Biochemical Pharmacology, Vol. 59, pp. 283–291, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00328-7

Comparison of DNA Lesions Produced by Tumor-inhibitory 1,2-Bis(sulfonyl)hydrazines and Chloroethylnitrosoureas

*Philip G. Penketh, Krishnamurthy Shyam and Alan C. Sartorelli**

Department of Pharmacology and Developmental Therapeutics Program, Cancer Center, Yale University School of Medicine, New Haven, CT 06520, U.S.A.

ABSTRACT. 1,2-Bis(sulfonyl)hydrazine derivatives, designed to generate several of the electrophilic species classically believed to be responsible for the alkylating (chloroethylating) and/or carbamoylating activities of the chloroethylnitrosoureas (CNUs), were compared with respect to the cross-linking and nicking of T7 DNA to that caused by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and 1-(2-chloroethyl)-3-(4-*trans*-methylcyclohexyl)-1-nitrosourea (MeCCNU). In the case of BCNU, a large proportion of T7 DNA strand nicking was found to be due to the generation of 2-chloroethylamine, produced from the hydrolysis of 2-chloroethylisocyanate, in turn formed during the decomposition of the parental nitrosourea. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (compound 1) gave a greater yield of DNA cross-links than the CNUs. Compound 1, as well as its derivatives that were incapable of generating 2-chloroethylisocyanate, did not produce detectable levels of strand nicking, indicating that N^7-alkylation of guanine did not occur to a significant extent with these agents. Since compound 1 and its derivatives are believed to generate chloronium and chloroethyldiazonium ions, it would appear that these species could not be significantly involved in the N^7-alkylation of guanine caused by the CNUs. The relatively low level of N^7-alkylation of guanine residues and the relatively high yield of cross-links generated by some of the 1,2-bis(sulfonyl)-1-(2-chloroethyl)hydrazine derivatives implies that they are more exclusive O^6-guanine chloroethylating agents than the CNUs. O^6-Guanine chloroethylation is believed to be the therapeutically relevant event produced by the CNUs; therefore, compound 1 derivatives represent promising new cancer chemotherapeutic agents, since they appear to generate lower quantities of therapeutically unimportant, yet carcinogenic lesions, and more of the therapeutically relevant O^6-guanine chloroethylation than the CNUs. BIOCHEM PHARMACOL **59**;3:283–291, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. antitumor agents; cross-linking; alkylating agents; nitrosoureas; bis(sulfonyl)hydrazines; DNA

The cross-linking of DNA by chemotherapeutic agents is thought to be the major event responsible for the anticancer activity of many clinically used alkylating agents [1]. BCH† derivatives have the capacity to act as bifunctional alkylating agents and cross-link DNA [2]. We have synthesized prodrugs of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazine (compound 1) which are further substituted at the N-2 position, blocking the normally very rapid series of activation reactions ($t_{1/2}$ 30 to 40 sec under normal conditions) that lead to the primary alkylation events [3–5]. These modifications greatly improved the efficacy of these drugs as antineoplastic agents, presumably by allowing time for their optimum distribution. Among the synthesized prodrugs are a series of aminocarbonyl derivatives that were designed to generate not only chloroethylating species, but also a carbamoylating agent (i.e. an isocyanate) analogous to that produced by the CNUs [5]. We have also synthesized a 1,2-bis(sulfonyl)hydrazine derivative lacking alkylating activity, but possessing the BCNU-like ability to generate chloroethylisocyanate. This agent provides an experimentally useful tool since it allows an independent comparison, when used with other compounds of this class, of the effects of the chloroethylating species and chloroethylisocyanate (the carbamoylating species).

The BCHs, as a class, can potentially form four chloroethylating species, namely $ClCH_2CH_2N{=}NSO_2R$, 2-chloroethyldiazohydroxide, 2-chloroethyldiazonium, and chloronium ions, via the mechanism shown in Fig. 1 [6]. In keeping with this, when allowed to decompose in Tris buffer in the neutral pH range, compound 1 gives a high yield (88%) of 2-chloroethanol [6], which is formed as a consequence of the chloroethylation of water. This finding contrasts with that produced by agents such as BCNU,

* Corresponding author: Dr. Alan C. Sartorelli, Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8066, U.S.A. Tel. (203) 785-4533; FAX (203) 737-2045; E-mail alan.sartorelli@yale.edu

† *Abbreviations:* BCH, 1,2-bis(sulfonyl)-1-(2-chloroethyl)hydrazine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CNU, chloroethylnitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(4-*trans*-methylcyclohexyl)-1-nitrosourea; G-C cross-link, 1-(3-cytosinyl),2-(1-guanyl)ethane; and H33258 Hoechst 33258.

Received 30 March 1999; accepted 3 August 1999.

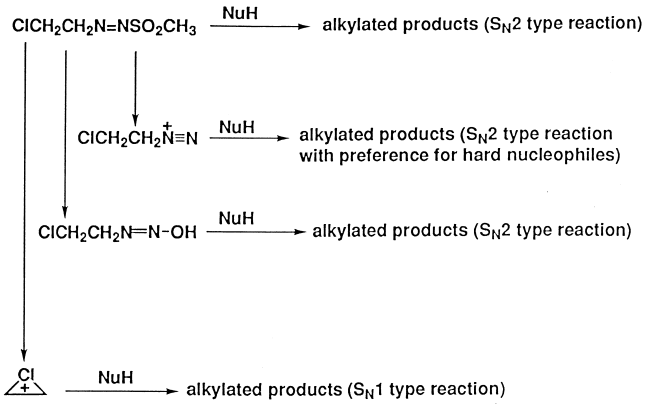


FIG. 1. Suggested pathways for the generation of reactive electrophiles from compound 1.

which give much smaller yields of 2-chloroethanol [7] due to multiple competing side reactions. Of the four potential chloroethylating species generated by compound 1, three (i.e. 2-chloroethyldiazohydroxide, 2-chloroethyldiazonium ions, and chloronium ions) have been classically implicated in the mode of action of the CNUs [8], while the generated species $ClCH_2CH_2N{=}NSO_2CH_3$ is unique to compound 1. $ClCH_2CH_2N{=}NSO_2CH_3$ and 2-chloroethyldiazohydroxide are expected to be the softest of the four electrophilic species potentially generated from compound 1 and should undergo S_N2 type reactions at the softest nucleophilic sites in DNA, primarily the N^7-position of guanine. In contrast, the chloroethyldiazonium and chloronium ions should have an affinity for harder nucleophilic sites such as the O^6-position of guanine and the phosphate groups in the DNA backbone. Haloethylation of the O^6-position of guanine leads to the formation of cross-links (Fig. 2), which result from the relatively rapid formation of O^6,N^1-ethanoguanine, via cyclization and halide loss, followed by the slower reaction (this process is essentially complete within 12 hrs) of this intermediate with a complementary cytosine, to form a G-C cross-link [9]. In contrast to alkylation at the O^6-position of guanine, N^7-alkylation of the purine ring leads to slow depurination followed by sugar-phosphate chain hydrolysis, resulting in a single-strand nick (Fig. 2) [10]. The $t_{1/2}$ for the hydrolysis of 7-alkylguanine residues to give apurinic sites at pH 7.0 is about 7 to 16 hrs at 37° [11]. This chemistry has been well described and is exploited in the Maxam and Gilbert chemical DNA sequencing method [12].

A much wider array of electrophilic species appears to be generated by the CNUs [7–9, 13]. In addition to the chloroethylating species (i.e. 2-chloroethyldiazohydroxide, 2-chloroethyldiazonium ions, and chloronium ions), the CNUs are capable of producing large quantities of hydroxyethylating, vinylating, and carbamoylating (isocyanates) species [13]. The possible presence of these species in the cells is based upon decomposition studies of the CNUs conducted in buffered solutions. The hydroxyethylating species is thought to be formed from 4,5-dihydro-1,2,3-oxadiazole, which itself results from an internal cyclization reaction involving the *N*-nitroso group. The CNUs are also able to chloroethylate and hydroxyethylate DNA by a more direct mechanism. In this reaction, the carbon adjacent to the halide becomes attached to the N^7-position of guanine, and the halide either migrates to the neighboring carbon or is replaced by a hydroxide or halide group from the bulk phase. A mechanism involving the formation of a cyclic nitrosooxazolidine has been proposed to account for the unusual features observed in this alkylation (Fig. 3) [14]. The direct alkylation appears to account for the majority of the alkylations that occur at the N^7-position of guanine [14]. Comparable mechanisms that could lead to hydroxyethylation or a direct alkylation of DNA are not obviously apparent for the BCH derivatives. With BCNU, an additional alkylating species (i.e. a monofunctional mustard) can also be generated [9]. Chloroethylisocyanate, derived from this CNU, hydrolyzes rapidly in aqueous solutions to generate 2-chloroethylamine, which in turn can cyclize to form a one-armed nitrogen mustard. In the present study, we compared the interaction of the various 1,2-bis(sulfonyl)hydrazine derivatives, shown in Table 1, with T7 DNA to that of BCNU, CCNU, and MeCCNU.

MATERIALS AND METHODS

T7 DNA and all chemicals were purchased from the Sigma Chemical Company, except where specified. H33258 was obtained from Molecular Probes, Inc. BCNU, CCNU, and MeCCNU were gifts from Bristol-Myers Squibb, Inc. Compound 1, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(2-chloroethyl)amino]carbonyl]hydrazine (compound 2), and 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (compound 4) were synthesized in this laboratory as previously described [5]. 1,2-Bis(methylsulfonyl)-1-[[(2-chloroethyl)amino]carbonyl]hydrazine (compound 3) was synthesized using a procedure similar to that described for compounds 2 and 4, by substituting 1,2-bis(methylsulfonyl)hydrazine for compound 1. The synthesis of compound 3 will be reported elsewhere.

The cross-linking of DNA was determined utilizing an assay based upon the snap cooling of thermally denatured T7 DNA under neutral pH conditions as previously described by this laboratory [15]. Only one cross-link is required per DNA molecule to allow the DNA to rapidly renature under snap-cooling conditions [15]. Scaled-up volumes were employed in the incubation of 100 μg/mL of T7 DNA (in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 buffer) with alkylating and/or carbamoylating agents (final concentration 0.2–0.4 mM) to allow for longer time–course determinations. The concentrations of alkylating agents were chosen to give a maximum of approximately 20–40% of the DNA being cross-linked (at least 1 cross-link in 20–40% of the molecules). At lower levels of cross-linking, the fluorescent signal becomes small, reducing the signal to noise ratio. At high levels of cross-linking, most of the molecules will have multiple cross-links, saturating the assay and making only large changes in the

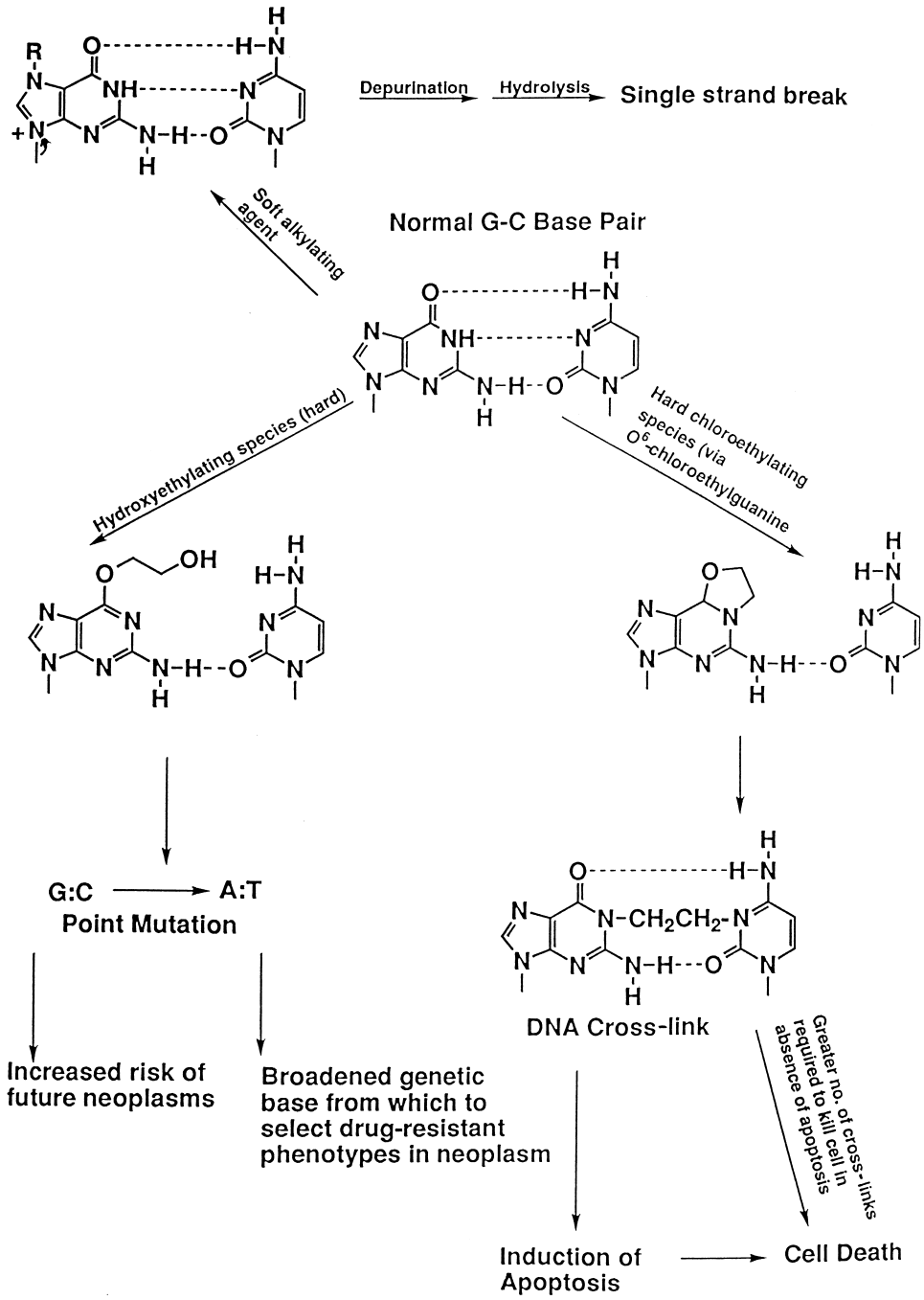


FIG. 2. Ramifications of guanine alkylation. G:C and A:T represent the guanine/cytosine and adenine/thymine base pairs, respectively.

number of cross-links readily determinable. Samples were removed at various times, diluted 100-fold by mixing with fluorescent probe solution (0.1 μg/mL of H33258 in 5 mM Tris–HCl/0.5 mM EDTA buffer at pH 8.0), and the fluorescence determined before and after a heating (96°) and chilling (0°) cycle. The fluorescence measurements were performed using a Hoefer Scientific Instruments TKO 100 Mini-fluorometer. The spermidine competition experiments were performed identically to the cross-linking experiments except for the inclusion of 10 μM spermidine in the initial mixture containing DNA and alkylating agent.

The nicking activity of selected agents was determined by the methodology previously described [15]. This method involves following the loss in fluorescence versus time of stably pre-cross-linked T7 DNA in the presence of H33258 after a heating and chilling cycle [15]. Nicking of the DNA results in a decrease in the fluorescence, since nicked DNA requires more cross-links to fully renature the molecules [15, 16]. The pre-cross-linked T7 DNA was prepared by treating the DNA with 0.2 mM compound 1 in 10 mM Tris–HCl and 1 mM EDTA buffer (pH 8.0) for 24 hrs at 37° [15].

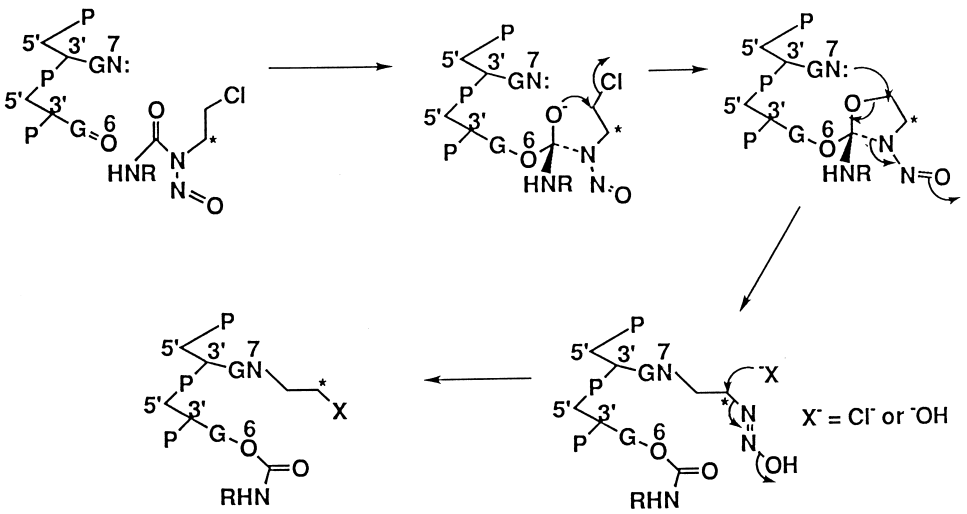


FIG. 3. Mechanism adapted from that proposed by Naghipur *et al.* [14].

The expected generation of isocyanate during the decomposition of isocyanate precursor 1,2-bis(sulfonyl)hydrazine derivatives was examined by trapping the isocyanate with benzylamine in the form of a benzylurea. A mixture of 1.0 mL of benzylamine and 10 mL of a 1.7% (w/v) sodium bicarbonate solution in water (pH 8.1) was added to 0.5 mmol of the agent to be tested and the mixture stirred for 16 hr. Sodium bicarbonate buffer was used to maximize the trapping efficiency, since isocyanates can readily react with other buffering reagents. The reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate (2 × 50 mL). The combined ethyl acetate layers were washed with dilute hydrochloric acid (2 × 10 mL), dried over anhydrous sodium sulfate, filtered and the filtrate was evaporated to dryness. The residue was chromatographed on silica gel (preparative TLC, 2000 microns) to isolate the trapped isocyanates as ureas. These ureas were examined by ^{1}H NMR in acetone-d_6 and the NMR spectra compared to those obtained for authentic samples synthesized by reacting either methylisocyanate or chloroethylisocyanate with benzylamine. The half-lives of the various BCH derivatives were determined at pH 7.4 and 37° using an acidification assay previously described [6].

RESULTS

The production of methylisocyanate (from compound 4) and 2-chloroethylisocyanate (from compounds 2 and 3) during the decomposition of isocyanate precursors was confirmed by isolating, identifying, and quantifying the benzylamine adducts, namely 1-benzyl-3-methylurea and 1-benzyl-3-(2-chloroethyl)urea, which were generated from compounds 4, 2, and 3 with yields of approximately 50%, 80%, and 50%, respectively. The actual yields of isocyanates are probably higher than the indicated values, since the yields of the ureas depend not only on the yields of the isocyanates but also on their trapping efficiency. ^{1}H NMR in acetone-d_6 gave identical NMR spectra to those obtained for authentic samples synthesized by reacting either methylisocyanate or 2-chloroethylisocyanate with benzylamine.

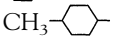
The major reactive moieties generated by the various 1,2-bis(sulfonyl)hydrazine derivatives and CNUs studied and their half-lives at pH 7.4 and 37° are given in Table 1. The half-lives for the BCHs were measured, and those for the CNUs were taken from the literature [17]. It can be seen that the kinetics of cross-linking and subsequent changes (Fig. 4, A–F) are relatively slow and do not reflect the relatively fast decomposition/primary alkylation kinetics of these agents. The cross-linking of DNA by compound 1 increased progressively with time, tending towards a maximum stable value at about 12 hr (Fig. 4A). The apparent cross-linking of DNA at 37° was approximately constant over a monitored period of 48 hr (data not shown). Samples of DNA cross-linked by compound 1 and stored at 4° retained their maximum apparent number of cross-links for several months. This finding suggests that compound 1 did not induce many strand nicks under these conditions. This result contrasts with that observed with BCNU, which showed a large decrease in apparent DNA cross-linking that became pronounced after 6 hr of incubation (Fig. 4D). 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[(2-chloroethyl)amino]carbonyl]hydrazine (compound 2), which can be regarded as being BCNU-like in that it generates both alkylating and functionally equivalent carbamoylating species, exhibited a more BCNU-like increase and decrease in apparent DNA cross-linking (Fig. 4B), although the decrease was not as pronounced as that observed with BCNU. Compound 4, which also generates a chloroethylating moiety but, in contrast to compound 2, produces methylisocyanate instead of chloroethylisocyanate as the carbamoylating species, did not produce measurable nicking and gave cross-linking kinetics similar to those observed with compound 1 (Fig. 4C). Both CCNU and MeCCNU (Fig. 4, E and F) also caused decreases in the amount of apparent cross-linking of DNA after 8 to 10 hr

TABLE 1. Comparison of the half-lives and the major reactive moieties generated by CNUs and 1,2-bis(sulfonyl)hydrazine derivatives

Hydrazine derivatives $CH_3SO_2-N(Y)-N(SO_2CH_3)-X$

Compound	X	Y	Chloroethylating agent	Carbamoylating agent	$T_{1/2}$ Hours
1	$ClCH_2CH_2-$	H–	YES	NO	0.01
2	$ClCH_2CH_2-$	$ClCH_2CH_2NHCO-$	YES	YES	0.25
3	H–	$ClCH_2CH_2NHCO-$	NO	YES	0.06
4	$ClCH_2CH_2-$	CH_3NHCO-	YES	YES	0.98

Nitrosoureas $X-N(NO)CONH-Y$

Compound	X	Y	Chloroethylating agent	Carbamoylating agent	$T_{1/2}$ Hours
BCNU	$ClCH_2CH_2-$	$ClCH_2CH_2-$	YES	YES	0.72*
CCNU	$ClCH_2CH_2-$		YES	YES	0.88*
MeCCNU	$ClCH_2CH_2-$	CH_3 	YES	YES	0.88*

*Wheeler *et al.* [17].

of incubation, but these changes were not as pronounced as those recorded for BCNU.

Since the only major difference in the activation pathways of compounds 1 and 2 is the formation of a carbamoylating chloroethylisocyanate species by compound 2, the effects of compound 3, which generates 2-chloroethylisocyanate, a carbamoylating agent, but possesses no chloroethylating activity, on pre-cross-linked DNA were examined (Fig. 5). Compound 3 caused a significant time-dependent decrease in the apparent cross-linking of the pre-cross-linked T7 DNA, indicating that single-strand nicks were introduced. There were no comparable changes when control pre-cross-linked DNA was incubated for this period of time in the absence of compound 3 (Fig. 5). 2-Chloroethylamine, a product of the hydrolysis of 2-chloroethylisocyanate, was also incubated with pre-cross-linked DNA; this reactive species produced a time-dependent decrease in apparent cross-linking similar to that caused by an equivalent concentration of compound 3 (Fig. 5).

The comparative effects of spermidine, a DNA-binding soft polynucleophile, on the cross-linking of DNA by BCNU and compound 1 were also examined (Fig. 6). The cross-linking of T7 DNA by BCNU was much more strongly inhibited by the presence of spermidine than that produced by compound 1.

DISCUSSION

The decomposition kinetics of these agents were much faster than the DNA cross-linking and nicking kinetics observed. This finding indicates that the time–course of the physical changes in the DNA (i.e. the cross-linking and nicking) reflects chemical changes subsequent to the primary alkylation events. The isocyanate trapping experiments confirmed the anticipated generation of isocyanates in good yields during the decomposition of the isocyanate precursors. In contrast to BCNU, compound 1 produced cross-linked DNA in the absence of significant strand nicking. However, compound 2, which in addition to the chloroethylating species generated by compound 1, was also capable of producing the chloroethylisocyanate carbamoylating species [5], gave a more BCNU-like rise and fall in the measured cross-linking. These results suggest that 2-chloroethylisocyanate is involved in DNA nicking. One mechanism by which 2-chloroethylisocyanate could give rise to nicking is by hydrolysis to form 2-chloroethylamine, followed by cyclization to generate a one-armed nitrogen mustard, which could then alkylate (aminoethylate) the N^7-position of guanine and produce a strand nick (Fig. 2). This possibility was tested in three ways: (a) by following the cross-linking of T7 DNA by compound 4, which is identical to compound 2 except that it generates methylisocyanate instead of chloroethylisocyanate. Methylisocyanate is incapable of causing strand nicks by the mechanism described, but would be able to nick the DNA if the presence of an isocyanate group was required for nicking; (b) by incubating pre-cross-linked DNA with compound 3, a precursor of chloroethylisocyanate and 2-chloroethylamine, which lacks chloroethylating activity; and (c) by incubating pre-cross-linked DNA with 2-chloroethylamine alone. In each case, the introduction of strand nicks was followed by monitoring the apparent decrease in the cross-linking.

Compound 4, like compound 1, produces stably cross-

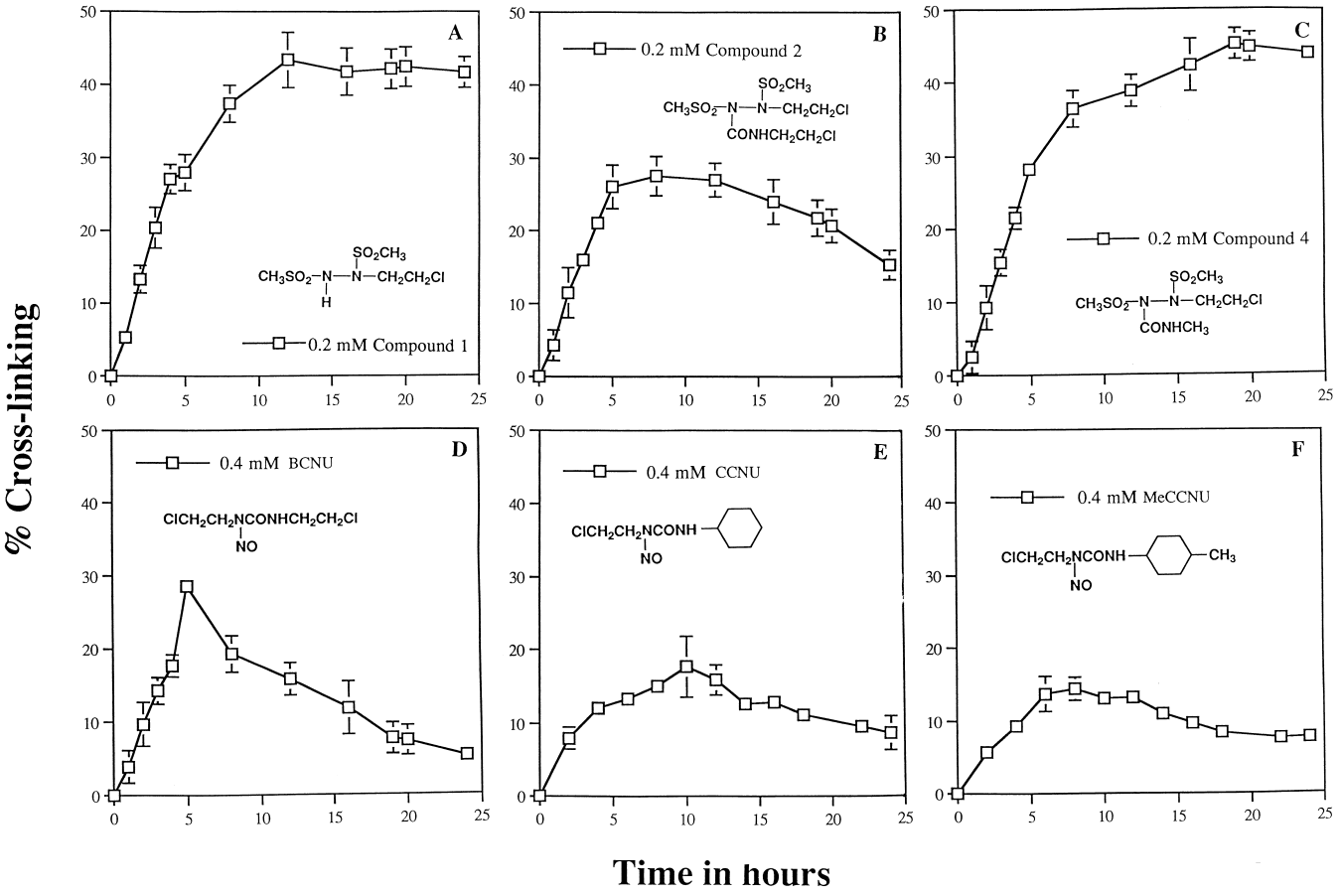


FIG. 4. The effects of incubation at 37° and pH 8.0 of various CNUs and BSHs on the apparent cross-linking of T7 DNA versus time.

linked DNA and does not cause the apparent nicking observed with compound 2 (Fig. 4C). This finding is consistent with the hypothesis that 2-chloroethylamine or a precursor thereof (i.e. 2-chloroethylisocyanate) is required to produce nicking. Moreover, incubation of stably pre-cross-linked T7 DNA with compound 3 or 2-chloroethylamine itself resulted in a comparable decrease in cross-linking (Fig. 3). Therefore, it appears that the nicking of DNA observed with compound 2 is due to the generation of a one-armed nitrogen mustard from 2-chloroethylisocyanate via 2-chloroethylamine. It should be noted that since BCNU was used at 0.4 mM and compound 2 was employed at 0.2 mM in an attempt to produce similar levels of DNA cross-linking, twice as much 2-chloroethylamine could be generated in the experiments with BCNU than in experiments with compound 2. The introduction of strand nicks by 2-chloroethylamine raises the question as to what proportion of the DNA strand nicking observed with BCNU is due to the generation of chloroethylamine. To gain information on this question, we compared the kinetics of cross-linking and subsequent fragmentation of T7 DNA by BCNU, CCNU, and MeCCNU. CCNU and MeCCNU, which cannot generate a one-armed nitrogen mustard, both caused DNA fragmentation, although the rate at which this occurred was less than one-half of that of BCNU (Fig. 4, E and F). Since the CNUs produce substantial amounts of alkylation of the N^7-position of guanine through both chloroethylation and hydroxyethylation, one would expect these agents to produce nicks in the absence of chloroethylisocyanate formation [9, 14].

The absence of significant strand nicking observed with compound 1 demonstrates that the N^7-position of guanine is not attacked by this agent and that compound 1 does not generate a significant quantity of a soft alkylating species. Alkylation of phosphate results in the production of strong alkali-labile chain-breaking sites, and the nitrosoureas produce substantial levels of these lesions [18]. Since phosphate groups are the hardest nucleophilic sites in DNA, it is likely that phosphotriesters are created by interaction of phosphate groups with chloronium and/or chloroethyldiazonium ions and therefore should be generated by both the BCHs and CNUs. Alkylphosphotriesters are highly stable under conditions of neutral pH [18–20]; therefore, it would be difficult to explain the observed differences in strand stability in terms of differences in phosphotriester formation, particularly under the pH conditions used. Further support for the lack of guanine N^7-derivatization by BCH derivatives lacking the ability to generate chloroethyliso-

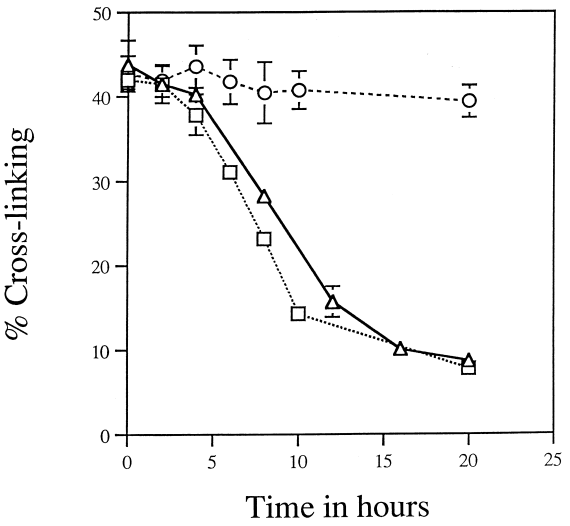


FIG. 5. The effects of the incubation at 37° and pH 8.0 of compound 3 (△), 2-chloroethylamine (□), and no additional chemical treatment (○) on the apparent cross-linking of pre-cross-linked T7 DNA versus time.

cyanate comes from earlier studies in this laboratory using alkaline elution, which also failed to detect the production of single-strand nicks by agents of this class [21]. These investigations also showed a large differential between cells expressing mer^+ and mer^- phenotypes in their susceptibility to the cytodestructive actions of the BCHs, supporting the hypothesis that the BCHs modify the O^6-position of guanine [21, 22].

Decomposition studies in aqueous buffer indicated that compound 1 gives a higher yield of 2-chloroethanol (88%) than the CNUs (26%), produced as a consequence of the chloroethylation of water, suggesting a high yield of chloroethylating species [6, 7]. This finding is consistent with the greater degree of cross-linking observed with the BCHs than with the CNUs (Fig. 4), despite the fact that the BCHs were evaluated at one-half of the concentration of that used for the CNUs. These findings all point to BCH derivatives being more selective chloroethylators of the O^6-position of guanine than the CNUs.

The alkylation of the N^7-position of guanine by the CNUs, by non-isocyanate-derived moieties, must involve species not produced by the BCHs, and this/these species must be relatively soft electrophiles to favor this site. The alkylation of the N^7-position of guanine by the CNUs has been characterized in some detail [9, 14]. The classically invoked CNU-derived electrophiles used to account for the decomposition products of the CNUs in aqueous buffers cannot explain several of the unusual features of this alkylation. Therefore, a direct alkylation mechanism that accounts for all of the discrepancies has been proposed [14] (Fig. 3). These features include: (a) sequence specificity, in that CNUs alkylate with a strong preference for the central guanine in the sequence 5′-dGdGdN-3′ (where N is any base). One would expect less specificity from the reactive intermediates classically believed to be responsible for the actions of the CNUs than from very soft electrophiles such as dimethylsulfate; however, dimethylsulfate has been found to alkylate randomly, whereas the CNUs do not [23]; (b) differences in the ratios of the modified bases produced by the different CNUs (if the CNUs all generated the same electrophilic species, they should behave identically with respect to base modification [24]); (c) isotopic studies of haloethylated and hydroxyethylated guanine, which strongly imply that the carbon to which the halogen is attached in the CNUs is the one that is attached to the N^7-position of guanine and that the carbon to which the halogen or hydroxide is attached is the one that was originally attached to N^1 in the parent molecule [14]; and (d) the fact that the halide can be exchanged and the yield of hydroxyethylated products decreased considerably when the reaction is carried out in buffers containing high concentrations of bromide [25].

The soft site-targeted direct alkylation mechanism proposed for the CNUs [14] has no obvious counterpart in the BCH derivatives. This alkylation mechanism may be another weakness inherent in the CNUs and may explain the very strong inhibition by spermidine of the cross-linking of DNA by BCNU compared to compound 1. The addition of a soft polynucleophile (i.e. spermidine) may have diverted a significant proportion of BCNU towards the alkylation of spermidine via a direct soft site-targeted alkylation mechanism, thereby reducing the amount available to generate a hard electrophilic chloroethylating species capable of cross-linking DNA. Changes in the conformation of DNA as a result of the binding of spermidine provide an alternative mechanism by which this polyamine could selectively influence the level of cross-linking through altering the reaction site preference.

A comparison of the DNA adducts remaining after a 5-hr repair period in CNU-resistant and -sensitive cell lines after

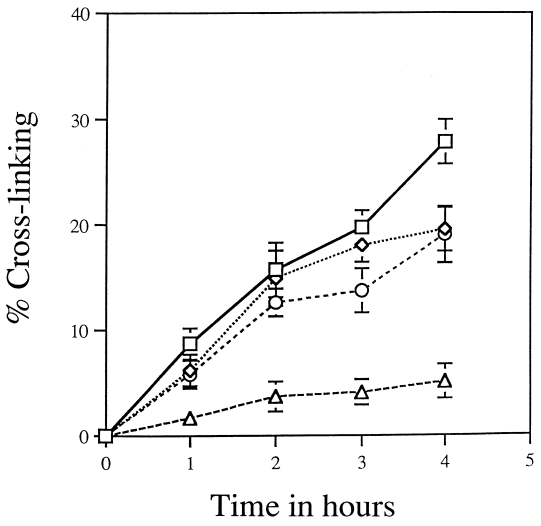


FIG. 6. The effects of the addition of spermidine (10 μM) on the cross-linking kinetics of BCNU and compound 1 versus time at 37° and pH 8.0. Compound 1 (□), compound 1 plus spermidine (◊), BCNU (○), BCNU plus spermidine (△).

exposure to the CNUs indicated that a highly significant decrease in the levels of the G-C cross-links occurred in resistant cells. Only minor differences were observed in the levels of guanine N^7-adducts and phosphotriesters [26]. These findings strongly imply that the G-C cross-links are the most therapeutically relevant lesions created by the CNUs. This interpretation is in keeping with results obtained with other chemotherapeutic agents, where cross-links appear to be of the greatest importance for therapeutic effects [1]. The cross-linking of DNA by BCH derivatives probably involves hard 2-chloroethyldiazonium ions and/or chloronium ions as the major electrophilic species, and these species probably account for the extensive and broad-spectrum anticancer activity exhibited by the BCHs against transplanted animal tumors [3–5]. It is likely that these species are also responsible for most of the therapeutic responses produced by the CNUs. The direct alkylation mechanism would not be expected to favor the alkylation of the O^6-position of guanine or to give rise to cross-links or to much of a therapeutic response.

Other covalent DNA modifications which do not lead to cross-links may have deleterious effects in addition to those stemming from the production of new neoplasms [22]. For instance, O^6-alkylation of guanine residues is extremely mutagenic [27] and these lesions increase the genetic variability of the neoplasm, providing a wider base from which resistant cells might be selected. When the O^6,N^1-ethanoguanine intermediate formed after the chloroethylation of the O^6-position of guanine interacts with nucleophiles, N^1-guanine adducts are formed [9]. However, if the initial alkylation event at the O^6-position of guanine involves hydroxyethylation, as can occur with the CNUs, such rearrangements are not possible and a difficult to repair (compared to methylation), highly mutagenic O^6-guanine lesion persists (Fig. 2). Moreover, hydroxyethylating agents, while toxic, are devoid of anticancer activity [28]. A similar argument can be made in the case of the modification of the N^7-position of guanine by the CNUs via a direct alkylation mechanism and by a one-armed mustard generated from 2-chloroethylamine. It has long been known that, while one-armed mustards have only about 1/30th of the anticancer activity of their two-armed crosslinking counterparts, they do have similar mutagenicity [29].

It would appear advantageous to design agents that minimize (a) the generation of reactive isocyanates and (b) the formation of DNA lesions with little or no therapeutic benefit, while maximizing the generation of O^6-haloethylguanine residues in DNA. The BCHs, in contrast to the CNUs, appear to represent a class of agents possessing these favorable properties.

This research was supported in part by U.S. Public Health Service Grant CA-74970 from the National Cancer Institute.

References

1. Lawley PD and Brookes P, Interstrand cross-linking of DNA by difunctional alkylating agents. *J Mol Biol* **25:** 143–160, 1967.
2. Shyam K, Hrubiec RT, Furubayashi R, Cosby LA and Sartorelli AC, 1,2-Bis(sulfonyl)hydrazines. 3. Effects of structural modification on antineoplastic activity. *J Med Chem* **30:** 2157–2161, 1987.
3. Shyam K, Penketh PG, Divo AA, Loomis RH, Patton CL and Sartorelli AC, Synthesis and evaluation of 1,2,2-tris(sulfonyl)-hydrazines as antineoplastic and trypanocidal agents. *J Med Chem* **33:** 2259–2264, 1990.
4. Shyam K, Penketh PG, Divo AA, Loomis RH, Rose WC and Sartorelli AC, Synthesis and evaluation of 1-acyl-1,2-bis-(methylsulfonyl)-2-(2-chloroethyl)hydrazines as antineoplastic agents. *J Med Chem* **36:** 3496–3502, 1993.
5. Shyam K, Penketh PG, Loomis RH, Rose WC and Sartorelli AC, Antitumor 2-(aminocarbonyl)-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. *J Med Chem* **39:** 796–801, 1996.
6. Penketh PG, Shyam K and Sartorelli AC, Studies on the mechanism of decomposition and structural factors affecting the aqueous stability of 1,2-bis(sulfonyl)-1-alkylhydrazines. *J Med Chem* **37:** 2912–2917, 1994.
7. Weinkam RJ and Lin HS, Reactions of 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in aqueous solution. *J Med Chem* **22:** 1193–1198, 1979.
8. Brundrett RB, Cowers JW and Colvin M, Chemistry of nitrosoureas. Decomposition of deuterated 1,3-bis(2-chloroethyl)-1-nitrosoureas. *J Med Chem* **19:** 958–961, 1976.
9. Ludlum DB, The chloroethylnitrosoureas: Sensitivity and resistance to cancer chemotherapy at the molecular level. *Cancer Invest* **15:** 588–598, 1997.
10. Brookes P and Lawley PD, The reactions of mustard gas with nucleic acids *in vitro* and *in vivo*. *Biochem J* **77:** 478–484, 1960.
11. Lawley PD and Brookes P, Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem J* **89:** 127–138, 1963.
12. Maxam MM and Gilbert W, Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* **65:** 499–560, 1980.
13. Weinkam RJ and Lin HS, Chloroethylnitrosourea cancer chemotherapeutic agents. *Adv Pharmacol Chemother* **19:** 1–33, 1982.
14. Naghipur A, Ikonomou MG, Kebarle P and Lown JW, Mechanism of action of (2-haloethyl)nitrosoureas on DNA: discrimination between alternative pathways of DNA base modification by 1,3-bis(2-fluoroethyl)-1-nitrosourea by using specific deuterium labeling and identification of reaction products by HPLC/tandem mass spectrometry. *J Am Chem Soc* **112:** 3178–3187, 1990.
15. Penketh PG, Shyam K and Sartorelli AC, Fluorometric assay for the determination of DNA–DNA cross-links utilizing Hoechst 33258 at neutral pH values. *Anal Biochem* **252:** 210–213, 1997.
16. Morgan AR, Pulleyblank DE, Murray NL and Evans DH, Ethidium fluorescence assays. Part 1. Physiochemical studies. *Nucleic Acids Res* **7:** 547–569, 1979.
17. Wheeler GP, Bowdon BJ, Grimsley JA and Lloyd HH, Interrelationships of some chemical, physicochemical and biological activities of several 1-(2-haloethyl)-1-nitrosoureas. *Cancer Res* **34:** 194–200, 1974.
18. Lown JW and McLaughlin W, Nitrosourea-induced DNA single-strand breaks. *Biochem Pharmacol* **28:** 1631–1638, 1979.

19. Shooter KV, The kinetics of the alkaline hydrolysis of phosphotriesters in DNA. *Chem Biol Interact* **13:** 151–163, 1976.
20. Shooter KV and Merrifield RK, The rate of hydrolysis of methyl phosphotriesters in DNA under conditions used in alkaline sucrose gradients. *Biochim Biophys Acta* **521:** 155–159, 1978.
21. Pratviel G, Shyam K and Sartorelli AC, Cytotoxic and DNA-damaging effects of 1,2-bis(sulfonyl)hydrazines on human cells of the Mer^+ and Mer^- phenotype. *Cancer Biochem Biophys* **10:** 365–375, 1989.
22. Penketh PG, Shyam K and Sartorelli AC, Mechanisms of resistance to alkylating agents. In: *Drug Resistance* (Ed. Hait WN), pp. 65–81. Kluwer Academic Publishers, Dordrecht, Boston, 1996.
23. Hartley JA, Gibson NW, Kohn KW and Mattes WB, DNA sequence selectivity of guanine-N7 alkylation by three antitumor chloroethylating agents. *Cancer Res* **46:** 1943–1947, 1986.
24. Tong WP, Kohn KW and Ludlum DB, Modification of DNA by different haloethylnitrosoureas. *Cancer Res* **42:** 4460–4464, 1982.
25. Parker S, Kirk MC, Ludlum DB, Koganty RR and Lown JW, Reaction of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) with guanosine: Evidence for a new mechanism of DNA modification. *Biochem Biophys Res Commun* **139:** 31–36, 1986.
26. Bodell WJ, Tokuda K and Ludlum DB, Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with *N*-(2-chloroethyl)-*N*-nitrosourea. *Cancer Res* **48:** 4489–4492, 1988.
27. Loveless A, Possible relevance of O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature* **223:** 206–207, 1969.
28. Swanson DH, Frei J and Lawley PD, Synthesis of 1-(2-hydroxyethyl)-1-nitrosourea and comparison of its carcinogenicity with 1-ethyl-1-nitrosourea. *J Natl Cancer Inst* **63:** 1469–1473, 1979.
29. Brookes P and Lawley PD, The reaction of mono and di-functional alkylating agents with nucleic acids. *Biochem J* **80:** 496–503, 1961.