λ_{max} 227 nm (ϵ 4.1 × 10^4), 260 (5.5 × 10^4), 307 (2.1 × 10^4); MS calcd for $C_{25}H_{26}O_2$, 358.1933; found, 358.1938.

Biological Assays. The experimental protocols used for the reversal of keratinization assay and the inhibition of the induction of ornithine decarboxylase assay were essentially those described by the groups of Sporn[3a] and Verma and Boutwell,[6] respectively.

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Registry No. 1, 7567-87-5; **2**, 56013-01-5; **3** (*E* isomer), 86471-12-7; **3** (*Z* isomer), 86471-17-2; 4 (*E* isomer), 86471-13-8; **5**, 27452-17-1; **6**, 86471-14-9; **7**, 86471-15-0; 8, 86471-16-1; ornithine decarboxylase, 9024-60-6.

An Analysis of 1-(2-Chloroethyl)-1-nitrosourea Activity at the Cellular Level†

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The effect of five different 1-(2-chloroethyl)-1-nitrosoureas on the growth of cultured P388 cells has been analyzed in terms of physical, chemical, and kinetic parameters that are related to the mechanism of action of this class of cancer chemotherapeutic agent. This study correlates structure with activity at the cellular level by using a dose function that is related to the amount of active species, the (2-chloroethyl)diazonium ion, that is formed during the period of exposure of cells to drug rather than to the initial drug dose. 1-(2-Chloroethyl)-1-nitrosourea analogues that rapidly enter the P388 cells are shown to have the same activity relative to the amount of active species formed. When analyzed in this way, activity is not influenced by the structure of the N-3 substituent, lipophilicity, or carbamoylating activity. The agents 1-(2-chloroethyl)-1-nitrosourea (CNU), 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (PCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) all produce a 50% cell growth inhibition at 6 to 7 μM active species formed per cell volume. Chlorozotocin required a twofold higher effective dose to produce the same toxic effect. This decreased activity is attributed to the slow uptake of the water-soluble chlorozotocin into P388 and L1210 cells relative to the rate of chlorozotocin conversion to active species in medium. The yields to 2-chloroethanol from CNU, BCNU, and chlorozotocin were shown to be the same, indicating that these agents generate the same yield of alkylating intermediate at 37 °C and pH 7.4.

The 1-(2-chloroethyl)-1-nitrosoureas are a class of chemotherapeutic agents with demonstrated clinical activity against a variety of malignant diseases,[1-3] particularly brain tumors[4,5] and lymphomas.[6] The mechanism of action of these agents involves the chemical conversion of the parent nitrosourea to active alkylating species, the (2-chloroethyl)diazonium ion, and substituted isocyanates.[7-9]

Quantitative structure–activity relationships have been developed for a large number of 3-substituted 1-(2-chloroethyl)-1-nitrosoureas by using intraperitoneally and intracerebrally inoculated L1210 mouse leukemia[10-12] and Lewis lung carcinoma.[13] These studies show that in vivo activity correlates with only one physical parameter, lipophilicity, and structural indicators. A further examination of 17 active nitrosoureas suggested that carbamoylating activity, as well as lipophilicity, contributed to the toxicity in L1210 leukemic mice. Alkylating activity was thought to be a major factor in determining the quantity of compound required to give a therapeutic response.[14] Subsequent studies demonstrated a significant correlation between in vivo toxicity and alkylating activity; no correlation was found with carbamoylating activity, however. Neither parameter showed any correlation with antitumor activity.[15] The ultimate biological effect of the 1-(2-chloroethyl)-1-nitrosoureas is now thought to result from covalent binding of alkylating and carbamoylating species in cellular macromolecules. There is much evidence that the antitumor effect is due to alkylation and cross-linking of DNA by (2-chloroethyl)diazonium ion.[16-20] The

† This work has been presented in part. See *Proc. Am. Assoc. Cancer Res.* **1982**, *23*, 163.

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Table I. Chemical and Biological Properties of 1-(2-Chloroethyl)-1-nitrosoureas

compd (NSC no.)	k_2,[a] min^{-1}	alkylating act.[b]	carbamoylating act.[b]	log P[a]	ED_{50} C_0,[c] μM	ED_{50} ΔC,[d] μM
CCNU (79037)	0.013	100	100	2.8	13.0	6.7
BCNU (409962)	0.014	260-300	70	1.5	13.3	6.3
CNU (97547)	0.53	1000	20-60	0.57	6.0	5.7
PCNU (95466)	0.027	360	24	0.37	8.1	6.5
chlorozotocin (178248)	0.033	450-640	2-4	-1.02	17.0	11.7

[a] From ref 25. [b] A range of values relative to CCNU (= 100) from ref 15 and 26. [c] Results are the mean value for the exposure of P388 cells to the drug for a 60-min period. Values for PCNU were extrapolated from 40-min exposure experiments. [d] Results are the mean value for the exposure of P388 cells to the drug following a variety of treatment schedules at pH 7.4 and 37 °C. The percent standard deviations were less than 35%.

isocyanates formed have been found to inhibit DNA repair.[21]

In the present study the cytotoxic activity of five (2-chloroethyl)nitrosoureas, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-1-nitrosourea (CNU), 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (PCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and chlorozotocin, was determined against P388 cells in culture. The cytotoxicity was analyzed with respect to chemical and physical properties in an effort to define those parameters that contribute to the toxic effect at the cellular level. Data were analyzed by using a dose function that contained the following terms: initial drug concentration, duration of exposure of the cell to the drug, and the rate of conversion of parent compound to active alkylating intermediate.[22] Physical properties of the drug that may affect the rate of partitioning between medium and the cell interior were also considered. Results show that for lipophilic 1-(2-chloroethyl)-1-nitrosourea analogues the toxic effect was related to the amount of active species generated during the exposure period and was not related to parent drug structure. The water-soluble analogue chlorozotocin was found to be less active than lipophilic analogues. This may be due to its slow cellular uptake.

Results and Discussion

1-(2-Chloroethyl)-1-nitrosourea analogues were analyzed for activity by an assay in which suspended P388 or L1210 cells were treated with drug by using different treatment schedules, either varying the exposure period of a given drug concentration or varying the concentration over a given period of exposure. The assay end point was the increase in cell number of exponentially growing cells that occurred within 48 h of treated relative to nontreated cells. Data are reported as ED_{50} values, the dose that produces a 50% decrease in cell number.

It was observed that the relative activity of these agents appeared to depend on the treatment schedule that was followed. Thus, CNU activity approached that of other 1-(2-chloroethyl)-1-nitrosoureas as the incubation period increased. Representative cytotoxicity curves are shown in Figure 1 where graded concentrations of CNU, PCNU, and chlorozotocin are incubated with cells for 40 min. CNU appears to be significantly more active than PCNU and chlorozotocin. A different picture emerges if the cell culture cytotoxicity data are analyzed according to a dose function that combines initial concentration (C_0), exposure time (t), and chemical activation rate constants (k_2)[22] (Figure 2). This function, eq 1, is consistent with the kinetic model shown in eq 2, where C and C' represent the

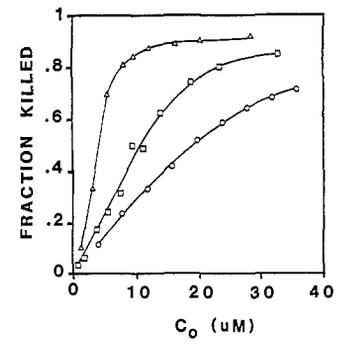


Figure 1. The fraction of P388 mouse leukemia cells killed after a 40-min exposure to various concentrations of CNU (△), PCNU (□), and chlorozotocin (○) at pH 7.4 and 37 °C is plotted against initial concentration of parent drug. Each curve represents the average of two experiments.

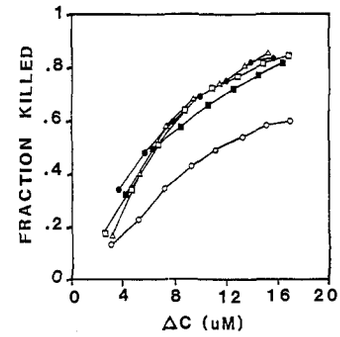
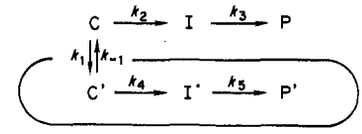


Figure 2. The fraction of P388 mouse leukemia cells killed after exposure to BCNU (●), CNU (△), PCNU (□), CCNU (■), and chlorozotocin (○) following a variety of cell treatment schedules at pH 7.4 and 37 °C are plotted against ΔC dose function. Each curve represents the average of at least four experiments.

drug concentration in medium and in the aqueous portion of the cell interior. 1-(2-Chloroethyl)-1-nitrosoureas are

$$\Delta C = C_0(1 - e^{-k_2 t}) \quad (1)$$



(2)

not active agents but react in a first-order chemical reaction to give active alkylating intermediates, I and I', and alkylation or rearrangement products, P and P'. Since I is a (2-chloroethyl)diazonium ion, k_3 and k_5 are very large in aqueous solution, and k_2 and/or k_4 are the rate-controlling step for activation. The values of k_2 measured in aqueous buffer or human serum at pH 7.4 and 37 °C are shown in Table I.

Lipophilic agents are known to partition into suspended cells rapidly so that the concentration of drug in the cell

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interior equals that of the medium, $C' = C$, within a few seconds.[23] Partitioning of agents into cell lipids in suspension culture does not, in contrast to the tissue environment, decrease free drug concentration or affect the activation of the 1-(2-chloroethyl)-1-nitrosoureas. In suspended cell cultures, the medium has a volume approximately 4500 times greater than the cell so that only a small fraction of drug would partition into lipid membranes. 1-(2-Chloroethyl)-1-nitrosoureas do not react in the lipid matrix.[24] The ΔC term can be used to represent the amount of active species formed during the period of exposure for drugs that have the above properties. This term may be related to the biological activity of the agent if the intracellular rate constant, k_4, is equal to or proportional to k_2, the rate constant measured in media. Correlations using different treatment schedules and different 1-(2-chloroethyl)-1-nitrosoureas have been described.[22]

When the ΔC term is used as the dose function, four of the five analogues have the same response curves (Figure 2). In each case the ED_{50} value is 6 to 7 μM, indicating identical activity at the cellular level. Chlorozotocin is twofold less active with an ED_{50} of 12 μM. The ΔC ED_{50} and C_0 ED_{50} terms may be related to various chemical and physical parameters (Table I).

The alkylating activity, measured by the extent of alkylation of 4-(4-nitrobenzyl)pyridine that occurs within 120 min at 37 °C and pH 6, has been reported for the 1-(2-chloroethyl)-1-nitrosoureas.[15,26] Alkylating activity represents both the rate of decomposition under these conditions and the yield of alkylating species formed during the decomposition. Both BCNU and CCNU are reported to give equivalent amounts of 2-chloroethanol, the major product formed from (2-chloroethyl)diazonium ion decomposition in aqueous solution. Other identified products were also derived from the diazonium ion, indicating that this reaction pathway accounts for at least 95% of the decomposition reaction.[7,27] It appears that the extent of alkylation measured at pH 6 using the 4-(4-nitrobenzyl)-pyridine procedure does not necessarily correspond to alkylation measured at pH 7.4, since CCNU and BCNU are reported to have significantly different alkylating activity when this procedure is followed. It follows that a comparison of ED_{50} values with alkylating activity (Table I) may not be relevant.

Carbamoylating activity is a measure of the amount of product formed upon incubation of [^{14}C]lysine with the nitrosourea for 6 h at 37 °C.[15,26] All of the 1-(2-chloroethyl)-1-nitrosoureas studied decompose to different isocyanates and have different carbamoylating activities. There is no correlation between ED_{50} C_0 and carbamoylating activity. Four of the five agents have the same ED_{50} ΔC, yet they represent a wide range of carbamoylating activity relative to CCNU (= 100). This broad range of carbamoylating activity suggests that the structure of the isocyanate is not important for activity at the cellular level.

A parameter that may effect the activity of 1-(2-chloroethyl)-1-nitrosoureas is lipophilicity. CNU and PCNU have log P values of 0.57 and 0.37, respectively, and

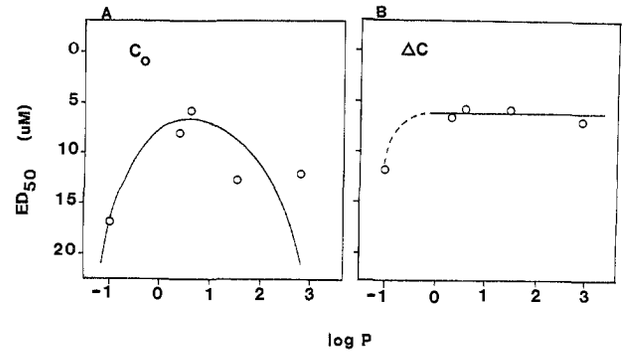

Figure 3. (A) The ED_{50} C_0 values and (B) the ED_{50} ΔC values are plotted against log P, where P is the octanol–water partition coefficient of the drug.

are the most active when considering the ED_{50} C_0 term (Figure 3a). The optimum log P using the ED_{50} C_0 plot is between 0.5 and 0.6, which is consistent with in vivo data obtained from intraperitoneally implanted L1210 cells.[28] If the ΔC term is used to measure dose, the apparent low activity of the more lipophilic analogues BCNU and CCNU shown in the ED_{50} C_0 curves is found to be an artifact of their slower activation rate (Figure 3B). Thus, the activity of the lipophilic 1-(2-chloroethyl)-1-nitrosourea analogues correlates with the amount of active species formed, not with parent structural parameters.

Chlorozotocin has a lower log P value, -1.02, and is significantly less active than the other analogues. The lower activity observed for chlorozotocin was further analyzed by repeating the cytotoxicity assay with L1210 leukemia cells. The same ED_{50} ΔC values were obtained: 6.6 ± 1.2 μM for BCNU and 12.2 ± 3.3 μM for chlorozotocin.

To determine if the yield of alkylating product formed from chlorozotocin was significantly different than the other 1-(2-chloroethyl)-1-nitrosoureas, we analyzed the amount of 2-chloroethanol, the major decomposition product of (2-chloroethyl)diazonium ion, by gas chromatography for BCNU, CNU, and chlorozotocin. All agents were found to yield 45% 2-chloroethanol, suggesting that the lower activity observed with chlorozotocin is not due to competing decomposition reactions.

The less lipophilic analogue chlorozotocin may not follow the proposed dose function. Reports indicate that chlorozotocin cellular uptake is slower than BCNU. Use of the ΔC dose function requires that equilibrium between cytosol and medium occurs before significant amounts of drug decompose. A study of the time course of uptake of intact BCNU and CCNU in L5178Y cells, by TLC analysis of extracellular and intracellular drug concentration, showed that equilibrium was established within a minute.[29] In these same cells, the ratio of intracellular chlorozotocin concentration to extracellular concentration was still increasing at 60 min.[30] Similar results were found for chlorozotocin in L1210 cells.[31] If this is the case, a significant amount of chlorozotocin can decompose in the medium before entering the cell, leaving less drug available for intracellular alkylation. This could explain the lower observed activity.

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We interpret these data to indicate that all of the lipophilic analogues at the cellular level have an activity related to the amount of active species formed within the cell and that this activity may be essentially independent of the structural parameters of the parent compound. This property would be consistent with the random chemical reaction of the parent drug in the aqueous environment of the cell to liberate the active alkylating intermediate. The parent drug structure does not influence activity, which suggests that there is no significant binding of parent drug to cellular macromolecules in the cytotoxic process. Interactions of this type are expected to be structure specific. The observation that 1-(2-chloroethyl)-1-nitrosourea analogues have the same activity at the cellular level allows the observed differences in in vivo activity to be explained primarily by differences in biodistribution. For a better understanding of the structure–activity relationship at the cellular level, the amount of active species formed during the exposure period can be considered for agents that act through a common intermediate. This approach may provide new insight into the understanding of structure–activity relationships.

Experimental Section

Chemicals. BCNU, CCNU, PCNU, CNU, and chlorozotocin were obtained from Dr. Robert Engle of the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, and stored at −20 °C. Drug solutions were prepared immediately before use by dissolving samples in ethanol and then diluted to the desired volume with distilled water.

Cells. P388 and L1210 mouse leukemia cells were obtained from EG and G Mason Research Institute. The cell lines were maintained in suspension culture by using Fischers growth medium supplemented with 10% (v/v) horse serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin G. Stock cultures were grown in stationary bottles at 37 °C, under 5% CO_2/humidified air, and maintained by diluting to 5×10^4 cells/mL every 3 to 4 days with fresh media. Approximately 24 h prior to treatment, cells were planted in 250-mL spinner flasks at a density of 3×10^5 cells/mL. Experiments were performed on cells in log phase growth (P388 doubling time, 10 to 11 h; L1210 doubling time, 7 to 8 h).

Exposure of Cells to 1-(2-Chloroethyl)-1-nitrosoureas. Immediately prior to the experiment, the cells were counted on a hemocytometer and adjusted to a concentration of 1×10^6 cells/mL. Two different types of experiments were conducted, either varying the initial drug concentration at a defined exposure period or varying the exposure period, during which a defined initial drug concentration is incubated at 37 °C and pH 7.4. In the first set of experiments, 2.0 mL of cells was added to a series of sterile glass tubes capped with a rubber stopper. Drug was added in varying amounts to duplicate tubes and allowed to incubate for the given exposure period. No drug was added to the first four tubes; however, an amount of ethanol/water equivalent to that used in the drug solution was added for the incubation period (less than 0.1% ethanol). After the exposure period, a 0.27-mL aliquot of cells from each tube was diluted in 3.8 mL of fresh media to attain a final cell concentration of 6.6×10^4 cells/mL. Cells were incubated at 37 °C for 48 h and then counted on a Coulter counter. The fraction of cells killed after exposure to the drug relative to untreated cells is correlated to the initial concentration of drug and to the amount of active alkylating species formed in the exposure period. In the second set of experiments in which the exposure time rather than initial concentration was varied, a known concentration of drug was added to 18.9 mL of cells. At various time intervals, duplicate 0.27-mL aliquots were removed and added to 3.8 mL of fresh medium. Blanks were taken from the same cells before drug was added and diluted as described above. The fraction of cells killed after exposure to drug is correlated to the time of exposure for a given initial concentration and to the amount of active alkylating species formed during each exposure period.

1-(2-Chloroethyl)-1-nitrosourea Conversion to 2-Chloroethanol. A stock solution of chlorozotocin, BCNU, or CNU dissolved in Me_2SO was added to preheated (37 °C) 0.025 M phosphate buffer to give a final concentration of 7.5 mM. Reactions were carried out in a septum capped vial at 37 °C for 2.5 to 4 h or until 90% decomposition has occurred. Ethanol was used as an internal standard. At the end of the reaction period, an aliquot was removed and analyzed by gas chromatography. 2-Chloroethanol was quantified from the peak height ratios by reference to a standard 2-chloroethanol/ethanol curve.

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Registry No. 1-(2-Chloroethyl)-1-nitrosourea, 2365-30-2; 1,3-bis(2-chloroethyl)-1-nitrosourea, 154-93-8; 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea, 13909-02-9; 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, 13010-47-4; chlorozotocin, 54749-90-5.

Antidiabetic Activity of Some 1-Substituted 3,5-Dimethylpyrazoles

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Several new 1-substituted 3,5-dimethylpyrazoles were prepared for testing as hypoglycemic agents. A number of these containing para-substituted 1-carbonylphenylurea and para-substituted 1-carbamoylbenzenesulfonylurea derivatives were found to possess potent hypoglycemic activity.

At present, it has been estimated that the incidence of persons with diabetogenic genes stands at one in every four and that the rate of increase of diabetes is approximately three times that of the population in general.[1] If this trend continues, the problems of diagnosis and treatment of the disease and accompanying cardiovascular problems will also increase.

Grunwald[2] stated that there is still hope and need for oral hypoglycemic agents with mechanisms of action other than those of the compounds presently available. Preliminary work on animals revealed that 5-methylpyrazole-3-carboxylic acid derivatives very nearly approached this goal.[3-5]

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