Chapter 6
Novel \textit{N}-nitrosourea and carbamate
6.1 INTRODUCTION

In the past years, our laboratory have synthesized a series of DNA-directed alkylating agents in which \( N \)-mustard derivatives linked to the DNA-affinic molecules such as acridine, 9-anilinoacridines, quinoline, 4-anilinoquinazoline and 4-anilinoquinoline chromophore via various alkyl spacers, urea and carbamates linker\(^1\text{-}^4\). These studies demonstrated that the strategy to design DNA-directed alkylating agents has high possibility in finding new potential anticancer agents. To find a new DNA-directed alkylating agent with potential antitumor activity, we then proposed to replace \( N \)-mustard pharmacophore with \( N \)-nitrosourea or \( N \)-nitrosocarbamate residue.

Nitrosoureas and nitrosocarbamate derivatives which are extremely active class of antitumor agents that is effective against solid tumors, as well as leukemia. In particular, 2-chloro ethyl derivatives and some of their metabolites show great promises as effective anti tumor agents\(^5\text{-}^6\). For the treatment of number of experimental and clinical tumors, several \( N-(2\text{-chloroethyl})\)-\( N \)-nitrosoureas have successfully been applied as chemotherapeutics agents. Not only do this drug show the ability to inhibit the growth and spread of many form of solid tumors in man and animals, but some of them, such as \( N,N \)-bis(2-chloroethyl)-\( N \)-nitrosourea \(5\) (BCNU) and \( N-(2\text{-chloroethyl})\)-\( N \)-cyclohexyl-\( N \)-nitrosourea \(3\) (CCNU), also have been found to rapidly enter the cerebrospinal fluid and control meningeal tumor implants. As a result they have been used in the treatment of brain tumors and menigeal leukemia. The drug decomposes spontaneously in the body to form two active compounds and alkylaing agents and a carbamolyting agent. The organic isocyanate which is formed carbamoylates lysine residue in proteins and may inactive DNA repair enzymes. The alkylating agent reacts initially with the O-6 position of a guanine moiety in one strand of DNA, then with the N-3 position of cytosine in the other strand to produce interstrand cross linking. Nitrosourea or nitrosocarbamates which utilized either a quinoline or quianazoline ring as a carrier group were synthesized and evaluated for anticancer activity.

During a random screening program of anticancer agents conducted at the Chemotherapy National Service Center (CCNSC) compound 1-methyl-3-nitro-1-nitrosoguanidine\(^7\) \(1\) showed very weak antileukemic activity\(^8\text{-}^9\). Assay of analogs of this compound led to the discovery of the antitumor activity of 1-methyl-1-
nitrosourea\textsuperscript{10} 2, was tested and shown\textsuperscript{8} to be more effective than 1 in increasing the life span of mice with ip-inoculated leukemia. It was soon discovered that introduction of a 2-chloroethyl chain on the nitrogen atom bearing the nitroso group (CNU$s$) led to much increased activity\textsuperscript{11}. These chloroethyl derivatives were lipophilic enough to cross the blood–brain barrier and therefore were useful in the treatment of brain tumors, which led to the synthesis of a large number of nitrosoureas, including lomustine 3 (CCNU) and its methyl derivative semustine 4, carmustine 5 (BCNU), nimustine 6 (ACNU), the water-soluble tauromustine 7 and fotemustine 8, but toxicity problems have prevented their widespread use. In 1967, streptozotocin (streptozocin) 9, a hydrophilic natural nitrosourea, was isolated from a strain of \textit{S. achromogenes}. This compound was chosen as a lead because initial SAR studies suggested that hydrophilic nitrosoureas were more potent and less toxic, and a number of analogs, like chlorozotocin 10, were prepared. Currently, the most clinically important nitrosoureas are CCNU, BCNU, ACNU, and streptozotocin. Nitrosoureas have been widely studied from a mechanistic point of view.

![Image of chemical structures of some Nitrosourea derivatives.](image)

\textit{Figure 1. Chemical structures of some Nitrosourea derivatives.}

The presence of the nitroso group labilizes the nitrogen-carbon bond, leading to two electrophiles, an isocyanate 12 and a diazene hydroxide 14, which has been detected in some cases by electrospray ionization mass spectroscopy.\textsuperscript{12} This intermediate in
turn generates a diazonium salt $15^{13}$ (Fig. 2). Alkylation seems to be the main reaction responsible for antitumor activity, while carbamoylation takes place primarily on amino groups in proteins, leading to inhibition of several DNA repair mechanisms. $N$-Nitrosoamides and $N$-nitrosocarbamates, which can behave as alkylating (but not carbamoylating) agents have also antitumor activity, which supports the above statement.$^{14}$ The above mechanism was based mainly on studies of the thermal decomposition of nitrosoureas under anhydrous conditions,$^{13}$ but in water solution the reaction is much more complex and has been explained by the mechanism shown in Fig. 3. Addition of a molecule of water to the nitrosourea, in its tautomeric form,$^{15}$ gives the tetrahedral intermediate $19$, which is decomposed into a primary amine, carbon dioxide, and $14$. This elimination requires an antiperiplanar conformation for $25$. Addition of a nucleophile other than water to the nitrosourea tautomer explains the isolation of carbamoylated products, formed by elimination of $14$.  

Most nitrosoureas contain one chloroethyl chain on the nitrosated nitrogen, which allows them to act as DNA cross-linking agents. Reaction of electrophilic diazonium species $25$ with guanine is assumed to take place on O-6 to give $27$. In fact, addition of O6-alkylguanine-DNA alkyltransferase, an enzyme that breaks O-6 guanine adducts, prevents cross-linking. This monoalkylated product reacts subsequently with the N-3 atom of the cytosine unit in the complementary DNA strand, by anchimeric assistance of the guanine N-1 atom through intermediate $28$, giving the cross-linked product 30 (Fig. 4).
In an alternative mechanism, intact nitrosourea molecules rather than diazonium species can directly alkylate DNA. Thus, nucleophilic attack of guanine O-6 to the nitrosourea tautomer 31 gives intermediate 33. Although alternative mechanisms have been proposed, according to labeling experiments it is probable that 33 cyclizes to the nitrosoisoxazolidine 34, which is attacked by another O-6 atom of a guanine unit.
neighboring in the DNA sequence to give 35. In this adduct, the O-6 of the first guanine is carbamoylated and the O-6 of the second guanine is alkylated with a 2-hydroxydiaoethyl group (Fig. 5). Diazonium generation and attack of N-3 from a cytosine of the opposite DNA strand, with anchimeric assistance from guanine N-1, finally gives the carbamoylated cross-linked product 36.

![Diagram of DNA cross-linking by nitrosoureas](image)

**Figure 5.** Alternative mechanism for DNA cross-linking by nitrosoureas.

Streptozotocin differs from other nitrosoureas in that it does not cross the blood–brain barrier because of its high hydrophilicity, and it also shows a relatively low myelosuppression because of decreased entry into bone marrow cells. Its main cytotoxicity is exerted on the pancreas β cells because their glucose carrier facilitates drug uptake to the islets. Therefore, the main applications of streptozotocin are the
induction of diabetes mellitus in experimental animals and treatment of islet cell pancreatic tumors, normally in association with nicotinamide for reasons that will be explained below. As expected from its nitrosourea structure, streptozotocin methylates DNA, specially at the guanine N-7 and O-6 positions, but there is also much evidence that shows that free radicals play an essential role in its cytotoxicity. It has been shown that streptozotocin induces the generation of nitric oxide, superoxide and hydroxyl radicals, and also that association with oxygen radical scavengers, such as nicotinamide, prevents streptozotocin induced cleavage of islet DNA.

6.2 Aliphatic analogs

Johnston et. Al. have synthesized a group of monosubstituted $N$-nitrosoureas, the substitution with either the methyl or 2-chloroethyl group, i.e. compounds 2 and 36, resulted in higher anticancer activity than either a substitution with longer carbon chains or an unsaturated group. Substitution at both nitrogen of the urea with the 2-chloroethyl moiety resulted in the bis($N$-2-chloroethyl)-$N$-nitrosourea (BCNU, carmustine, 5) which was found to be the most active agent of a large series of such analogs and more active than the N1 methyl analogs 37. Compounds 36 and 5 were clearly superior to 2 against both the ip and ic-implanted L1210 leukemia.

Same group have synthesized 1,3-bis(2-haloethyl)-1-nitrosoureas 38-42 for their anticancer activity, the bis-fluoroethyl (BFNU, 38) and 39 were active against both ip- and ic-implanted L1210 leukemia, whereas the bis-bromoethyl compound (BBNU, 40) and the bromo-chloro compound 41 were only active against ip-implanted L1210 and the bis-iodoethyl compound (BINU, 42) was inactive against both ip- and ic implanted L1210. The order of reactivity of the 2-haloethyl compound F, Cl > Br > I is reversed in the C-X bond strengths, i.e. I < Br < Cl < F. Thus, the iodo and bromo analogs 42 and 40 should be more susceptible to nucleophilic attack and, hence, could undergo decomposition in the plasma before reaching the interior of the cells.

Much attention has been devoted to the synthesis and biological testing of bisnitrosoureas. The first reported bis-$N$-methylnitrosoamides 43-45 were somewhat more active than the simple analog 37.
Similarly, the biological evaluation\textsuperscript{23,24,25} of the bis-\(N\)-(2-chloroethyl)-\(N\)-nitrosoureas \textsuperscript{46-50} against the rat leukemia L5222 revealed little change from that of the parent compound BCNU (\textsuperscript{5}), and no relationship could be found\textsuperscript{23,24} between the polymethylene chain length linking the CENU moieties and their anticancer activities.

Tauromustine (TCNU, \textsuperscript{51}), a analog of the amino acid taurine, as well as its two probable metabolites \textsuperscript{52} and \textsuperscript{53} were synthesized. The anticancer activity of compounds \textsuperscript{51-53} against L1210 leukemia, Walker mammary carcinoma, Lewis lung carcinoma, Harding-Passey melanoma, and colon carcinoma C was equal to or better than that of BCNU (\textsuperscript{5}), and several other \(N\)-nitrosoureas. Several nitroso-carbamate derivatives were synthesized and tested for anticancer activity\textsuperscript{26}. Compound \textsuperscript{54}, \textsuperscript{55} were found to be highly active against both the ip- and ic-inoculated L1210
6.3 Alicyclic Analogs

Early in the history of nitrosourea research it was discovered\textsuperscript{22} that the $N$-cyclohexyl-$N'$-(2-haloethyl)-$N'$-nitrosoureas (FCNU, 56) and CCNU (lomustine, 3) had excellent activities against both the ip- and ic-implanted L1210 leukemia, as was demonstrated by the number of survivors on day $^27$. As a result of this work a large number of alkyl-substituted cyclohexyl analogs 57-64 were synthesized and screened\textsuperscript{22,28} against the L1210 cell line by means of the log kill and therapeutic ratio ED50/ LD10 criteria. A number of analogs which included substituted cyclohexyl 57-60, cyclopentyl 61, methylcyclopentyl 62, and 2-indanyl 63, bornyl 64, were found\textsuperscript{22,28} to be highly active against both the ip- and ic-inoculated L1210, with their therapeutic ratios ranging from 0.28 to 0.77.

![Figure 7](image)

6.4 Aromatic analogs

Kim and co-worker have synthesized a large series of ortho-, meta-, and para-substituted phenyl analogs of $N$-methyl-$N$-nitrosourea and $N$-(2-chloroethyl)-$N$-nitrosourea and tested in vitro\textsuperscript{29,30} for inhibitory activity against the L1210 leukemia. However, the in vivo testing of various ortho-, meta-, para-substituted aryl
nitrosourea analogs and aryl bis-nitrosoureas revealed\textsuperscript{22,21,28,31,32} that only a few compounds such as 65-70 possessed activities against the ip-inoculated L1210, albeit all were found to be inactive against the ic-inoculated L1210. The conclusion drawn\textsuperscript{22} from these results was that an aromatic ring prevents the passage of these drugs across the blood-brain barrier.

Bigler et. al. have synthesized\textsuperscript{33} Several nitrosourea analogs of CCNU, 71-74 and tested for anticancer activity. The congeners 71, 72, and 74 had moderate and compound 73 high activity against the ip inoculated P388 and L1210 leukemias over a wide dose range. The 4-phenyl-4'-fluorophenyl analog 73 also exhibited high activity against solid cancers, including the B16 melanoma, colon adenocarcinoma, and Lewis lung carcinoma, but either low or no activity against the Harding-Passey melanoma and the ependymoblastoma brain tumor.\textsuperscript{33} the (2-chloroethyl)nitrosocarbamate have been synthesized and tested for anticancer activity. Compound 75 and 76 were showed very good in vitro activity against NCI-H23 (lung) and SNB-7 (CNS).

### 6.5 Heterocyclic analogs

Several \textit{para} substituted derivatives of phensuximide (77) were known to be good anticonvulsant agents,\textsuperscript{34} and a relationship was established\textsuperscript{35} between anticonvulsant activity and the ability to penetrate the central nervous system (CNS). On the basis of these results, several \textit{N}-nitrosourea analogs 78-82 of phensuximide were synthesized and tested\textsuperscript{36} as potential CNS anticancer agents. The 2-chloroethyl derivative 82 was the only analog with a significant activity of 96\% ILS against ip-L1210 in mice.\textsuperscript{36} The compound was found to have a moderate activity against the CNS cancer ependymoblastoma with a 140\% ILS, but the clinical drugs BCNU (5) and MeCCNU (58) were much more effective against both cancer lines. By contrast, the glutarimide analog 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1- nitrosourea (PCNU, 83) had a high activity against both the ip- and ic-inoculated L1210 in mice.\textsuperscript{37,38}

Substitution of the cyclohexyl ring of CCNU (3) with either piperidine, morpholine, or 2,6-dimethylmorpholine rings yielded the corresponding CENU semicarbazides 84, 85, and 86, respectively.\textsuperscript{39,40} The water-insoluble piperidine analog 84 was very active against the rat leukemia L5222 and Yoshida sarcoma in the rat similarly to the water-soluble morpholino congeners 85 and 86.\textsuperscript{39-41} The unsubstituted morpholine CENU 85 was more active than 86 against two neurogenic cancers but both were less active than
the clinical drug cyclophosphamide.\textsuperscript{41} A pyridine analogs \textsuperscript{87} were synthesized and tested\textsuperscript{39} for anticancer activity. The 3-picolyl analog \textsuperscript{87} was very active against P388 leukemia in vivo.

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Filippatos and co-workers have synthesized 8-quinolylnitrosourea 88 and various tricyclic xanthen-9-yl- and thioxanthen-9-ylnitrosoureas 89 and 90.\textsuperscript{42} The compound 89 and 90 was only weakly active against the P388 leukemia in vivo. Recently, Domarkas et. al. have synthesized several nitroso urea using 4-anilinoquinazoline\textsuperscript{5}. Compound 91 shown good EGFR TK inhibitor and presented an anomalously long half-life in serum-containing media (t\textsubscript{1/2} = 41 h). Reynolds et. al. have synthesized\textsuperscript{43} some (2-chloroethyl)nitrosocarbamate as potential anticancer alkylating agents. Compound 92a,b were found very good active against DLD-1 (colon) cancer cell lines.

**Figure 9**
6.6 Hydroxyalkyl Analogs

Eisenbrand and co-workers have synthesized water-soluble analogs 93-95 possessed dramatically different anticancer activities.\textsuperscript{23,24} Thus, the anticancer evaluation of 3-(2-hydroxyethyl)-1-(2-chloroethyl)-1-nitrosourea (HECNU, 93) resulted in 90% cures against the ip-inoculated rat L5222 leukemia and an 85% cancer weight reduction against sc-implanted Walker carcinoma. However, the corresponding figures for 94 and 95 were 10.5% and 5.0%, respectively. Further studies revealed\textsuperscript{24,25} that HECNU (93) was more active than BCNU (5) against both the ip- and ic-implanted L5222 leukemia. A number of esters 96 and ethers 97 and 98 of HECNU have been found\textsuperscript{44} to have high activity against the rat L5222 leukemia and rat glioma G616. In particular, the methanesulfonate analog HECNU-MS (96) possessed excellent antileukemic activity.

On the basis of this, Heal and co-worker have synthesized several polyhydroxy-CENU analogs 99-102 and displayed as high activity as that of BCNU (5) against the L1210 leukemia. The water-soluble polyhydroxy analogs were strongly myelosuppressive, as measured by the depression of peripheral blood neutrophil count on day three, the nadir of white blood cell suppression.\textsuperscript{45}
6.7 Carbohydrate Analogs

Several CENU analogs of 1-deoxyaldopentose, namely the ribofuranosyl-CENU (RFCNU, 103), ribopyranosyl-CENU (RPCNU, 104), and xylopyranosyl-CENU (XPCNU, 105) were synthesized and shown to have some activity in vivo against the L1210. In these studies the prolongation of survival (PS) values are given as $\infty$ when more than 50% of the animals are cured. These compounds were less toxic and had greater therapeutic indices than CCNU (3) or MeCCNU (58). Of all compounds tested, only RFCNU (103) was not immunosuppressive in the hemolytic plaqueforming cell (PFC) test, either before or after the addition of the antigen in the form of fresh sheep red blood cells administered ip to mice. The other compounds 104 and 105 were immunosuppressive whether given before or after the antigen.

Compound 106 had the highest %ILS and greatest reduction of the tumor volume of any member of this series as well as CCNU (3) and RFCNU (103). The percentage of mice developing the melanoma after 39 days was 42% for 106 and 65% for CCNU, but at 90 days the values were approximately 65% for both compounds.
**6.8 Amino acid and peptide analogs**

The structure-activity studies of \(N\)-2-(chloroethyl)- \(N\)-nitrosourea (CENU) analogs of hydroxyalkyl compounds, such as HECNU 93, and of carbohydrates indicated that such water-soluble analogs possessed greatly reduced bone marrow toxicity and improved therapeutic indices. The attachment of L-amino acids to the CENU moiety could add desirable hydrophilic properties.

Suami and other group have synthesized CENU amino acid primary amides 108-11351,52 and tested anticancer activity in vivo against the rat L5222 and murine L1210 leukemias. Among the primary amides the CENU sarcosinamide 112 was particularly attractive because of a combination of high anticancer activity of 711% ILS, very high chemical stability with a half-life of 330 min, and low toxicity with a LD50 of 392 mg/kg.458 The L-proline analog 113 also had high chemical stability and low toxicity but also a greatly reduced anticancer activity, so there was no obvious correlation between the % ILS and the chemical half-life values. The CENU L-serinamide (111) had excellent therapeutic indices equal to 40.51

A large number of CENU analogs of carboxylic acids and amides of amino acids 114, 115, dipeptides 116, 117, 118, and tripeptides 119, 120 were screened for in vivo activity against three transplantable mouse adenosarcomas of the colon (MAC)53-55 and MNU induced mammary carcinoma56. The amide derivatives 114, 115, and 117 had higher activity against the sc-administered solid tumors MAC 13 and MAC 26 than the acid analogs 115. The free acid dipeptide analog CENU-Ala-Ala (115) was highly active against the ascitic MAC 15A tumor line but was either weakly active or inactive against the sc-administered MAC 13 and MAC 26 tumor lines.53,54
6.9 Steroid Analogs

The discovery of estrogen receptors (ER) in human breast cancer has led to significant progress in the management of the disease. A series of androgen-linked nitrosocarbamates which are related to the estrogen analog estramustine (121) were synthesized. From this series the \( N \)-(2-chloroethyl)-\( N \)-nitrosocarbamate of 19-nortestosterone (122) was studied in detail. Compound 122 exhibited excellent in vitro activity against the L1210 leukemia but it had only a low in vivo activity against the L1210, Ehrlich ascites, and Walker carcinoma. Compound 122 possessed alkylating but no carbamoylating properties. The administration of 122 caused a dose-dependent reduction of the growth of dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats, and a greater reduction of tumor growth and tumor DNA synthesis than either administration of 19-nortestosterone or CCNU (3).

Carroll et. al. have synthesized\(^{59}\) steroid-linked \( N \)-nitrosourea 123 caused, at a daily dose of 40 mg/kg, an 80-100% inhibition of a rat mammary tumor. The first estrogen-linked \( N \)-nitrosoureas 124, 125 were synthesized and tested anticancer activity. The CENU analogs 124 at a daily dose of 40 mg/kg, were found\(^{60}\) to inhibit the growth of DMBA-induced rat transplantable mammary cancer by factors of 85% and 100%, respectively whereas the corresponding \( N \)-methyl-\( N \)-nitrosoureas 125 at the same daily doses inhibited the cancer growth by only 23% and 15%, respectively.
6.10 Nucleoside Analogs

Most of the reported work on the nucleoside compounds has focused on the attachment of the \(N\)-nitrosourea group to the 3'- and 5'-positions of the carbohydrate portion of the molecules. The C3' \(N\)-methyl- and \(N\)-chloroethyl-\(N\)-nitrosourea containing compounds 126 and 127 had approximately an equal growth inhibitory effect on the H.Ep-2 cells while the C5' \(N\)-chloroethyl analog 129, but not the \(N\)-methyl analog 128, was shown to have good in vitro activity. No clear correlation was found between the cytotoxicity of compounds 126-129 and their carbamoylating and alkylating activities relative to BCNU (33). Thus, the C3' compounds 126 and 127 had widely differing alkylating activities but nearly equal growth inhibitory properties. The C5' analogs 128 and 129 had low alkylating activities but only 129 was cytotoxic.
6.11 Aim of the current work

The goals of the current study are finding new anticancer agents with high therapeutic efficacy, low toxicity, good bioavailability, and effective against various multi-drug resistant tumor cells. In the past years, we have focused on the research and development of DNA-alkylating agents as anticancer agents. The following class of DNA-alkylating have been designed and synthesized for antitumor evaluation:

I. Synthesis and characterization of several new (2-chloroethyl) nitrosocarbamates derivatives

The chemical synthesis and characterization of Nitrosocarbamate derivatives are described in the Chapter 7.