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A STRUCTURE–ACTIVITY STUDY OF SEVEN NEW WATER SOLUBLE NITROSOUREAS*

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Abstract—The *in vitro* alkylating activity, carbamoylating activity, decomposition rates and octanol–water partition coefficients (Log P) of seven water soluble chloroethylnitrosourea antitumor agents and a reference lipid soluble analog were correlated with their biological activities in mice. The alkylating activity of each compound demonstrated a significant inverse linear correlation with both the decomposition rate in 0.1 M sodium phosphate buffer, pH 7.4 ($r = -0.92$, $P < 0.01$), and the molar LD_{10} dose ($r = -0.87$, $P < 0.01$). A direct relationship was found between the Log P values and both the alkylating activity ($r = -0.86$, $P < 0.01$) and the molar LD_{10} dose ($r = 0.77$, $P < 0.025$). However, the addition of the variable, Log P, in multiple regression analysis did not contribute significantly to any of the direct correlations of chemical parameters with biological variables. In comparison, carbamoylating activity did not function as an independent variable for the relative myelotoxicity or lethality of each compound. All water soluble drugs except for chlorozotocin and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea, the two analogs with glucose carriers, produced a significant reduction in circulating neutrophils at their respective LD_{10} doses. There was no correlation between relative myelotoxicity and alkylating activity, carbamoylating activity or Log P. The glucose moiety appears to function as an independent variable for reducing nitrosourea cytotoxicity to bone marrow cells without significantly altering antitumor activity.

The chloroethyl nitrosoureas are now recognized as an important class of antitumor agents that have established clinical antitumor activity for a broad range of human malignancies [1]. However, these same agents produce delayed and cumulative bone marrow toxicity which limits seriously their clinical application [2, 3].

The biochemical pharmacology of the nitrosoureas has been the subject of extensive study. Under physiological conditions, these compounds undergo spontaneous decomposition [4, 5]. The chemical half-lives of the individual nitrosoureas in 0.1 M sodium phosphate buffer, pH 7.4, vary from 5 min to as long as 2 hr [6]. In the process of decomposition, a series of alkylating moieties are formed of which an alkyldiazohydroxide precursor and the chloroethyl carbonium ion are considered to be the most important. Organic isocyanates are also generated which carbamoylate the lysine residues of proteins [7, 8].

In addition to their spontaneous chemical dissociation, the nitrosoureas are now known to be metabolized by the liver microsomal mixed function oxidase system [9–13]. In the case of CCNU‡ the ring structure is hydroxylated rapidly. The major metabolite in rodents is the *cis*-4 hydroxy form which accounts for approximately 50 per cent of administered CCNU [9]. Studies of metabolism in man have been conducted using CCNU given by slow intravenous infusion. Walker and Hilton [10] found that approximately 75% of the plasma drug concentration, after 1 hr, was in the *trans*-4 and *cis*-4 hydroxylated forms, and that hydroxylation proceeded faster than the drug infusion rate. Current data indicate that the rate of metabolic hydroxylation of CCNU exceeds the rate of chemical dissociation and that, as a result, it is probable that the hydroxylated metabolites are the immediate precursors of the toxic moieties.

The demonstration that reduced myelosuppression in man and animals [14, 15] was associated with the attachment of the methyl nitrosoureido group to a glucose moiety led to the synthesis of chlorozotocin, in which the more active chloroethyl cytotoxic group is also linked to a glucose carrier [16]. This compound has curative antitumor activity for murine L1210 leukemia. Unlike other chloroethylnitrosoureas in active clinical use, chlorozotocin is water soluble, possesses low carbamoylating activity, and is non-myelosuppressive when given at an LD_{10} dose in mice [17]. To evaluate the chemical features of chlorozotocin that may contribute to its bone marrow sparing effect, in particular the property of water solubility, structure–activity studies have been carried out with six new water soluble nitrosoureas with varying carbamoylating activities. GANU, a C-1 substituted glucose derivative [18], and ACNU, a pyrimidine analog, are two compounds currently in clinical trial in Japan [19]. DSI incorporates an inositol carrier moiety while TE6

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‡ Abbreviations used: Log P: logarithm of the distribution coefficients in an octanol–water system; LD_{10}: dose resulting in death of 10 per cent of normal mice; GANU: 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea; ACNU: 1-(2-chloroethyl)-3-(4-amino-2-methylpyrimidine-5-yl)methyl-1-nitrosourea; CCNU: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; ED_{50}: dose causing 50 per cent of mice to survive at least 45 days after i.p. implantation of 10^5 L1210 cells; DSI: 1-(2-chloroethyl)-3-(1-deoxyscyllo-inositol)-1-nitrosourea; *cis*-2-OH CCNU: 1-(2-chloroethyl)-3-(*cis*-2-hydroxycyclohexyl)-1-nitrosourea; *trans*-2-OH CCNU: 1-(2-chloroethyl)-3-(*trans*-2-hydroxycyclohexyl)-1-nitrosourea; TE6: (1,2,3,4/5)-5-[3-(2-chloroethyl)-3-nitrosoureido]-1,2,3,4-cylopentantetrol; and chlorozotocin: 1-(2-chloroethyl)-3-(2α-D-glucopyranosyl)-1-nitrosourea.

is a cyclopentanetetrol derivative. Two isomers of CCNU, the *trans*-2 and *cis*-2 hydroxylated derivatives, were also evaluated. Both have been shown to have greater water solubility than CCNU and on a molar basis to be more active against L1210 leukemia than the parent compound [20].

In the present structure–activity study, the contribution of water solubility, alkylating activity, carbamoylating activity and the decomposition rate of these seven water soluble and one reference lipid soluble compounds have been correlated by linear regression analysis with their essential biological activities: lethal toxicity, bone marrow depression and antitumor activity.

MATERIALS AND METHODS

Animal studies

Male BALB/c X DBA/2 F_1 mice (hereafter called $CD2F_1$) weighing 17–25 g, and maintained on Lablox Laboratory chow pellets and water *ad lib.*, were used throughout. Each group consisted of ten mice.

The following drugs were investigated: chlorozotocin (NSC 178248), GANU (NSC D254157), DSI (NSC 275618), CCNU (NSC 79037), *cis*-2-OH CCNU (NSC 253946), *trans*-2-OH CCNU (NSC 253947), ACNU (NSC D245382) and TE6. CCNU, chlorozotocin, GANU and ACNU were kindly supplied by Dr. Harry Wood, Drug Development Branch, National Cancer Institute, Bethesda, MD. TE6 was obtained from William Bradner of Bristol Laboratories Syracuse, NY. The hydroxylated CCNU derivatives were synthesized by Dr. George S. McCaleb and provided by Dr. Thomas P. Johnston of the Southern Research Institute, Birmingham, AL.

CCNU was suspended in an aqueous solution of 5.0% (v/v) polyethoxylated vegetable oil, 5.0% (v/v) ethanol and 90% (v/v) 0.01 M citrate buffer, pH 4.0. The hydroxylated derivatives were dissolved in a 5.0% (v/v) alcohol solution in 0.01 M citrate buffer, pH 4.0. The other nitrosoureas were soluble in 0.01 M citrate buffer, pH 4.0. All drugs were given intraperitoneally as a single dose in a volume of 0.1 ml/10 g body weight.

The mouse L1210 leukemia system was used to assess antitumor activity. Treatment was administered as a single LD_{10} dose (dose resulting in death of 10 per cent of normal mice) on day 2 after intraperitoneal (i.p.) implantation of 1×10^5 L1210 leukemia cells. The percentage increase in life span was calculated from the survival of control tumor-bearing animals that received appropriate volumes of the respective diluants [21]. Groups of ten normal $CD2F_1$ mice received a single i.p. dose of drug. Serial white cell counts were performed over a 28-day period: a 20 μl sample of retro-orbital sinus blood was placed in 9.98 ml Isoton (Curtin Matheson, Washington, DC) and counted in a model ZB1 Coulter Counter after lysis of red blood cells with Zapoglobin (Curtin Matheson, Washington, DC). White blood cell differential counts were performed on Wright-stained blood smears taken on day 3 following treatment. This point was chosen because it corresponded with the nadir of the white blood cell count after nitrosourea treatment in this species [17]. The absolute neutrophil counts are presented as a per cent of control values.

BIOCHEMICAL STUDIES

Alkylating activity. The *in vitro* alkylating activity was measured by the method of Wheeler *et al.* [22], which depends on the capacity of the drug to alkylate 4-(*p*-nitrobenzyl) pyridine (N.B.P.) to the quarternary pyridinium which is highly colored in an alkaline medium. A 10 mM solution of each nitrosourea was freshly prepared in acetone. The acetone insoluble drugs, ACNU and DSI, were dissolved in 0.025 M acetate buffer, pH 6.0, and 0.5 to 2.0-μmole aliquots were incubated with 4.0 ml of 0.025 M acetate buffer, pH 6.0, and 1.5 ml of 5% (w/v) N.B.P. reagent in acetone (Fisher Chemicals) at 37° for 2 hr. At the end of the incubation, the reaction mixture was placed on ice in subdued light, 2 ml of acetone, 3 ml of ethylacetate and 1.5 ml of 0.25 N NaOH were added, and the test tube was shaken for 10 sec and then centrifuged at 3000 rev/min for 10 sec. The absorbance of the colored ethyl acetate layer was determined at 540 nm in a Coleman Junior II spectrophotometer. The μmoles of drug giving an O.D. of 0.5 was taken as the alkylating activity and this was expressed as a percentage of the value for CCNU, the compound with the lowest alkylating activity. As a result, the more potent alkylators have higher percentage figures.

Carbamoylating activity. Two μCi of [^{14}C]lysine (specific activity 300 mCi/m-mole) was diluted with 21 μmoles of unlabeled lysine in 200 μl of 0.1 M sodium phosphate buffer, pH 7.4, to give a specific activity of 95 μCi/m-mole. Then 4.2 μmoles of this mixture was added to 4.2 μmoles of the nitrosourea in 160 μl equal volumes of ethanol and 0.1 M sodium phosphate buffer, pH 7.4. The reaction mixture without drug served as the control. After incubation at 37° for 6 hr 10 μl of each sample was spotted on Whatman No. 3 chromatographic paper pre-wetted with 0.1 M sodium phosphate buffer, pH 6.0. The carbamoylated lysine was separated from unreacted lysine and drug by electrophoresis at 3000 volts for 75 min in a Savant high voltage flat plate tank using 0.1 M sodium phosphate buffer (pH 6.0). The dried paper was cut into 1 inch squares, immersed in 10 ml aqueous counting scintillant (Amersham/Searle, Arlington Heights, IL) and assayed for radioactivity in a Searle Mark III liquid scintillation counter that gave an efficiency for carbon 14 of 92 per cent. We have demonstrated previously that the peak of radioactivity not associated with the free [^{14}C]lysine results from the carbamoylated products [23]; these counts, expressed as a percentage of the total radioactivity, were termed the carbamoylating activity of the compound.

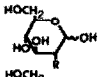
Chemical half-life

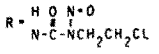
The half-life of the compounds in minutes was measured by following the change in absorbance at 230 nm during incubation at 37°. Solutions of the soluble drugs were made in 0.1 M sodium phosphate buffer, pH 7.4, to give an initial absorbance between 0.5 and 1.0 at 230 nm. CCNU was first dissolved in absolute ethanol and then diluted 20-fold with the sodium phosphate buffer, pH 7.4.

Octanol–water partition coefficient (Log P)

The logarithms of the distribution coefficients, in an octanol–water system, for chlorozotocin, CCNU, *cis*-

Table 1. Chemical and biological activities of water soluble nitrosoureas

Drug	Structure	Log P+	Alkylating Activity % Chlorozotocin	Carbamoylating Activity % ^{14}C-Lysine	$T_{\frac{1}{2}}$* Mins	LD_{10}† μmoles/kg	Absolute‡ Neutrophil Count % Control	ILS // % Control
Chlorozotocin		-1.02	100	2.0	48	64	110	282
GANU		-1.02	111	25.0	20	32	113	90
DSI		-2.13	108	0.6	64	35	47	158
ACNU		+0.39	61	3.0	75	97	15	492
TE6		-1.63	65	20.0	70	70	36	429
Cis-2-OH CCNU		+1.6	31	3.7	107	100	31	267
Trans-2-OH CCNU		+1.3	63	6.0	69	48	12	360
CCNU		+2.8	15	45.0	117	171	4	364



+ Logarithm of the distribution coefficient in an octanol–water system.

* Chemical half-life measured in 0.1 M sodium phosphate buffer, pH 7.4, at 37°.

† Dose killing 10 per cent of normal $CD2F_1$ mice.

‡ Per cent of control peripheral blood neutrophil count on day 3, the nadir of white blood cell suppression.

// Increased life span of $CD2F_1$ mice inoculated i.p. with 10^5 L1210 cells and treated i.p. on day 2 with an LD_{10} dose.

and *trans*-2-OH CCNU were taken from Wheeler *et al.* [20]. These are estimates from elution data derived from reversed phase high pressure liquid chromatography. Those for ACNU, DSI and TE6 were kindly calculated by Dr. Albert Leo (Pumona College Medicinal Chemistry Project). This technique has given good agreement previously between observed and calculated values [24]. However, there has been less experience with estimating Log P values for polyol derivatives; therefore, the figure for TE6 may not be as accurate.

Statistical analysis

A linear model was assumed and single or multiple linear regressions were calculated by the least squares method using an H.P. 97 calculator (Hewlitt Packard, Cupertino, CA). The polynomial regression analysis was done with a model 600 Wang calculator (Wang Labs Inc., Tewkesbury, NA). Statistical significance of white blood cell values was determined using the Turkey and Dunnett tests [25].

RESULTS AND DISCUSSION

Table 1 presents the structures, physico-chemical activities and biological effects of the eight chloroethylnitrosoureas studied. The data from this table have been used to determine the degree of correlation between each biological parameter and one or more of the chemical properties. Table 2 gives these equations, their correlation coefficients and statistical significance.

All the compounds tested have greater *in vitro* alkylating ability when compared to the lipid soluble reference compound, CCNU; this ranges from a 7-fold increased activity for the two glucose containing nitrosoureas, chlorozotocin and GANU, to a 2-fold increased activity for the *cis*-2 hydroxylated CCNU metabolite. A strong inverse correlation ($r = 0.92$, $P < 0.01$) exists between the alkylating activity and the decomposition rate of the drugs (equation 3); that is, the faster a nitrosourea breaks down the greater the alkylation. This correlation has been noted previously [6, 22]. A similar inverse correlation holds for the alkylating activity and the Log P value ($r = -0.86$, $P < 0.01$) (equation 1) but not with the carbamoylating activity and Log P (equation 2). The carrier molecule for the nitrosourea cytotoxic group may play an important role in determining the half-life and thus the relative alkylating ability of these drugs. Wheeler *et al.* [22] also noted a direct relationship between the half-life and the carbamoylating activity. We failed to confirm this with our smaller group of water soluble compounds (equation 4). These nitrosoureas appear to fall into two groups: (1) those with relatively high carbamoylating activity (20 to 45 per cent, mean 30 per cent), CCNU, GANU and TE6; and (2) those with low carbamoylating activity (0.6 to 6.0 per cent, mean 3.1 per cent), chlorozotocin, DSI, ACNU and the two hydroxylated metabolites of CCNU. Once generated, the availability of the isocyanate for carbamoylation of lysine or cellular constituents may be strongly influenced by whether there are sites accessible for intramolecular carbamoylation on the carrier moiety rather than the half-life of the parent molecule [23]. For ACNU, and intramolecular carbamoylated product formed non-enzymatically has been identified by Tanka *et al.* [26].

Table 2. Equations for linear regression analysis of biological and chemical parameters

Equation	No. of drugs	Correlation coefficient	Statistically significant at
(1) AA* = 69.88 − 17.89 (Log P)+	8	−0.86	P < 0.01
(2) CA‡ = 13.01 + 3.52 (Log P)	8	0.39	NS§
(3) $T\frac{1}{2}$‖ = 126.8 − 0.80 (AA)	8	−0.92	P < 0.01
(4) $T\frac{1}{2}$ = 64.91 + 0.48 (CA)	8	0.24	NS
(5) $T\frac{1}{2}$ = 70.78 + 12.89 (Log P)	8	0.74	P < 0.025
(6) LD_{10}¶ = 155.25 − 1.13 (AA)	8	−0.87	P < 0.01
(7) LD_{10} = 150.76 − 1.06 (AA) + 1.49 (Log P)	8	0.872	P < 0.05
(8) LD_{10} = 11.08 + 1.24 ($T\frac{1}{2}$)	8	0.83	P < 0.01
(9) LD_{10} = 55.24 + 1.66 (CA)	8	0.57	NS
(10) LD_{10} = 64.28 + 0.93 (CA) + 16.73 (Log P)	8	0.822	P < 0.10
(11) LD_{10} = 74.4 + 20 (Log P)	8	0.77	P < 0.025
(12) LD_{10} = 136.0 − 0.99 (AA) + 0.74 (CA)	8	0.902	P < 0.05
(12) LD_{10} = 134.44 − 0.97 (AA) + 0.74 (CA) + 0.55 (Log P)	8	0.902	P < 0.10
(14) ANC** = 18.85 + 0.94 (AA)	8	0.77	P < 0.05
(15) ANC = −36.92 + 1.19 (AA) + 6.01 (Log P)	8	0.783	P < 0.10
(16) ANC = 125.9 − 1.12 ($T\frac{1}{2}$)	8	−0.81	P < 0.05
(17) ANC = 50.87 − 0.37 (CA)	8	−0.14	NS
(18) ANC = 42.27 + 0.33 (CA) − 15.1 (Log P)	8	0.615	NS
(19) ANC = 46.5 − 14.76 (Log P)	8	0.61	NS
(20) ANC = 34.4 + 1.05 (AA) + 0.60 (CA)	8	0.799	P < 0.10
(21) ANC = −49.50 + 1.27 (AA) + 0.58 (CA) + 5.28 (Log P)	8	0.806	NS
(22) ILS++ = 460.4 − 2.24 (AA)	8	0.59	NS
(23) ILS = 589.84 − 4.09 (AA) − 43.06 (Log P)	8	0.652	NS
(24) ILS = 150.25 + 2.18 ($T\frac{1}{2}$)	8	0.50	NS
(25) ILS = 302.58 + 0.2 (CA)	8	0.02	NS
(26) ILS = 320.02 − 1.21 (CA) + 32.28 (Log P)	8	0.388	NS
(27) ILS = 304.2 − 28.02 (Log P)	8	0.37	NS
(28) ILS = 518.9 − 2.26 (AA) − 2.26 (CA)	8	0.635	NS
(29) ILS = 635.31 − 4.35 (AA) − 2.06 (CA) − 40.43 (Log P)	8	0.688	NS
(30) ILS = 181.0 + 9.0 (AA) − 0.09 (AA^2)	8	0.87	P < 0.05

* Alkylating activity as a percent of chlorozotocin.
+ Octanol–water partition coefficient.
‡ Carbamoylating activity as per cent [^{14}C]lysine.
§ Not statistically significant.
‖ Chemical half-life in minutes.
¶ Dose, in μmoles/kg, resulting in death of 10 per cent of normal $CD2F_1$ mice.
** Absolute neutrophil count on day 3 after a single i.p. LD_{10} dose.
++ Per cent increased life span compared to untreated i.p. L1210 tumored controls.

Figure 1 plots the LD_{10} dose of the compounds against their respective alkylating activities. A significant inverse correlation ($r = -0.87$, $P < 0.01$) exists between this *in vitro* test and the lethal toxicity in mice (equation 6). The most active alkylators, chlorozotocin and GANU, produce lethal toxicity with the lowest molar doses, while a considerably higher molar dose of CCNU is tolerated. We have found this relationship between alkylating activity and the LD_{10} dose to be of practical value in estimating the dose for lethal toxicity of newer compounds. The more water soluble drugs also had greater toxicity, as determined by the molar LD_{10} dose, although this relationship was less strong ($r = 0.77$, $P < 0.025$) (equation 11), and may reflect the correlation of alkylating activity and Log P. No such association exists between the LD_{10} dose and carbamoylating activity (equation 9), and the combination of alkylating activity and carbamoylating activity did not give a significantly higher correlation with the LD_{10} dose than that demonstrated with alkylating activity alone (equation 12). Similarly, inclusion of the Log P value did not improve the correlation coefficients of either lethal toxicity and alkylating activity (equations 6 and 7) or in the multiple correlation of LD_{10} with

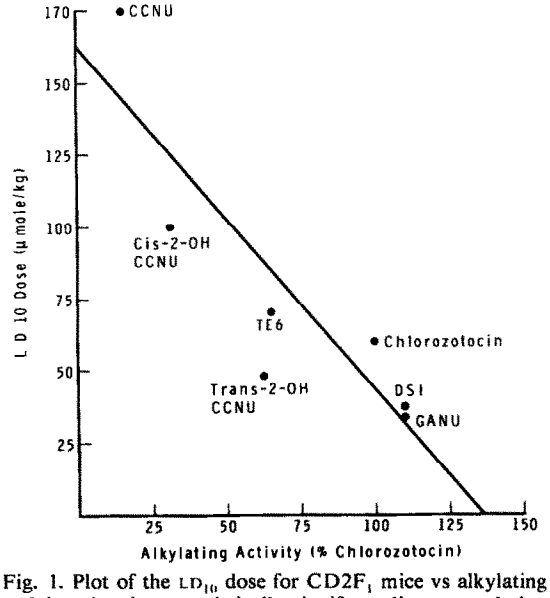


Fig. 1. Plot of the LD_{10} dose for $CD2F_1$ mice vs alkylating activity, showing a statistically significant linear correlation with a coefficient of 0.92 ($P < 0.01$).

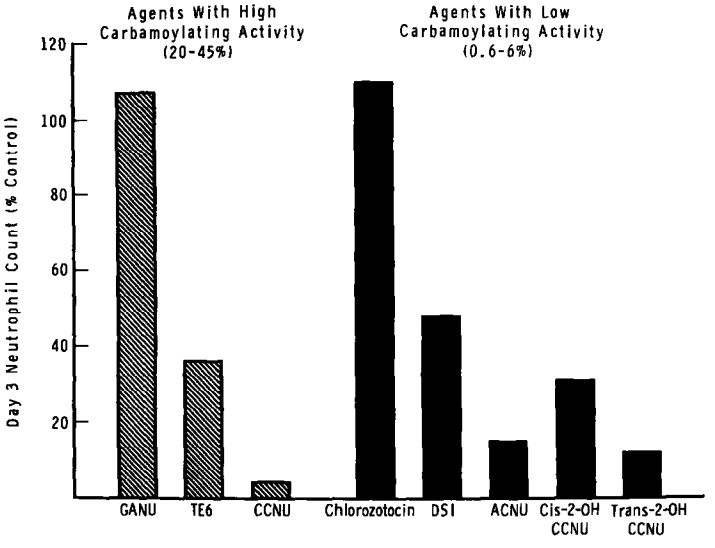


Fig. 2. Comparison of the neutrophil nadirs in $CD2F_1$ mice produced by nitrosoureas of high or low carbamoylating activity. An LD_{10} dose of each drug was given as a single i.p. injection.

alkylating activity and carbamoylating activity (equations 12 and 13). The only regression for which Log P adds significantly is the equation using LD_{10} and carbamoylating activity (equations 9 and 10). This may just reflect the correlation of alkylating activity with LD_{10} and Log P.

Of the eight drugs tested, only GANU and chlorozotocin were non-myelosuppressive when administered at an LD_{10} dose; their respective absolute neutrophil counts were 113 and 110 per cent of control values on day 3, the time of white blood cell nadir after nitrosourea administration in this species [18]. Water solubility itself is not the mediator of the bone marrow sparing property of these two glucose nitrosoureas. ACNU, TE6 and DSI, three very water soluble drugs, have bone marrow toxicity comparable to the lipid soluble CCNU, and the more polar hydroxylated metabolites of CCNU are no less myelosuppressive than the parent compound. No significant linear correlation was found between the Log P values and myelotoxicity ($r = -0.61$) (equation 19).

Earlier studies proposed that carbamoylating activity contributed significantly to nitrosourea toxicity [24]. However, more recently, data with both the methylnitrosourea [23] and a group of chloroethylnitrosoureas [6] suggest a more modest role for this chemical activity. Figure 2 demonstrates that drugs with minimal carbamoylating activity, ACNU and DSI, are as toxic to the bone marrow as the high carbamoylator CCNU. GANU has strong carbamoylating activity but is not myelosuppressive at an LD_{10} dose. A weak correlation ($r = 0.77$, $P < 0.05$) exists between alkylating activity and neutrophil suppression (equation 14), (Fig. 3). The addition of a glucose carrier to the cytotoxic nitrosoureido moiety appears to reduce selective bone marrow toxicity at the LD_{10} dose, without significantly altering antitumor activity. With both methyl and chloroethyl nitrosoureas, the position

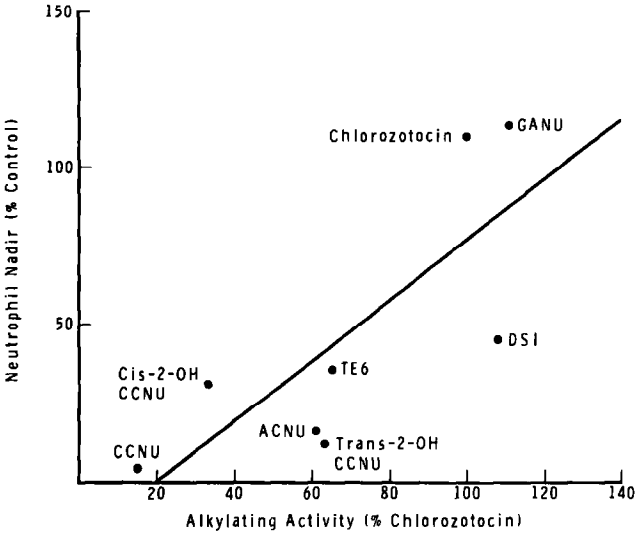


Fig. 3. Plot of *in vitro* alkylating activity vs the neutrophil nadir occurring in $CD2F_1$ mice after a single i.p. LD_{10} dose of each compound, showing a linear correlation coefficient of 0.77 ($P < 0.05$).

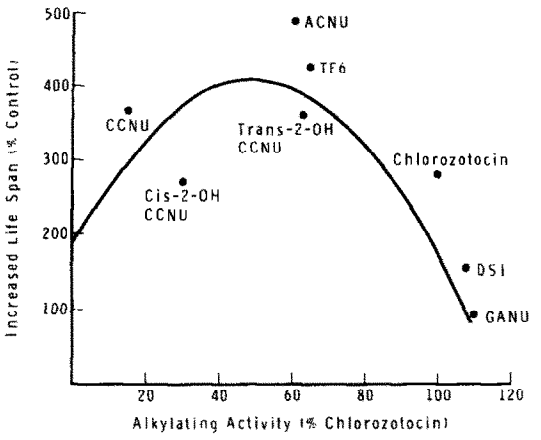


Fig. 4. Plot of increased life span of tumored mice against the alkylating activity of each nitrosourea. $CD2F_1$ mice were inoculated with 10^5 L1210 leukemia cells. Drugs were given i.p. at an LD_{10} dose on day 2 of tumor growth.

of the cytotoxic group on either the C1 or C2 of the glucose molecule is not critical for the reduction of myelotoxicity [19, 23]. However, a small change in the molecular structure of glucose, as with the inositol carrier group (DSI), restores the prominent bone marrow depression of nitrosoureas. The poor correlation of bone marrow suppression with any of the chemical parameters was not improved by including Log P in the equations (equations 14, 15, 17, 18, 20 and 21).

None of the parameters studied correlated linearly with antitumor activity, and increase in life span and water solubility could not be fitted to either a cubic or quadratic equation. However, a parabolic relationship was demonstrated between alkylating activity and antitumor effect for the eight compounds tested ($P < 0.05$), with optimal tumor cell kill occurring at an alkylating activity 60 per cent of chlorozotocin (Fig. 4).

The present structure–activity studies confirm our initial findings that the relative bone marrow sparing property of chlorozotocin is not related either to its water solubility or to low carbamoylating activity [6]. The glucose moiety appears to function as an independent variable for reducing nitrosourea cytotoxicity to bone marrow cells without significantly altering antitumor activity.

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