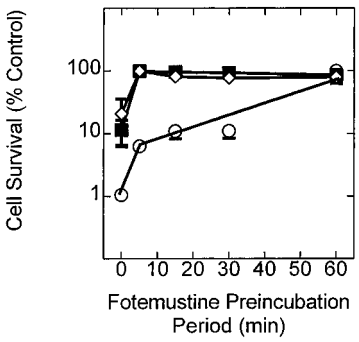

FIGURE 1: Effect of preincubating FM in medium on survival of MM96L cells. Treatments were ◇, 25 μg/mL; ■, 50 μg/mL; ○, 100 μg/mL. Points are mean ± SD ($n = 4$). Cell treatment time after preincubation was 1 h. The medium was then replaced and the plates were incubated at 36.5 °C in a 5% CO_2 atmosphere until control wells were confluent (5 days; see Materials and Methods).

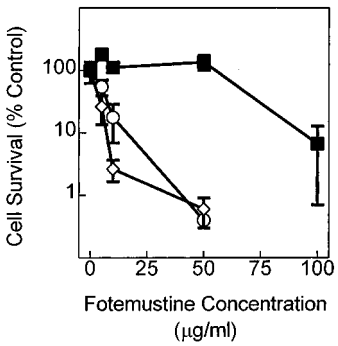

FIGURE 2: Selective toxicity of FM for Mer$^-$ cell lines (◇, HeLa; ○, MM253c1) compared with Mer$^+$ (■, MM96L) cells. Points are mean ± SD ($n = 4$). After drug treatment the medium was replaced and the plates were incubated at 36.5 °C in a 5% CO_2 atmosphere until control wells were confluent (5 days; see Materials and Methods).

Electrospray Ionization Mass Spectrometry. An ethanol solution of FM was freshly prepared and added to 10 mM TE buffer solution (pH 7.2) to give a final concentration of 1 mM FM and 1% ethanol, comparable to solutions used for DNA alkylation and cross-linking studies. The solution was analyzed by flow injection analysis via an electrospray source with a Finnigan SSQ710 mass spectrometer. The solution was sampled as 10 μL aliquots with a Waters 712 autosampler into a 0.3 mL/min 50% methanol/water solvent stream which was passed directly to the electrospray head. A blank sample of TE/ethanol was used for comparison. Initial samples were measured 2 min after addition of FM to the TE buffer and then every 8 min for 100 min. Signal intensity vs time for the atomic weights of interest were integrated at each time point to provide semiquantitative data on the rate of increase or decrease of each species.

RESULTS

Bioassay of FM Stability in Culture and Selectivity for Mer$^-$ Cells. Preincubation of FM in medium for 5 min prior to addition to MM96L cells resulted in a sharp loss of cytotoxicity (Figure 1). The zero time point for this experiment was obtained by adding the drug directly to medium in wells as an alcohol solution (final alcohol concentration 1% for all wells). After the first 5 min, further loss of activity with longer preincubation times was slow (Figure 1). Note that cell survival increased after 5 min preincubation to 100% from 10 and 20% for the 50 and 25 μg/mL treatments respectively. Figure 2 shows the sensitivity of Mer$^-$ cell lines for FM in comparison to Mer$^+$ line. In this experiment, unlike in Figure 1, the drug was first mixed with media prior to the addition to cells to emulate limb perfusion studies (Hayes *et al.*, 1997) leading to the loss of activity of the drug at 25 and 50 μg/mL treatments.

Reactivity of FM with N^7-Guanine. Figure 3a shows an autoradiogram of the kinetics of the piperidine-induced strand cleavage pattern arising from N^7-guanine alkylation, with or without preincubation of fotemustine. The drug alkylated all guanines, with preference for runs of guanines (bases 80−81, 115−116, 123−124, and 129−131). The autoradiograph was intentionally underexposed for quantitation purposes. For clearer results see also Figure 4. In the absence of preincubation (lanes 2−5), there was a biphasic increase in the band intensities, a rapid increase in the first 5 min, followed by a slow rise up to 1 h. Preincubating the drug for 5 min led to a significant decrease in the band intensities in the first time point (lane 6). No further loss of intensity occurred at longer incubation times (lanes 7−9). The intensity of the nonincubated sample was 31% greater than that of the preincubated sample after 1 h. The results are presented in graphical form in Figure 3b, which demonstrates the disappearance of the fast component in the sample preincubated for 5 min. To test the effect of preincubation time on alkylation by FM, DNA was incubated with the drug for 1 h after preincubation of FM in TE buffer (pH 7.4) for increasing periods. The graph in Figure 3c (gel not shown) shows that increasing the preincubation beyond 5 min did not lead to further reduction in DNA alkylation. The overall alkylation rate of the drug was slow compared to 2-chloroethylnitrosourea, which reaches completion in under 15 min (Prakash & Gibson, 1992). The average loss of N^7-alkylation activity with 5 min preincubation followed by 1 h incubation was 31 ± 8% from three separate experiments. Guanine bands 81, 112, 115, 116, 121, 123, 124, 129, 130, and 131 were used for quantitation, and each band was quantitated by integrating the area vs density (volume) using Image Quant software. The mean values obtained from these bands were used in Figure 3, panels b and c. Normalization of bands with respect to the total activity in each lane was not required since there was no overall change in the alkylation pattern.

Ellagic acid has been shown to increase the level of N^7-guanine alkylation by bifunctional CENU's. It has been suggested that ellagic acid does this by forcing the O^6-chloroethyl-guanine lesion to react intramolecularly with the N^7 rather than the N^1 (Prakash & Gibson 1992). Figure 4 is an autoradiogram showing the effect of 250 μM ellagic acid on DNA alkylation by 1 mM FM and SM. SM was used for comparison as a lipophilic, CENU anticancer drug. Ellagic acid increased the band intensities by an average of 12 and 11% (±3%), respectively, for the two compounds. Reduction in the intensity of the bands corresponding to unmodified DNA observed at the top of the lanes is a further indication of increased alkylation in the presence of ellagic acid. The difference between ellagic acid treatment can best be seen at guanine bands 51, 53, 64, 81, 104, and 112.

DNA Interstrand Cross-Linking. Figure 5 is an autoradiogram from a cross-linking experiment which showed increasing ISC with increasing length of exposure to FM and SM (lanes F1−F4 and S1−S4). Short preincubation (5

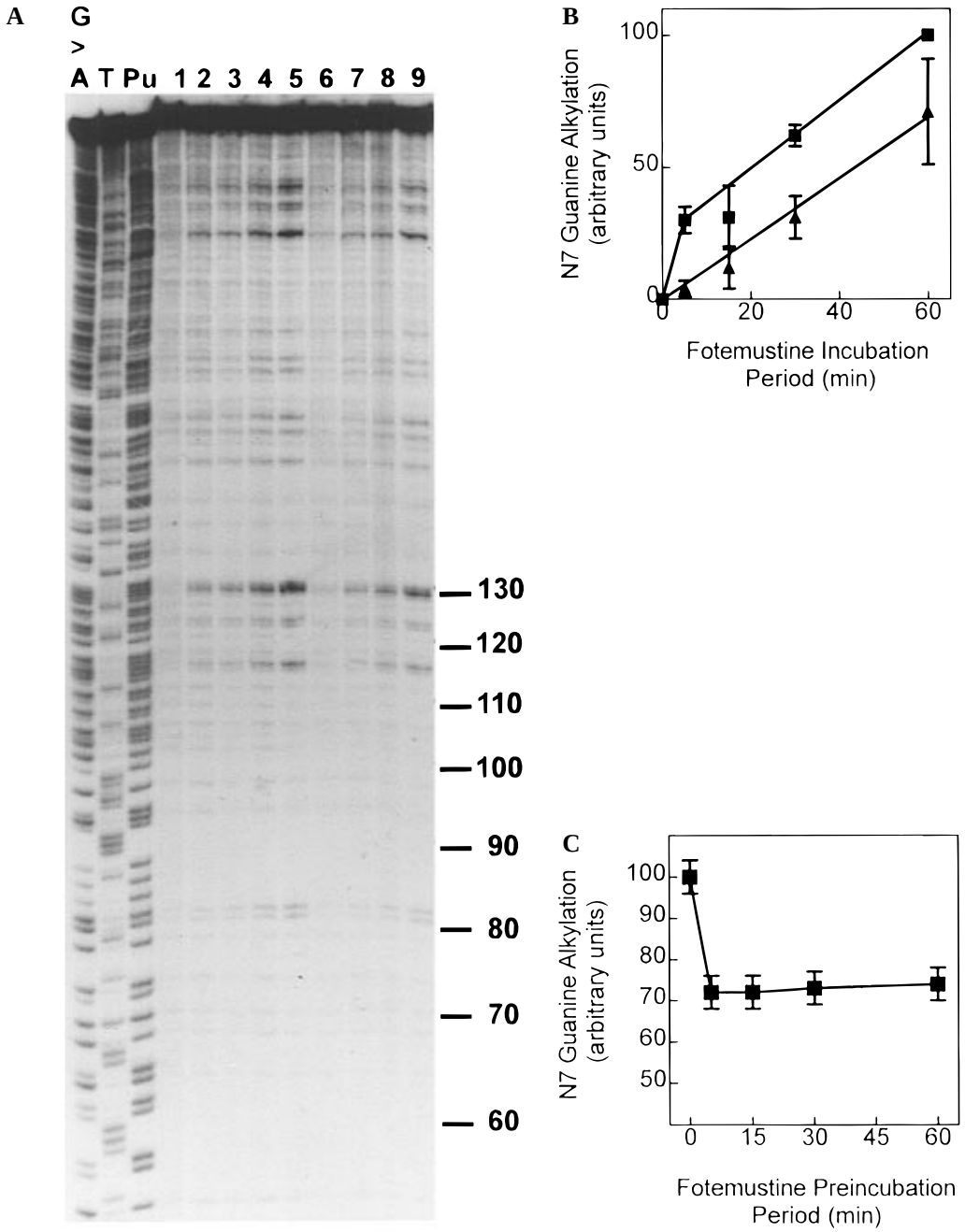

FIGURE 3: (A) Autoradiogram of a 6% denaturing polyacrylamide gel showing DNA strand cleavage of 3′ end labeled 375 bp *Eco*RI/*Bam*HI fragment of pBR322 DNA. Lanes G > A, T, and Pu are Maxam-Gilbert sequencing lanes. Lane 1, control; lanes 2−5, no preincubation with varying incubation times (5, 15, 30, and 60 min) of DNA with 1 mM FM; lanes 6−9, 5 min preincubation followed by 5, 15, 30, and 60 min incubation of DNA with 1 mM FM. (B) Graphical representation of the autoradiogram in panel A, showing the effect of incubation time on the kinetics of N^7-guanine alkylation of DNA. ■, No preincubation; ▲, 5 min preincubation in 10 mM TE buffer pH 7.2. (C) Effect of the preincubation period on N^7-guanine alkylation of DNA treated for 1 h, taken from a different gel (not shown).

min) of FM in buffer decreased the level of cross-linking (lane F5) but preincubation of SM produced a much less pronounced effect (lane S5). Coincubation of ellagic acid with FM also reduced the level of ISC sharply (lane F6) but, again, coincubation with SM did not affect the level of ISC significantly (lane S6). In addition, FM treatments for 30 and 60 min and the preincubation and ellagic acid treatments (lanes F3−F6) showed significant reductions in the level of single-stranded DNA, not seen for SM (lanes S3−S6).

Dependence of Cell Survival on the Length of Exposure to FM. To explore the observed rapid formation of the precross-link lesion and its potential significance to cell killing activity of the drug, different exposure times were tested using MM96L (Mer^+) and HeLa (Mer^-) cell lines (Figure 6). In this assay, the cells were treated with drug for various times as shown in figure followed by post-drug incubation until control wells were confluent (5 days). Note that a 15 min exposure (50 μg/mL FM) had little effect on MM96L but virtually eliminated HeLa, the Mer^- cell line.

ESI-MS Analysis. In order to study the decomposition profile of FM in aqueous medium, we analyzed the fate of FM in TE buffer (pH 7.2). Figure 7a shows possible breakdown products and their *m/e* values while Figure 7b shows the spectral data for FM after 5 min incubation (upper panel) and 100 min incubation (lower panel). Relative quantitation for each species was obtained by integrating signal intensity over time. Four types of ion were found,

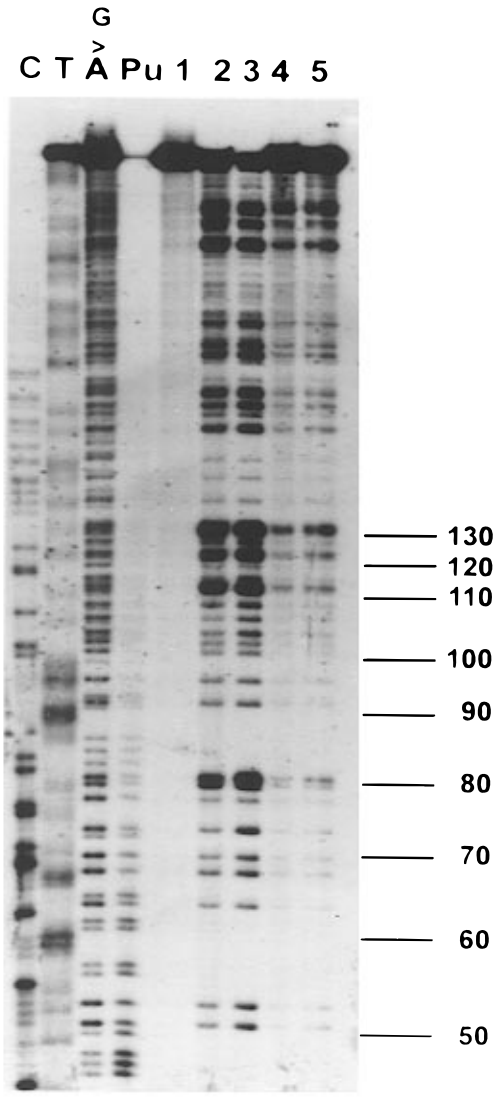

FIGURE 4: Enhancement of N^7 G alkylation by ellagic acid. Autoradiogram of a 6% denaturing polyacrylamide gel showing DNA strand cleavage of 3′- end labeled 375 bp *Eco*RI/*Bam*HI fragment of pBR322 DNA. DNA was subjected to piperidine treatment after exposure to FM. Lanes C, T, G > A, and Pu are Maxam−Gilbert sequencing lanes. Lane 1, control; lanes 2 and 4, 1 mM FM and SM, respectively, lanes 3 and 5, 1 mM FM and SM respectively in the presence of 250 μM ellagic acid. The samples were incubated for 1 h at 37 °C.

relevant to DNA alkylation. A strong peak at *m/e* = 338, corresponding to either the parent drug− Na^+ complex or a rearranged sodiated intermediate of the parent drug with the same *m/e* value; and a moderately strong peak at *m/e* = 437 corresponding to a Tris-FM complex. These two peaks persisted for a long time, reducing in intensity by about 50% at 100 min. Upon increasing the voltage by 30 V, the 437 peak completely disappeared (data not shown), suggesting that it was a gas phase complex produced in the detection chamber. Second, moderately strong peaks at 389 and 411 corresponded to sodium and Tris adducts of diethyl (1-[2-(hydroxyethyl)-ureido]ethyl)-phosphonate (HODEP), resulting from hydrolysis of the chloro group. This material was not present in the starting solution but increased in intensity over time. Third, 2-chloroethyldiazohydroxide was observed as a very small, unstable peak at the first injection at 2 min, as well as the other product from loss of the diazohydroxide, the Tris adduct of diethyl(1-isocyanatoethyl) phosphonate (DEPISO). No oxadiazole or chloroethanol peaks were noted, suggesting that 2-chloroethanol is not a major product under these conditions and that the oxadiazole is either not formed or breaks down too rapidly to be seen in the mass spectrometer. Finally, the product from hydrolysis of DEPISO, and the hydrogen, sodium, and Tris adducts of diethyl(1-aminoethyl) phosphonate (DAP) were seen at 182, 204, 303, and 329.

DISCUSSION

Tapiero *et al*. (1989) found that FM, SM, and BCNU had similar cytotoxicities in a murine cell line. They also found that FM lost half its activity in 30 min and virtually all activity within 120 min. *In vivo* pharmacokinetic studies on this drug have been conducted by a number of authors (Gordon *et al*., 1989; Ings *et al.*, 1990; Bartosek *et al.* 1991; Tranchand *et al*., 1993). Estimates of the half-life of the intact drug in plasma have ranged from 12 min in *cynomolus* monkeys (drug administered as a bolus) to 23 min in a human (drug administered as an intravenous infusion over a 1 h period (Gordon *et al*., 1989). In HEPES buffer, the half-life was estimated to be 24−40 min (Brakenhoff *et al*., 1994). Our studies showed a biphasic effect with a substantial loss in cytotoxic activity, N^7-guanine alkylation and DNA interstrand cross-linking occurring in the first 5 min of addition of drug to aqueous medium. This is the first experimental evidence for the presence of two DNA-reactive intermediates from a CENU.

Comparison of survival of Mer^+ and Mer^- cell lines following a 1 h FM treatment (Figure 2) shows that approximately 9% of the concentration of drug used for Mer^+ is required to produce the same toxicity in the Mer^- cell line. These results are consistent with those reported by Tapeiro *et al.* (1989), indicating that alkyl guanine transferase deficiency enhances the toxicity of FM, and implicating O^6-guanine lesions as central to the cell killing activity of the drug. FM also alkylated the N^7 position of guanine in a time-dependent manner, with a preference for runs of guanines. These results are in agreement with other studies on the alkylation patterns of CENUs (Briscoe & Duarte, 1988; Briscoe *et al.*, 1990; Prakash & Gibson, 1992; Hartley *et al.*, 1986). However, in contrast to 2-chloroethylnitrosourea (CNU), which showed no further reactivity after 15 min (Prakash & Gibson, 1992), FM had a much slower reaction rate, alkylation being incomplete even after 1 h.

Because FM rapidly lost cytotoxic activity when preincubated in the medium (Figure 1) in the first 5 min, preincubation studies were included in the alkylation and cross-linking experiments. This approach provided evidence for the presence of two reactive intermediates. The fast component dissipated within 5 min of addition of the drug to the buffer, after which incubation with DNA produced about 31% less total N^7-G alkylation and resulted in a significant reduction in cross-linking.

O^6-Chloroethylguanine lesions are thought to be the main mechanism for cytotoxicity of these agents, with an intramolecular reaction to initially produce an N1-O^6-ethanoguanine intermediate which subsequently reacts with the adjacent cytosine to form a GC cross-link (Ludlum, 1986). However, the piperidine treatment, which converts N^7-guanine lesions (both chloroethyl and hydroxyethyl adducts) to strand breaks, is not capable of discerning lesions at the O^6 position of

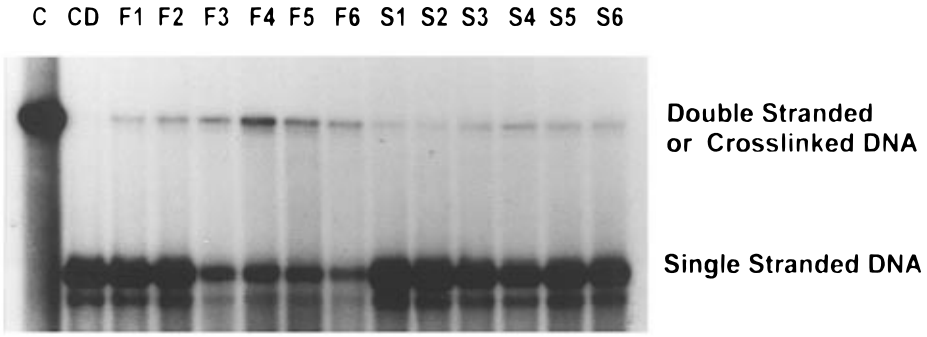

FIGURE 5: Autoradiograph of a 1% agarose gel showing DNA interstrand cross-linking of a 3′ end labeled linear pBR322 plasmid. C, native control DNA; CD, denatured control DNA; lanes F1−F4 and S1−S4 are 5, 15, 30, and 60 min exposures to 1 mM FM and SM, respectively; lanes F5 and S5, FM and SM, respectively, were preincubated for 5 min before treatment of DNA for 1 h; lanes F6 and S6, FM and SM were coincubated with ellagic acid and DNA for 1 h. All samples were precipitated at the end of treatment, then resuspended in 10 mM TE buffer and incubated for a further 3 h to allow cross-link formation.

Table 1: Modification of CENU Activities by Coincubation with Ellagic Acid or Preincubation in Medium

		CENU		
treatment	CENU activity	FM	SM	CNU[a]
ellagic acid (250 μM)	N^7 G alkylation	↑ (12%)	↑ (11%)	↑
	DNA cross-linking	↓ (70%)	marginal ↓	negligible
preincubation (5−60 min, 37 °C)	N^7 G alkylation	↓ (31%)	not done	not done
preincubation (5 min, 37 °C)	DNA cross-linking	↓ (40%)	marginal ↓	not done

[a] Prakash and Gibson (1992).

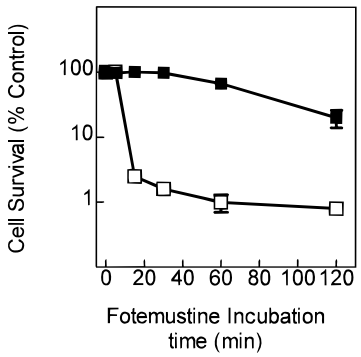

FIGURE 6: Effect of treatment time on FM toxicity (50 μg/mL) to MM96L (■, Mer^+) and HeLa (□, Mer^-) cell lines. Points are mean ± SD ($n = 3$). After drug treatment the medium was replaced and the plates were incubated at 36.5 °C in a 5% CO_2 atmosphere until control wells were confluent (5 days; see Materials and Methods).

guanine. We therefore used a protocol developed by Prakash and Gibson (1992) who found that ellagic acid converts the O^6-chloroethyl guanine adduct to an N^7-hydroxyethyl lesion via an O^6-N^7-ethanoguanine intermediate. Ellagic acid increased total N^7-guanine alkylation by 12 and 11% for FM and SM, respectively, indicative of preformed O^6-guanine chloroethyl adducts.

DNA interstrand cross-linking provided another means of examining the formation of the O^6-chloroethylguanine lesion. Figure 5 shows that a 5 min preincubation reduced the ISC by 40% (compare lanes F4, no preincubation, and F5, Figure 5). Coincubation with ellagic acid also reduced ISC considerably (compare lanes F4 and F6, Figure 5), suggesting that the source of this ISC is the initial O^6-guanine lesion. The results of both the ISC and DNA alkylation experiments are consistent with the drug forming two DNA reactive species. The first species is short lived, capable of alkylating guanines at both the N^7 and O^6 positions and contributes about 31% of the total N^7-guanine alkylation and 40% of ISC. The second component is also capable of producing ISC but takes a longer time to form the initial precross-link lesion (compare lanes F2 and F3 to F4, Figure 5).

Table 1 shows that FM reactions with DNA are different from those of SM and CNU. While coincubation with ellagic acid increased N^7 alkylation for all three drugs, it reduced ISC for FM but not SM. Preincubation in buffer before addition to DNA also reduced the level of ISC for FM but SM was only slightly affected. These differences may be attributed to the shorter lifetimes of the fast component for SM and CNU, which would then be lost before reaction with DNA could occur.

The likely candidates for the reaction intermediates revealed by this work are not obvious. Briscoe *et al*. (1990) suggested pathways for breakdown of the chloroethyl moiety of CENUs based on BCNU; Brakenhoff *et al*. (1993, 1994) also provide a scheme for the breakdown of FM. These mechanisms involve the formation of 2-chloroethyldiazohydroxide, which is thought to be unstable and hence a reasonable candidate for the fast reaction component. ESI-MS studies gave direct evidence for formation of the unstable diazohydroxide (2 min after addition to buffer, Figure 7b) consistent with a rapid but limited hydrolysis of the drug. It is pertinent to note that the shortest preincubation time used in the DNA and cell studies was 5 min and is therefore an upper limit for detection of the fast component.

As for the slow intermediate, recent work (Chen *et al*., 1996) with CNU suggests that the formation of the Tris−CNU complex may be responsible for the slow reaction of CNU with DNA. In order to address this possibility for our slow component, we ran ESI-MS analysis on FM in Tris buffer over time (Figure 7b). The spectrum showed the formation of a voltage-dependent Tris−FM complex at *m/e* 437, suggesting that it is a gas phase complex formed in the detection chamber of the spectrometer. More importantly, it also showed the presence of a much bigger peak whose *m/e* value (338) is identical to that of a gas phase sodium complex of the parent drug. This peak reduced by about

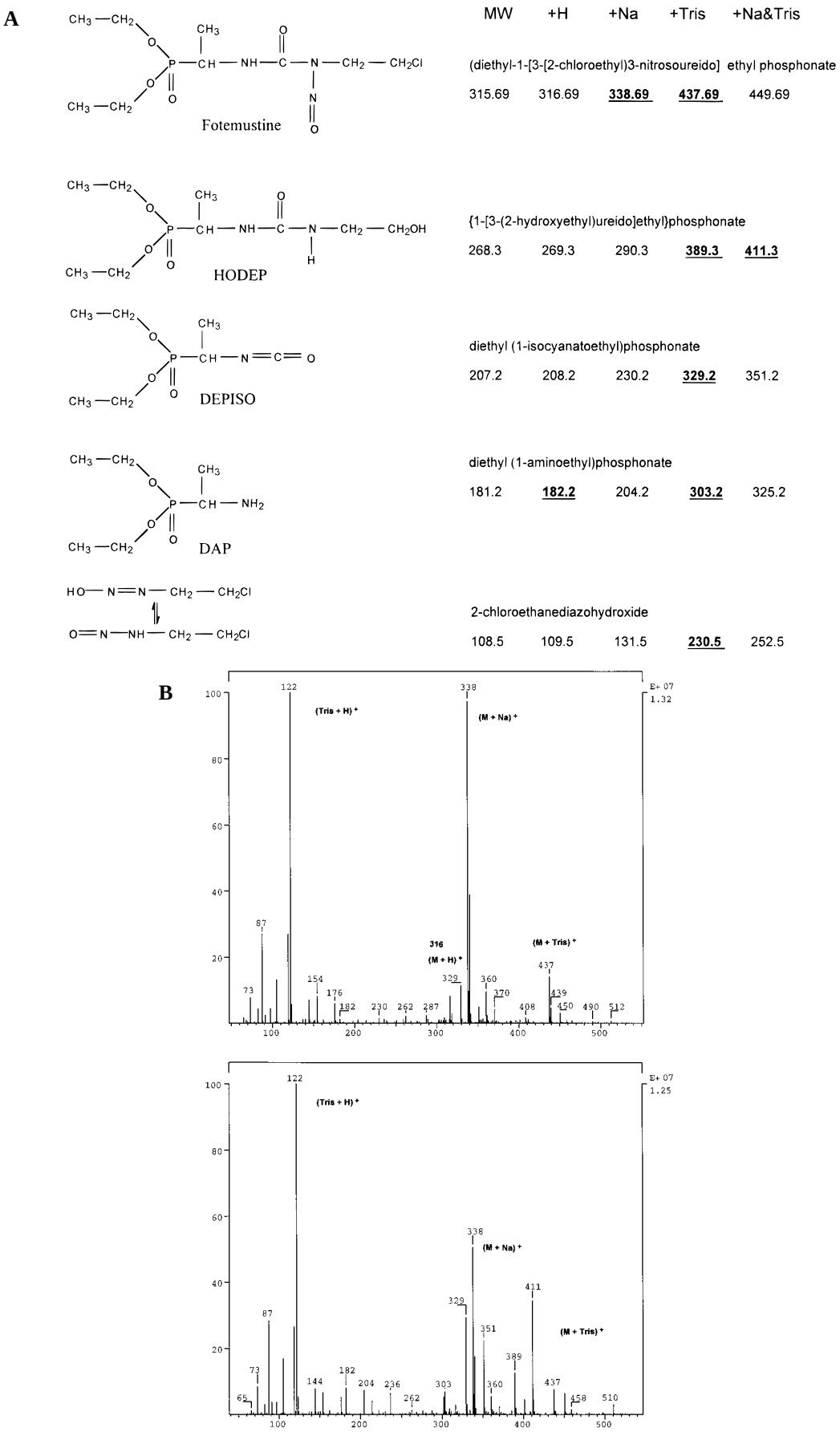

FIGURE 7: (A) Potential breakdown products of FM and possible ESI-MS *m/e* peaks. (B) ESI mass spectra at 2 min and 100 min after addition of FM to TE buffer (pH 7.2). The drug was incubated at room temperature (22 °C).

50% after the drug solution was kept at room temperature for 100 min. Since our alkylation kinetics studies indicate the presence of a short-lived intermediate, this peak more likely arises from a rearranged product of the parent drug

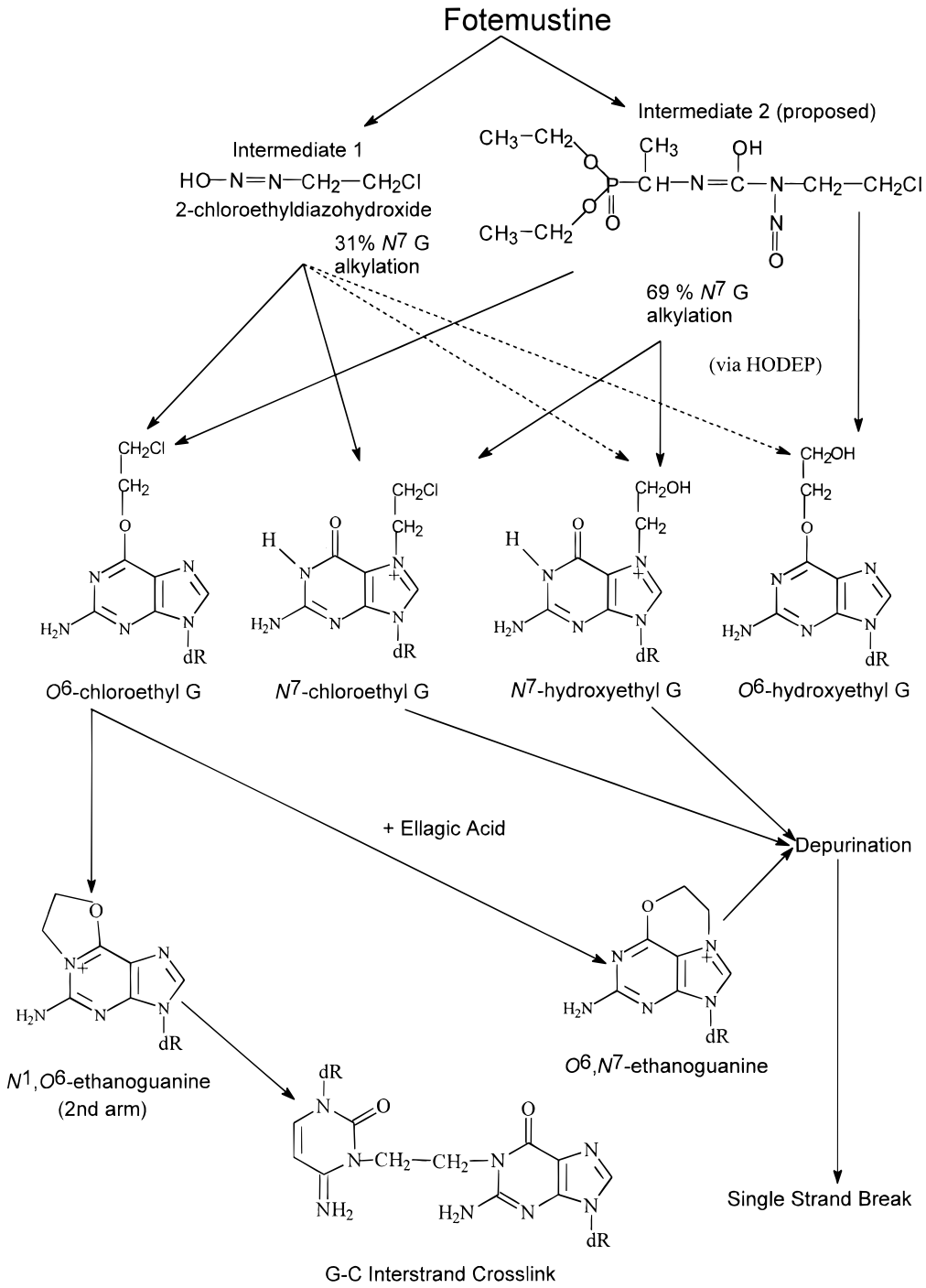

FIGURE 8: Proposed scheme for the short- and long-lived reaction components derived from FM.

with the same *m/e* value. In this regard it is pertinent to note that Buckley and Brent (1988) have proposed a similar model for CENUs in which the drug undergoes rearrangement in the presence of DNA, in order to explain the formation of O^6-chloroethylguanine adducts.

On the basis of our findings, we propose that at physiological pH, FM decomposes to form the fast component and a rearranged long lived isomer both of which are capable of alkylating guanine at both N^7 and O^6 sites either directly or through formation of HODEP (Figure 8). According to this scheme, the short-lived species produces O^6-chloroethylguanine adducts (which lead to biologically important GC cross-links) and up to 31% of the N^7-guanine alkylation. The long lived species reacts with both N^7 and O^6 sites on guanine as well, which explains the formation of remaining 60% ISC as observed in the cross-linking assay. The slow component also gives rise to the majority of the N^7-guanine alkylation, which eventually leads to depurination and subsequent strand breaks as observed in the cross-linking studies.

The implication of our findings in terms of clinical use of FM is that a large proportion of the biologically significant lesions occur within a very short period after drug exposure, allowing cell kill to be maximized by short exposures to high doses of the drug, particularly for the Mer^- phenotype. The differential response of Mer^+ and Mer^- cell types to brief, high concentrations of FM might usefully be exploited, since approximately 20−25% of melanomas are Mer^- and most normal tissues are Mer^+. In this regard, it is pertinent to note that although systemic doses of FM used in patients range 2−8 μg/ mL (Fety *et al.*, 1992), much higher doses

are feasible in isolated limb perfusion (Hayes *et al*., 1997). Future research will study the formation of the O^6 lesions using human recombinant MGMT, the characterization of fotemustine intermediates, and determination of conditions which would favor increased formation of the fast component in order to improve the efficacy of the drug.

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