Review

Cross-linkable Nitrosamines with a Chloroalkyl Group as Candidates for Anticancer Lead Compounds

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DNA alkylation and cross-linking are classical mechanisms underlying the activity of cancer chemotherapeutic agents such as procarbazine and mitomycin C, which are currently used in the clinic. Chloroethyl *N*-nitrosoureas is one group of anticancer cross-linking agents, and exert their biological activities by formation of DNA cross-links. Because anticancer chloroethyl *N*-nitrosoureas shows toxic side-effects due to protein carbamylation, many efforts have been exercised to reduce unwanted side-effects by design of new drugs with low carbamoylating activity. *N*-Nitrosamines, another category of *N*-nitroso compounds, alkylate cellular nucleophiles *in vivo*, and might induce gene mutation and lead to carcinogenesis by DNA alkylation. Its active form is released after elimination of aldehyde; i.e. *N*-nitrosamines do not carbamoylate proteins. We have an interest in structure modification of alkylating nitrosamines as useful candidates for anticancer lead compounds. Three *N*-nitroso-*N*-(acetoxymethyl)- ω -chloroalkylamines, chloroethyl, chloropropyl and chlorobutylamines have been synthesized and their chemical and biological properties were evaluated. The chloropropyl nitrosamine showed mutagenicity in *Salmonella typhimurium* TA92 that is positively responsive to DNA cross-linker, and also showed interstrand cross-linking activity towards plasmid DNA assayed by agarose gel electrophoresis. Then three-ring aromatic moieties were introduced into the structure of chloropropyl nitrosamine to potentiate its binding affinity to DNA molecules. Although three aromatic analogs intercalated to double-stranded DNA, only acridine analog had DNA cross-linking activity. In TA92 strain, the acridine analog showed the highest mutagenicity among all nine *N*-nitroso compounds used in this study. These results suggest that the aromatic ring moiety confers DNA-intercalating ability to the cross-linkable chloropropyl nitrosamine; the acridine analog that forms DNA cross-links efficiently might be a potential new anticancer lead compound. To assess the application of cross-linkable nitrosamines as new anticancer agents, further investigations such as that on DNA adducts or on their activity towards cancer cells are required.

Key words: nitrosamines, DNA cross-linker, DNA intercalator, anticancer agents

Introduction

Although DNA alkylation represents a mechanism of carcinogenesis induced by alkylating agents, it is also a classical mechanism of anticancer agents to target DNA molecule, such as procarbazine and dacarbazine, which are clinically used (1). Furthermore, DNA cross-linking is also a classical mechanism underlying the activity of cancer chemotherapeutic agents such as mitomycin C and cyclophosphamide, which are also currently used in the clinic. These DNA targeting agents are carcinogenic themselves, but they are quite effective for killing cancer cells under proliferation phase. *N*-Nitroso compounds are one group of DNA alkylators and after alkylation of DNA base they induce gene mutation to lead carcinogenesis. Therefore, it is supposed that alkylating *N*-nitroso compounds might have potential to act as anticancer agents in cancer cells, and we have proposed alkylating nitrosamines as useful candidates for anticancer agents in conjunction with anticancer nitrosoureas (2). This review describes our results on the DNA-alkylating and cross-linking properties of *N*-nitroso chloroalkylamines, and the prospective application of these compounds as anticancer lead compounds.

Mechanism of DNA Alkylation by *N*-Nitroso Compounds

N-Nitroso compounds are well known as strong carcinogens in experimental animals, and suspected to be human carcinogenesis. Many of these compounds are present in our environment: they can be formed from tobacco smoking and also from dietary precursors in the stomach under acidic conditions (3–5). They have also been reported to be formed from amines by activated macrophages under neutral conditions *in vivo* (6,7). Such *N*-nitroso compounds are divided into two groups: *N*-nitrosamines and *N*-nitrosamides. In general, *N*-nitrosamines such as *N*-nitrosodialkylamines need to be

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metabolized *in vivo* by cytochromes P450 to attain biological activity (8); by contrast, *N*-nitrosamides such as *N*-nitroso-*N*-alkylureas do not need metabolic conversion and spontaneously degrade to alkanediazohydroxide (9) (Fig. 1). Activated metabolites of *N*-nitrosodialkylamines—namely, α -hydroxy nitrosamines—decompose spontaneously to alkanediazohydroxides, which can react with biological nucleophiles such as nucleic acids and proteins (8). The induced DNA alkylation finally results in carcinogenesis or mutagenesis (Fig. 1).

Because α -hydroxy nitrosamines are relatively unstable in aqueous solution, α -acetoxy, α -methoxy, and α -phosphonoxy nitrosamines have been used as model precursors for α -hydroxy nitrosamines in studies on the chemical and biological activities of *N*-nitrosodialkylamines (10–13) (Fig. 2). α -Acetoxy nitrosamine is hydrolyzed by esterase *in vivo* to form α -hydroxy nitrosamine (14,15); thus, many studies have used α -acetoxy nitrosamine to investigate its chemical behavior (16–20), bacterial mutagenicity (20), and mutagenicity towards mammalian cells (21). In 1980, the α -hydroxy nitrosamines were isolated by deoxygenation of α -

Fig. 1. Activation of *N*-nitroso compounds. The character R or R' indicates any alkyl group.

Fig. 2. Model precursor compounds for unstable α -hydroxy nitrosamine. The character R or R' indicates any alkyl group.

hydroperoxy nitrosamines (22) and their chemical properties and mutagenicity have been elucidated (23,24). The results revealed that α -hydroxy nitrosamines are involved in the metabolic activation of *N*-nitrosamines. Subsequently, we studied chemical and biological properties of alkanediazotates—the model precursors of alkanediazohydroxides. The results support the idea that α -hydroxy nitrosamines degrade to alkanediazohydroxides, which alkylate DNA (25,26).

***N*-Nitroso Compounds as Anticancer Agents**

Clinically used chemotherapeutic agents such as cyclophosphamide alkylates DNA by reacting with one electrophilic center in the drug molecule, which enables a neighboring nucleophile within the alkylated DNA to react with a second electrophilic center to eventually form a DNA cross-link (27). Although many *N*-nitroso compounds are considered to be carcinogens, some *N*-nitrosoureas have anticancer activities (Fig. 3). For example, chloroethyl nitrosoureas (CENUs) are one group of clinically effective anticancer alkylating agents. CENUs exert their biological activities by alkylation and carbamylation, and protein carbamylation by released isocyanates from CENUs is thought to be the

Fig. 3. *N*-nitrosoureas used as anticancer agents. BCNU; *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (carmustine), CCNU; *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (lomustine), ACNU; *N'*-(4-amino-2-methylpyrimidin-5-yl)methyl-*N*-(2-chloroethyl)-*N*-nitrosourea (nimustine) and *N'*-(2-amino-2-deoxy-D-glucosyl)-*N*-(2-chloroethyl)-*N*-nitrosourea.

Fig. 4. Structures of chlorinated nitrosamines, *N*-nitroso-*N*-(acetoxymethyl)- ω -chloroalkylamines (**1–3**), and their parent non-chlorinated nitrosamines, *N*-nitroso-*N*-(acetoxymethyl)alkylamines (**4–6**).

main cause of their toxic side-effects (28,29). CENUs have a chloroethyl moiety functions as a common bifunctional group, and decompose under physiological conditions to generate chloroethyl diazohydroxides spontaneously in a manner similar to the decomposition of carcinogenic *N*-nitrosamines as shown in Fig. 1. Chloroethyl diazohydroxide reacts with both protein and DNA to form an alkylated products, and a second alkylation event on alkylated base can lead to DNA cross-links owing to the presence of the chloro leaving group (28,30). DNA interstrand cross-links prevent separation of the DNA double strands during the replication process, which eventually inhibits DNA synthesis, resulting in a cytotoxic effect (31). Therefore, among alkylation of protein and DNA by CENUs, DNA alkylation and cross-linking is thought to be more important for anticancer activity of CENUs. To date, many analogs have been developed in an effort to improve DNA-targeting reactivity and to reduce undesirable toxic side-effects (2).

Chloroalkyl Nitrosamines as DNA Cross-linkers

In contrast to *N*-nitrosamides, *N*-nitrosamines degrade *in vivo* by releasing aldehydes instead of isocyanates that cause protein carbamylation, then alkylate DNA to show their biological activity. Aldehyde molecules might react with DNA or protein, but resulting effects should differ from that of isocyanates. Among the various *N*-nitroso compounds, chloroethyl nitrosocarbamates have been developed as potential anticancer alkylators in addition to many *N*-nitrosoureas (32). However, *N*-nitrosamines have so far seldom considered as anticancer agents. Therefore, we have an interest in the potential of *N*-nitrosamines as lead compounds for novel anticancer agents, and planned to examine the behavior of nitrosamines with either a chloroethyl group or other chloroalkyl group with a longer carbon chain. On the basis of our studies on the chemical and biological properties of carcinogenic *N*-nitrosamines, we are interested in bifunctional nitrosamines that could act as DNA cross-linkers *in vivo*

Table 1. Half-lives of chlorinated nitrosamines **1–3** determined in aqueous buffer solutions.

	Half lives (min)		
	pH = 5.0	pH = 7.4	pH = 9.0
1	0.33	0.33	0.34
2	49.5	24.8	7.5
3	43.3	21.0	6.8

and possess anticancer activity.

The synthesis of *N*-nitroso-*N*-(acetoxymethyl)-2-chloroethylamine (**1**, 76215-00-4) has been reported together with its effect on increasing the life span of tumor-bearing mice (33), but little is known about other biological activity to date. Subsequently, we synthesized *N*-nitroso-*N*-(acetoxymethyl)-3-chloropropylamine (**2**, 312304-87-3) and *N*-nitroso-*N*-(acetoxymethyl)-4-chlorobutylamine (**3**, 312304-89-5) (Fig. 4), and compared the properties of **2** and **3** with those of **1**, as well as examining the effect of alkyl chain length and chloro leaving group (34). The rate of decomposition of **1–3** in aqueous solution was determined as one of the key chemical properties, and found that the decompositions were time-dependent. The half-lives of **2** and **3** and the effect of pH on those were clearly different from those of **1** (Table 1). Thus, it is suggested that the mechanism underlying the pH-dependent decomposition of **2** and **3** differs from that of **1**, which rapidly decomposed at a rate independent of the pH of the solution. All these compounds were mutagenic in *Salmonella typhimurium* TA92 (+*uvrB*, pKM101). Because TA92 strain can detect both alkylating agents and cross-linking agents (35), our results suggested that **1–3** hydrolyzed and decomposed to chloroalkyldiazohydroxides in aqueous solution, alkylated DNA and then formed DNA cross-links (34).

The ability of the chlorinated nitrosamines **1–3** to form DNA interstrand cross-links was determined using plasmid DNA (36). Electrophoresis coupled with DNA visualization showed that the intensity of the bands of

Fig. 5. Dose- and time-dependent cross-linking activity of chloropropyl analog **2** towards plasmid DNA. The compound dissolved in DMSO was treated with linearized pBluescript® II KS⁻ DNA in a phosphate buffered solution (pH 7.4) at 37°C. After reaction, the recovered DNA was denatured by heating then chilled immediately, and annealed DNA was electrophoresed on an agarose gel. The DNA bands were visualized and quantified as reported (Reference 37). ●, 24 h; ■, 48 h; ▲, 72 h; DS: double-stranded DNA, SS: single-stranded DNA.

cross-linked double-stranded DNA increased dose-dependently after treatment with the chloropropyl analog **2**. Densitometric analysis of the intensity of the bands of double-stranded DNA indicated that the cross-linking was both dose- and time-dependent (Fig. 5). The chlorobutyl analog **3** showed weaker activity than **2**, and the chloroethyl analog **1** showed no activity under the reaction conditions used. The three non-chlorinated α -acetoxy nitrosamines (**4–6**, Fig. 4) did not show any cross-linking activity (37).

Chlorinated nitrosamines have been reported only about a product of the nitrosation of spermidine in the presence of chloride ions (38), and about a product of the reaction of hydroxylated nitrosamine and chloride ions (39). Concerning to CENUs, haloethyl nitrosoureas form haloethyl diazotates as ultimate electrophiles, and *E*-2-chloroethyl diazotate exhibits suppressive activity against P388 leukemia *in vivo* (30). The above results indicate that not only a chloroethyl group but also a chloropropyl group could behave as an anticancer bifunctional group, and thus suggest the possibility that chlorinated nitrosamine derivatives could be applicable as lead compounds for anticancer agents.

Mutagenic Profiles of Chlorinated Nitrosamines

The mutagenicities of compounds **1–6** were assayed in following nine *Salmonella typhimurium* strains of various genotypes without S9 mix; TA1535 (*rfa*, Δ *uvrB*), YG7108 (*rfa*, Δ *uvrB*, Δ *ada*, Δ *ogt*), YG7100 (*rfa*, Δ *uvrB*, Δ *ada*, +*ogt*), YG7104 (*rfa*, Δ *uvrB*, +*ada*, Δ *ogt*), TA1975 (*rfa*, +*uvrB*), G46 (+*uvrB*), YG7155 (+*uvrB*, Δ *ada*, Δ *ogt*), TA92, TA100 (*rfa*, Δ *uvrB*, pKM101). The strains used have *hisG46* base change

Fig. 6. Specific mutagenicity of chlorinated nitrosamines, non-chlorinated nitrosamines and alkanediazotates in *Salmonella typhimurium* TA1535. ●, *N*-nitroso-*N*-(acetoxymethyl)- ω -chloro-alkylamines (**1–3**); ■, (*E*)-potassium alkanediazotates (Reference 25).

mutation. All the chlorinated nitrosamines have α -acetoxy structure (Fig. 4), and they do not need any metabolic activation. Comparison of the specific mutagenicities (revertants per μ mol of chemical) showed that mutagenicity was influenced by the alkyl chain length of the nitrosamines, but the effect of alkyl chain length differed between the chlorinated nitrosamines and the potassium alkanediazotates that are ultimate alkylating form of non-chlorinated nitrosamines (Fig. 6) (37). The results showed that all nitrosamines assayed were directly mutagenic in the TA1535 strain, where they damaged bacterial DNA and induced base-pair changes. With respect to *Salmonella* strains deficient in the alkylguanine-DNA alkyltransferase (AGT) genes *ada* and *ogt* (40), the nitrosamines showed stronger mutagenicities in YG7108 and in YG7104 than in TA1535 and in YG7100 (Fig. 7). These results indicate that Ogt protein, but not Ada protein, is responsible for repairing the DNA alkylation damage induced by nitrosamines with an alkyl group of ethyl, propyl and butyl. For the chlorinated nitrosamines (**1–3**), an increase in alkyl chain length resulted in an increase of mutagenicities in the TA1535 and YG7100 and TA100, which possess the *ogt* gene and are deficient in the *uvrB* gene. However, the chloropropyl analog **2** was the strongest in the other six strains, where *uvrB* is present or *uvrB* and *ogt* are absent. Conversely, among the non-chlorinated nitrosamines (**4–6**), an increase in alkyl chain length generally decreased mutagenicity (37).

The GC $\rightarrow$ AT transition mutation detected was mainly induced by *O*⁶-alkylguanine at the *hisG46* locus, which is repaired by Ogt protein. This observation indicates that the nitrosamines **1–3** alkylate the *O*⁶-

Fig. 7. Effects of AGT gene deficiency on the relative mutagenicity of chlorinated nitrosamines (1–3). ●, TA1535 (+ada, +ogt); ▲, YG7100 (Δ ada, +ogt); ○, YG7104 (+ada, Δ ogt); △, YG7108 (Δ ada, Δ ogt).

position of guanine as do classical *N*-nitroso compounds including non-chlorinated nitrosamines. The chlorinated nitrosamines 2 and 3 showed stronger mutagenicity than the non-chlorinated nitrosamines, which suggested that the chloro group enhanced the reactivity of nitrosamines towards biological nucleophiles. Chloroethyl nitrosamine 1 showed weaker mutagenicity than 4. This result could be explained by the replacement by chlorine enhanced the reactivity due to a neighboring group effect and rapid decomposition occurred in aqueous conditions. Evaluation of *in vitro* alkylating activity should be made to clarify the effect of chlorine atom to chemical reactivity of 1–3.

Among genotypes of tester strains, we assumed that *rfa* genotype did not affect to the incorporation of the nitrosamines because mutagenicity of 1–3 in TA1975 strain was the same as that in G46 strain (34). Further, comparison between the mutagenicity of nitrosamines in TA1535 and that in TA100 revealed that presence of plasmid pKM101 reduced nitrosamine-induced mutation (34).

Excision repair mechanisms are important in the repair of alkylated DNA (41). A deficiency in excision repair genes increased the mutagenicity of the nitrosamines, and the action of AGT was affected by the presence of excision repair. Nevertheless Ogt protein plays a major protective role against alkylating damage in *Salmonella typhimurium*, in the presence of the excision repair gene *uvrB*, the effect of *ogt* gene deficiency is small (37). Excision repair is also responsible for repairing mutations induced by cross-linking damage (34,35). The chlorinated nitrosamines induced revertants probably due to DNA cross-linking in

Table 2. Specific mutagenicity of chlorinated and non-chlorinated nitrosamines in *Salmonella typhimurium* TA92.

Compound	Specific mutagenicity (revertants/μmol)	Compound	Specific mutagenicity (revertants/μmol)
1	4.3	4	8.3
2	22.0	5	4.5
3	7.2	6	4.6

excision repair-proficient strains, such as TA1975 and TA92 strain. Despite the fact that the non-chlorinated nitrosamines have no cross-linking activity, they showed weak but clear mutagenicity in the TA92 strain. This observation might be explained by the production of more alkylated DNA base than could be repaired by the excision repair pathway. In comparing the mutagenicities of the non-chlorinated nitrosamines with those of the chlorinated nitrosamines, the influence of excision repair deficiency was most evident with exposure to the chloropropyl analog 2, and the mutagenicity of 2 in strain TA92 was the strongest among all nitrosamines tested (Table 2). From the results using TA1535 strain, the chlorinated nitrosamines induced revertants derived from DNA base alkylation, but in case of the chloropropyl analog 2, chloropropyl group in alkylated base might react to form cross-link, considering the above results of the plasmid DNA cross-linking assay. Although chloropropyl nitrosourea has been reported not to have cross-linking activity (30), the chloropropyl nitrosamine 2 showed clear mutagenicity towards three strains with an intact excision repair system: TA1975, G46 and TA92.

Chloroalkyl Nitrosamines as DNA Intercalating Cross-linkers

Anticancer alkylating and cross-linking agents are important for cancer chemotherapy, and new drugs with more effective and more selective activity are desired (42). Commonly, DNA cross-linking agents are not specific to DNA: they can react with other macromolecules such as proteins and cellular nucleophiles. By contrast, DNA intercalators and minor groove binders could target specific structures of DNA such as a planar aromatic ring system of base-pairs in the duplex of nucleic acids (43,44). Proflavine and amsacrine are well known intercalators with their acridine structure, and one of the minor-groove binders is distamycin A with its polypyrrrole structure (43). Recently, hybrid alkylating molecules with an intercalating or minor-groove-binding moiety have been produced to alter DNA binding affinity or sequence specificity of parent alkylators. For instance, intercalating acridine chromophore was introduced into aniline mustard moiety, and resulting molecule was more

cytotoxic than untargeted mustards (45). Further, Beraldi et al. reported that a series of distamycin A-bound uracil mustards showed enhanced anti-leukemic activity (46). Figure 8 shows examples of hybrid alkylating mustards combined with intercalating acridine or minor groove binding polypyrrole structure.

As mentioned above, DNA intercalation is thought to be DNA-selective, and compounds with intercalating activity were found to show stronger reactivity with DNA bases than simple cross-linking agents (45,47). Therefore, we planned to enhance the DNA binding affinity of the cross-linkable chloropropyl nitrosamine **2**. We designed chloropropyl nitrosamines with a three-ring aromatic system as the minimum structural moiety for the intercalator, then synthesized three nitrosamines with an anthracene- (48), acridine- (44), or anthraquinone- (49) carboxylate structure (Fig. 9) (50). The arylcarboxylates, *N*-nitroso-*N*-(9-anthracenecarboxyloxymethyl)-3-chloropropylamine (**7**; 736185-20-9), *N*-nitroso-*N*-(9-acridinecarboxyloxymethyl)-3-chloropropylamine (**8**; 736185-22-1), and *N*-nitroso-*N*-(2-anthraquinonecarboxyloxymethyl)-3-chloropropylamine (**9**; 736185-24-3) were more unstable in aqueous solution than were the acetate esters, and the acridine analog decomposed most rapidly: the half-lives of **7–9** at pH

Fig. 8. Hybrid alkylating molecules with an intercalating (right) or minor-groove-binding (left) moiety. Right; 9-[[4-(Acetylamino)-2-methoxyphenyl]amino]-*N*-[3-[4-[bis(2-chloroethyl)amino]phenyl]propyl]-4-acridinecarboxamide (Ref. 45), Left; 3-[1-Methyl-4-[1-methyl-4-[N1-[5-bis(2-chloroethyl)amino]-2,4-(1*H*,3*H*)pyrimidine-2,4-dione]acetylamino]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride (Ref. 46).

Fig. 9. Structures of *N*-nitroso-*N*-(arylcarboxyloxymethyl)-3-chloropropylamines (**7–9**).

7.4 were 1.6, 0.11, 3.7 h, respectively. Nitrosamines **7–9** were assayed for their DNA binding affinity as DNA intercalating activity by a competitive ethidium displacement assay (51). All nitrosamines intercalated with DNA, but their activities were weaker than that of amsacrine, a famous intercalator. Among the nitrosamines tested, **9** showed higher binding affinity than either **7** or **8**. The nitrosamine **2**, which does not have an intercalating moiety, showed no activity in this assay (Fig. 10). These results suggest that nitrosamines **7–9** have DNA intercalating activity owing to their aromatic ring systems.

The ability of nitrosamines to form DNA interstrand cross-links was determined using plasmid DNA. The acridine analog **8** clearly formed DNA interstrand cross-links in plasmid DNA, whereas the anthraquinone analog **9** showed no activity in this test system (50). The activity of **8** was weaker than that of cisplatin, a cross-linker, but was similar to that of the parent compound **2**. Furthermore, bacterial mutagenicity was assayed in the three *Salmonella typhimurium* strains TA100, TA98 and TA92. In TA100, **7–9** had positive activity: the activity of **9** was higher than that of **2** (Fig. 11A), followed by **7** and **8**. In TA98, only **9** showed a killing

Fig. 10. Competitive ethidium-DNA fluorescence displacement assay of *N*-nitroso-*N*-(arylcarboxyloxymethyl)-3-chloropropylamine. ◇, **2**; ●, **7**; ▲, **8**; ■, **9**; ◆, amsacrine.

effect, suggesting that **9** intercalates more effectively, which agreed with the results of the intercalating assay (37). In TA92, which is positively responsive to cross-linking agents, the acridine analog **8** showed higher mutagenicity than **2**, and the order of mutagenicity in strain TA92 agreed with the results of the cross-linking assay (Fig. 11B). From these results, the acridine analog **8** is expected to act as a more effective DNA cross-linker than its parent compound **2**, because of the introduction of the intercalating acridine structure.

Mechanism of DNA Damage Caused by Chlorinated Nitrosamines

The nitrosamines with the three-ring aromatic system were expected first to intercalate DNA and then to release an active form of nitrosamine by hydrolysis of the ester; finally, the alkyl diazonium ion was expected to alkylate DNA (Fig. 12). The arylcarboxylate we synthesized were characterized by the detection of activated nitrosamine, which is released only after ester

bond cleavage at the intercalation site.

Many factors affect the biological activity of nitrosamines, such as stability, intercalating activity, alkylating activity, and cross-linking activity. In our study, cross-linkable chloropropyl nitrosamines **8** demonstrated DNA binding affinity, but the activity or stability in an aqueous solution did not contribute to enhance the cross-linking activity. Nevertheless, cross-linking activity and bacterial mutagenicity did not well correlate when the structure of the ring system is different in the chloropropyl nitrosamines. The anthracene analog **9**, which had the highest intercalating activity and the highest mutagenicity in TA100 and TA98, was not a cross-linker. Whereas the acridine analog **8** had the strongest mutagenicity in TA92, and showed cross-linking activity to plasmid DNA. If the nitrosamines react according to the mechanism in Fig. 12, and if intercalating activity does not affect the activity of nitrosamines, no difference in alkylating and cross-linking activity among nitrosamines **7–9** should be expected. From the results that the activities of nitrosamines **8** differed from that of **9**, other causes such as the base sequence selectivity of DNA intercalation could be thought as explanation of the difference between **8** and **9**. Further study is needed to confirm the contribution of the structure of the bifunctional nitrosamines with aromatic ring systems to the base sequence specificity of DNA alkylation or to DNA cross-link formation (52).

Conclusion

DNA targeting agents are often carcinogenic themselves, but in cancer chemotherapy, they are quite effective for killing cancer cells under proliferation phase. We have synthesized activated nitrosamines with chloroalkyl groups, and found that a chloropropyl analog, *N*-nitroso-*N*-(acetoxymethyl)-3-chloropropylamine **2**, shows direct mutagenicity in *Salmonella typhimurium* TA92, which is positively responsive to

Fig. 11. Mutagenicity of *N*-nitroso-*N*-(arylcarboxyloxymethyl)-3-chloropropylamine in *Salmonella typhimurium* TA100 and TA92. ◇, **2**; ●, **7**; ▲, **8**; ■, **9**.

Fig. 12. Possible mechanism of intercalation, alkylation, and cross-linking of DNA by chloropropyl nitrosamines with an aromatic ring system.

DNA cross-linkers. Chloropropyl analog **2** also showed interstrand cross-linking activity towards plasmid DNA, suggesting that **2** acts on DNA to form interstrand cross-links. Furthermore, chloropropyl nitrosamines with three-ring aromatic ring systems were synthesized to enhance their DNA binding affinity. Three compounds **7–9** intercalated to double-stranded DNA, and their intercalating activities were affected by the ring structure. Only the acridine analog **8** formed cross-links towards plasmid DNA, and its ability was similar to that of the parent acetoxynitrosamine **2**. In a bacterial mutation assay, the acridine analog **8** had the highest mutagenicity in TA92 strain among six chloroalkyl nitrosamines we synthesized. These results suggested that the chloro group introduced at the end of alkyl chain may act as a second eliminating group, and that the three-ring aromatic moiety conferred DNA-intercalating ability on the cross-linkable chloropropyl nitrosamines. Thus, the acridine analog **8** that formed efficient DNA cross-links can be considered to have the potential as a new anticancer lead compound. From the result that only acridine analog **8** formed DNA cross-links among three intercalative chloropropyl nitrosamines **7–9**, DNA intercalation was not a driving force behind the formation of cross-links. Despite of higher mutagenicity of **8** in TA92 than **2**, their cross-linking activities were almost similar, suggesting that another factors such as differences in DNA alkylating activity or in base sequence specificity may contribute to the cross-linking activity of the nitrosamines *in vivo*. Definitely, further study about *in vivo* toxicity concerning to unwanted side effects by newly proposed compounds is needed. And investigations on DNA adduct and on the activity towards cancer cells are also required to assess the application of cross-linkable nitrosamines as candidates of anticancer agents.

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