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Cytostatic action of two nitrosoureas derived from cysteamine

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1 2-Chloroethyl nitrosocarbamoylcystamine or ICIG-1325 (CNCC) is a lipid-soluble isomeric mixture of nitrosoureas.

2 Its dose-effect relationship on L1210 leukaemia is characterized by a large maximally efficient dose-range (MEDR), greater than that of other nitrosoureas. CNCC also demonstrated significant therapeutic activity on intracerebrally (i.c.) transplanted L1210 leukaemia and on six transplanted solid tumours, TM2 mammary carcinoma, M555 ovarian carcinoma, B16 melanoma, glioma 26, 3LL, Lewis lung carcinoma and colon 26 carcinoma. It was inactive on fibrosarcoma ICIG-Ci4. Its antitumour activity spectrum is wider than that of the related compounds 2-[3-(2-chloroethyl) 3-nitrosoureido]D-glucopyranose (CZT), (chloro-2-ethyl)-1(ribofuranosyl-isopropylidene-2′-3′para-nitrobenzoate-5′)-3 nitrosourea (RFCNU), and (chloro-2-ethyl)-1 (ribopyranosyl triacetate-2′-3′-4′)-3 nitrosourea (RPCNU).

3 A study of its metabolic disposition in animals has shown that CNCC undergoes extensive first-pass metabolism leading to the formation of four main plasma metabolites.

4 These metabolites are water-soluble nitrosoureas that arose from the bioreduction of the disulphide bridge followed by the methylation and the oxidation of the thiol groups.

5 Experimental screening was performed with these chemically synthesized metabolites. Both N′-(2-chloroethyl)-N-[2-(methylsulphinyl)ethyl]-N′-nitrosourea ($CMSOEN_2$) and N′-(2-chloro-ethyl)-N-[2-(methylsulphonyl)ethyl]-N′-nitrosourea ($CMSO_2EN_2$) are very active on L1210 leukaemia grafted intraperitoneally (i.p.) and i.c., L40 leukaemia, B16 melanoma, glioma 26 and Lewis lung carcinoma. Their effectiveness is better than that of the parent compound CNCC. In addition, the percentage of mice cured after $CMSOEN_2$ or $CMSO_2EN_2$ treatment is increased especially on B16 melanoma and glioma 26.

6 Haematological toxicity of both active metabolites is lower than that of CNCC, particularly on platelets which is the main toxicity location due to nitrosoureas.

Introduction

Several nitrosourea analogues have already been detected by murine tumour experimental screenings, but the main problem with this class of compounds is their rather disappointing clinical effect (Schein *et al.*, 1984).

A first generation including 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) (Carter, 1973), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) (Wasserman *et al.*, 1974a), 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU) (Wasserman *et al.*, 1974b) has only limited value for the treatment of lymphomas and gliomas (Schein *et al.*, 1984), and colon and ovary carcinomas (Wasserman *et al.*, 1974a,b). A more recent generation of glucosyl analogues, (chloro-2-ethyl)-1 (ribofuranosyl-isopropylidene-2′-3′ *para*nitrobenzoate-5′)-3 nitrosourea (RFCNU) and (chloro-2-ethyl)-1 (ribopyranosyl triacetate-2′-3′-4′)-3 nitrosurea (RPCNU) (Montero *et al.*, 1977; Hayat *et al.*, 1979; Vlaeminck *et al.*, 1981; Mathé *et al.*, 1982a,b; 1983a; Imbach *et al.*, 1981) and 2-[3-(2-chloroethyl) 3-nitrosoureido] D-glucopyranose (CZT, Johnston *et al.*, 1975, Schein *et al.*, 1973; Van Amburg *et al.*, 1980; Mathé *et al.*, 1983b) and a pyrimidyl analogue ACNU (Ogawa, 1981) has only

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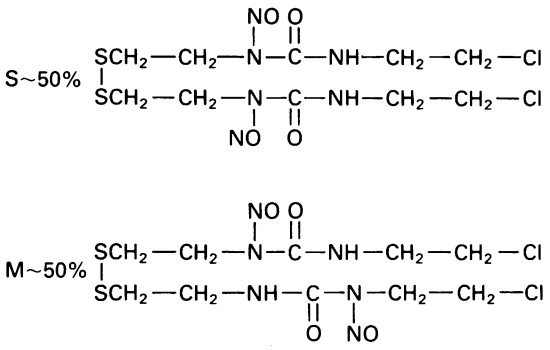

Figure 1 Structure of CNCC (2-chloroethyl nitrosocarbamoyl cystamine).

introduced a slight benefit, the first two agents by their moderate haematotoxicity (Mori *et al.*, 1980) but presenting the same clinical indications as CCNU (Wasserman *et al.*, 1974a), CZT by its discrete myelotoxicity and its effects against myeloproliferation (Schein *et al.*, 1973) and ACNU by its milder gastrointestinal toxicity (Ogawa, 1981).

A new series of sulphur-containing nitrosoureas has recently been developed in France. CNCC (2-chloroethyl nitrosocarbamoyl cystamine) is a mixture of isomers derived from cysteamine (Figure 1) and has showed very promising experimental antitumour activity, especially against murine glioma and melanoma (Maral *et al.*, 1983; 1984). Its metabolic fate has been investigated in rats (Godenèche *et al.*, 1985; Madelmont *et al.*, 1985). We showed that its plasma elimination half-life was less than 5 min. Four metabolites were identified: N'-(2-chloroethyl)-N-[2-(methylsulphinyl) ethyl]-N-nitrosourea ($CMSOEN_1$), N'-(2-chloroethyl)-N-[2-(methylsulphinyl)ethyl]-N'-nitrosourea ($CMSOEN_2$), N'-(2-chloroethyl)-N-[2-(methylsulphonyl) ethyl]-N-nitrosourea ($CMSO_2EN_1$) and N'-(2-chloroethyl)-N-[2-methylsulphonyl) ethyl]-N'-nitrosourea ($CMSO_2EN_2$) (Figure 2), which arise from the reduction of the disulphide bridge followed by methylation and oxidation of the thiol. They are water-soluble nitrosoureas unlike the parent compound and potentially active antitumour agents. The purpose of this study was to compare the cytostatic action of CNCC with that of its metabolites on experimental murine tumours and to determine whether these compounds were effective cytostatic agents.

Methods

Cytostatic activity on L1210 leukaemia

B6D2F1/O1a male mice, 3 months old, were injected with 10^5 L1210 leukaemia cells i.p. on day 0. As determined by preliminary results showing the range of toxicity of the four compounds, they received different dose levels: 5–70 mg kg^{-1} intravenously (i.v.) for $CMSOEN_1$, 20–160 mg kg^{-1} i.v. for

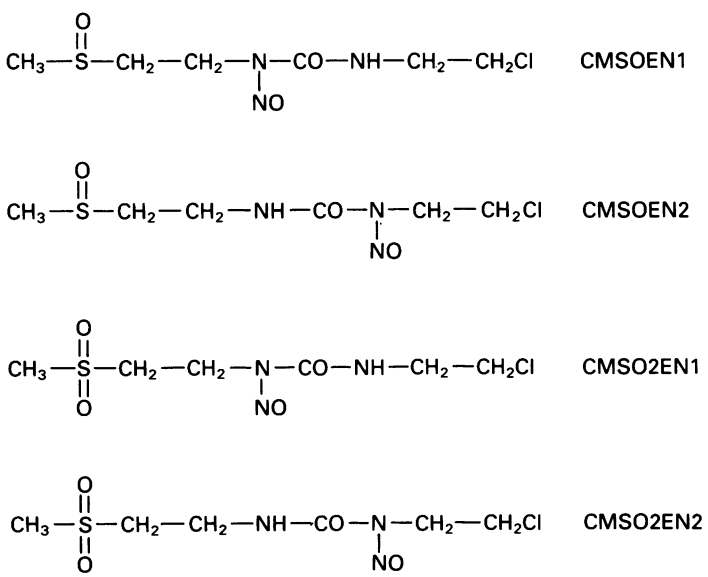

Figure 2 Structures of CNCC metabolites

$CMSO_2EN_1$, 1–50 mg kg^{-1} i.v. for both $CMSOEN_2$ and $CMSO_2EN_2$. Mice were individually weighed and 0.1 ml volumes of aqueous solution containing the compound were injected in their retro-orbital sinus per 20 g body weight. Eight dose-levels were administered for each compound, except for $CMSO_2EN_1$ which was not water soluble at doses greater than 160 mg kg^{-1}. Each treated group included eight mice.

An untreated control group (12 mice) received an equivalent volume of distilled water i.v., while a treated control group was administered the optimal dose of CNCC, the liposoluble parent drug, i.e. 30 mg kg^{-1} intraperitoneally.

Drug injections were repeated on days 5 and 9 in those mice presenting no manifestation of toxicity. Mortality was monitored daily and autopsies were performed to determine whether death was due to leukaemia or to a toxic action of the drugs. Surviving mice were observed for a period of 100 days after L1210 graft.

For each dose, the effect was expressed as an oncostatic index: $I = T/C \times 100$ where T was the median survival time of the treated group and C that of the control group.

Mortality range in the treated group was statistically compared to that of the control group according to the non-parametric Wilcoxon's W test. When the statistical test was significant ($P < 0.05$) and I greater than 125, the compound was considered active at the given dose.

For each compound, a graph was drawn representing the correlation between I and the dose. When $I = \infty$ (for doses in which more than 50% of the treated animals were alive at day 100), the curve presents a plateau called 'maximally efficient dose-range' (MEDR) (Mathé & Jasmin, 1979), the median of which is considered as the optimal dose.

Cytostatic activity on other murine tumours

Four other grafted leukaemia-lymphomas (L40 leukaemia, C1498 myeloid leukaemia, LGC and TLX lymphomas) and six grafted solid tumours (ICIG Ci-4 fibrosarcoma, glioma 26, B16 melanoma, 3LL Lewis lung carcinoma, MA–16C mammary carcinoma and colon 26 carcinoma) were studied according to the modalities shown in Table 1.

The same experiment was conducted on L1210 leukaemia grafted i.c., using 10^4 cells in 0.03 ml of saline and on BCNU-resistant TLX lymphoma. On days +1, +5 and +9 after the tumour graft, $CMSOEN_2$ and $CMSO_2EN_2$ were administered intravenously, and CNCC intraperitoneally as control. Three dosages were used: the optimal dose as defined in the assay on L1210 leukaemia, half-optimal dose, and double optimal dose, respectively 7.5, 3.25 and 15 mg kg^{-1} for $CMSOEN_2$, 15, 7.5 and 30 mg kg^{-1} for $CMSO_2EN_2$ and 30, 15 and 60 mg kg^{-1} for CNCC. Mortality was observed daily and survivors were examined for a period of 100 days.

Results were expressed by $I = T/C \times 100$ and the statistical Wilcoxon's W test was performed as in the L1210 assay.

Study of toxic effect on haemopoietic tissue after i.v. administration of optimal dose

In order to compare the toxicity of the two metabolites with that of CNCC, the following counts were done: white and red blood cells, platelets of peripheral blood, and bone marrow nucleated cells with an adjusted ZM Coulter counter (Coultronics) with a 70 μm tube aperture.

The optimal dose of $CMSOEN_2$ and $CMSO_2EN_2$ intravenously and of CNCC orally (p.o.) was adminis-

Table 1 Murine tumours used for this study

Tumour	*Form*	*Mouse strain*	*Graft route*	*Inoculum cells*
L1210 Leukaemia	Ascitic fluid	B6D2F1	i.p.	10^5
			i.c.	10^4
L40-AkR Leukaemia	Lymph nodes	AKR	i.p.	$\sim 10^6$
LGC C57 Bl/6 Lymphoma	Spleen	C57/B16	i.p.	$\sim 10^6$
TLX Lymphoma	Ascitic fluid	CBA	i.p.	$\sim 10^6$
TLX/BCNU Lymphoma	Ascitic fluid	CBA	i.p.	$\sim 10^6$
C1498 Leukaemia	Solid	C57/B16	s.c.	$\sim 10^6$
ICIG-Ci4-Fibrosarcoma	Solid	C57/B16	s.c.	$\sim 10^6$
Glioma 26	Solid	C57/B16	s.c.	$\sim 10^6$
B16 Melanoma	Solid	C57/B16	s.c.	$\sim 10^6$
3LL Lewis lung carcinoma	Solid	C57/B16	s.c.	$\sim 10^6$
MA16C Mammary carcinoma	Solid	C3H/He	s.c.	$\sim 10^6$
Colon 26 carcinoma	Solid	BALB/c	s.c.	$\sim 10^6$

tered on days 1 and 5 to 20 mice for each drug. Blood was collected from the retro orbital sinus in heparin-containing tubes. Counts were performed after appropriate dilutions (2×10^{-3} for leukocytes and 4×10^{-6} for erythrocytes). Blood 2×10^{-4} diluted was centrifuged and platelets in the supernatant were counted. Mice were then killed by cervical dislocation. One femur was cut off from each mouse and its content was flushed via a 25-gauge needle into saline. Counts were made after dilution (2.5×10^{-3}). Blood and femurs were taken from five mice treated with each compound per day and from five non-treated mice as controls. Cell counts of each mouse were carried out on days 0, 8, 12, 19 and 26. Means and standard deviations were calculated and graphs were drawn accordingly.

Chemicals

CNCC was synthesized by Oiry *et al.* (1984). $CMSOEN_1$, $CMSOEN_2$, $CMSO_2EN_1$ and $CMSO_2EN_2$ were prepared at INSERM U71 by Madelmont *et al.*, as described elsewhere (Madelmont *et al.*, 1985).

Results

Effect on L1210 leukaemia

The acute LD_{50} was found to be greater than 70 mg kg^{-1} for $CMSOEN_1$ and 160 mg kg^{-1} for $CMSO_2EN_1$. Both compounds were not active against L1210 leukaemia (data not shown).

On leukaemic mice, the acute LD_{50} by the i.v. route was 25 mg kg^{-1} for $CMSOEN_2$, 50 mg kg^{-1} for $CMSO_2EN_2$ compared to 75 mg kg^{-1} by the i.p. route for CNCC.

As shown in Figure 3, both $CMSOEN_2$ and $CMSO_2EN_2$ were very highly active against L1210 leukaemia ($0.01 > P > 0.001$) and have a large MEDR, which was greater for $CMSO_2EN_2$ than for $CMSOEN_2$.

Thus, the relationship acute LD_{50}/optimal dose is the same for $CMSOEN_2$ (25/7.5 = 3.3) and $CMSO_2EN_2$ (50/15 = 3.3). That of CNCC (75/30 = 2.5) is less important and indicates a greater beneficial effect for metabolites than for CNCC.

Effect on other leukaemia-lymphomas

$CMSOEN_2$ and $CMSO_2EN_2$ were active ($P = 0.001$) against L40 leukaemia, C1498 myeloid leukaemia and LGC lymphoma. As with CNCC, they were less active, at the limit of significance for some groups, on TLX lymphoma (Table 2). With all three compounds,

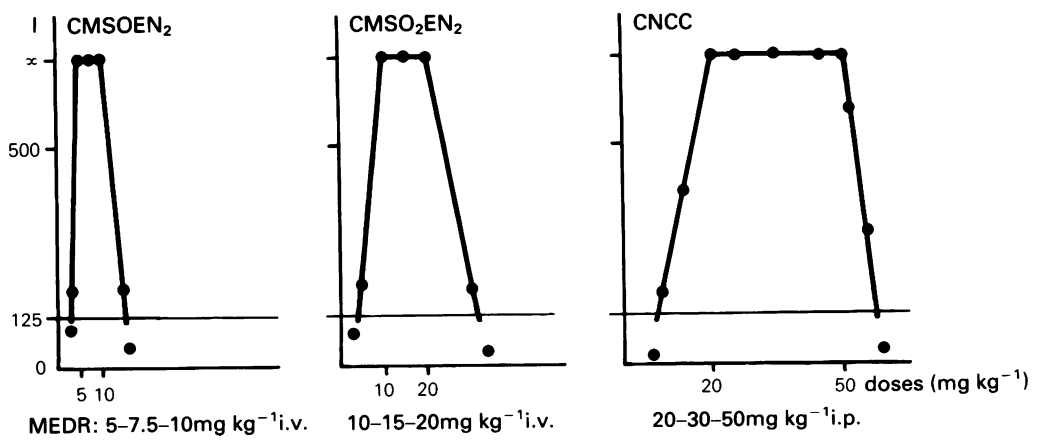

Figure 3 Determination of maximally efficient dose range (MEDR), of $CMSOEN_2$ and $CMSO_2EN_2$ (i.v. in aqueous solution) and CNCC (i.p. in oily suspension) on L1210 murine leukaemia (10^5 cells, i.p.). Drugs were administered 1, 5 and 9 days after leukaemia cells. Each mouse was weighed individually to determine dosage. Mice mortality was monitored daily and dead animals were systematically autopsied to determine whether the cause of death was toxicity or leukaemia. The oncostatic effect of each dose group (8 mice) is expressed as I (oncostatic index) = T/C × 100 where T represents the median survival time of the treated mice, C the median survival time of the control group; ∞ indicates that more than 50% of treated animals in the group were cured. $I > 125$ is statistically significant (non parametric Wilcoxon's test: $P < 0.05$).

Table2 Comparative effect of $CMSOEN_2$, $CMSO_2EN_2$ and CNCC on leukaemias and lymphomas

	Dose (mg kg^{-1})	*L40*		*LGC*		*TLX*		*TLX/BCNU*		*L1210 IC*		*C1498*	
		T/C × 100	% alive d.100	T/C × 100	% alive d.100	T/C × 100	% alive d.100	T/C ×[100	% alive d.100	T/C × 100	% alive d.100	T/C × 100	% alive d.100
$CMSOEN_2$	7.5	∞	91	∞	100	133	0	NA	0	∞	50	∞	87.5
i.v.	3.25	∞	88	∞	100	188	0	NA	0			125	0
$CMSO_2EN_2$	15	∞	100	∞	100	133	0	NA	0	∞	100	∞	87.5
i.v.	7.5	∞	100	∞	100	150	0	NA	0			∞	87.5
CNCC	30	∞	77	∞	62.5	133	0	NA	0	∞	55	∞	50
i.p.	15	∞	80	∞	87.5	211	37.5	NA	0				
BCNU	15					233	37.5	NA	0				
i.p.	7.5					211	12.5	NA	0				

Leukaemias and lymphomas were grafted at day 0 (mice, route and inoculum cells are indicated in Table 1). Compounds were administered 1, 5 and 9 days after grafting. Mortality was monitored daily. T/C × 100: T is treated median survival and C control median survival. Indicated numbers are statistically significant (non parametric Wilcoxon's test with $P < 0.05$). NA: not active.

the highest dose, equal to twice the optimal dose, was toxic in mice.

Effect on solid tumours

$CMSOEN_2$ and $CMSO_2EN_2$ were very active ($P = 0.001$ and $I = \infty$) on four of the solid tumours studied: B16 melanoma, 3LL and colon 26 carcinomas, and glioma 26 (Table 3). Conversely, they showed no activity on ICIG-Ci4 fibrosarcoma, or on MA-16C mammary carcinoma. The highest dose was toxic to the mice.

Effect on L1210 leukaemia grafted intracerebrally

$CMSOEN_2$ and $CMSO_2EN_2$ were maximally efficient on L1210 leukaemia grafted i.c.; $CMSO_2EN_2$ cured 100% of animals (Table 2).

Effect on BNCU-resistant lymphoma

Neither analogue was effective on the BCNU-resistant TLX lymphoma.

Comparison with CNCC

$CMSOEN_2$ and $CMSO_2EN_2$ were slightly active or inactive on the same tumours as CNCC (TLX and TLX/BCNU lymphomas, MA16C carcinoma, ICIG-Ci4 fibrosarcoma). However, both metabolites were at least as active as CNCC on the eight leukaemia-lymphomas or solid tumours studied. On the other hand, a difference appeared between the three compounds: most of the time, the percentage of long survivors in the groups treated with $CMSOEN_2$ and $CMSO_2EN_2$ was greater than in the CNCC-treated group. This result is particularly clear for $CMSO_2EN_2$ vs. CNCC on B16 melanoma, glioma 26 and i.c. grafted L1210 leukaemia.

Effect on blood and bone marrow

Red blood cells No effect was observed on red blood cells (data not shown).

Leukocytes The minimum number of leukocytes (nadir) was observed between days 8 and 12 (3 to 7 days after the last administration of compounds). Recovery was complete in $CMSOEN_2$- and $CMSO_2EN_2$-treated mice and took place on day 19 (Figure 4).

Platelets Whereas platelet counts of CNCC-treated mice were lowest, the plots for $CMSOEN_2$ and $CMSO_2EN_2$-treated groups were not different from that of control mice during the same period (Figure 4). There was no nadir.

Table 3 Comparative effects of $CMSOEN_2$, $CMSO_2EN_2$ and CNCC on solid tumours

	Dose (mg kg^{-1})	ICIG-Ci4	Glioma 26		B16		MA16C		3LL		Colon 26	
			T/C × 100	% alive d.100	T/C × 100	% alive d.100		T/C × 100	% alive d.100	T/C × 100	% alive d.100
$CMSOEN_2$ i.v.	7.5	NA	∞	50	∞	100	NA	∞	75	∞	100
	3.25	NA	NA		167	0	NA	150	0	∞	100
$CMSO_2EN_2$ i.v.	15	NA	∞	100	∞	100	NA	∞	100	∞	100
	7.5	NA	∞	100	∞	100	NA	NA		∞	100
CNCC i.p.	30	NA	200	33	156	0	NA	∞	100	∞	87.5
	15	NA			145	0	NA				

Solid tumours were grafted at day 0 (mice, route and inoculum cells are indicated in Table 1). Compounds were administered 1, 5 and 9 days after grafting.
Mortality was monitored daily. T/C × 100: T is treated median survival and C control median survival.
Indicated numbers are statistically significant (non parametric Wilcoxon's test with $P < 0.05$). NA: not active.

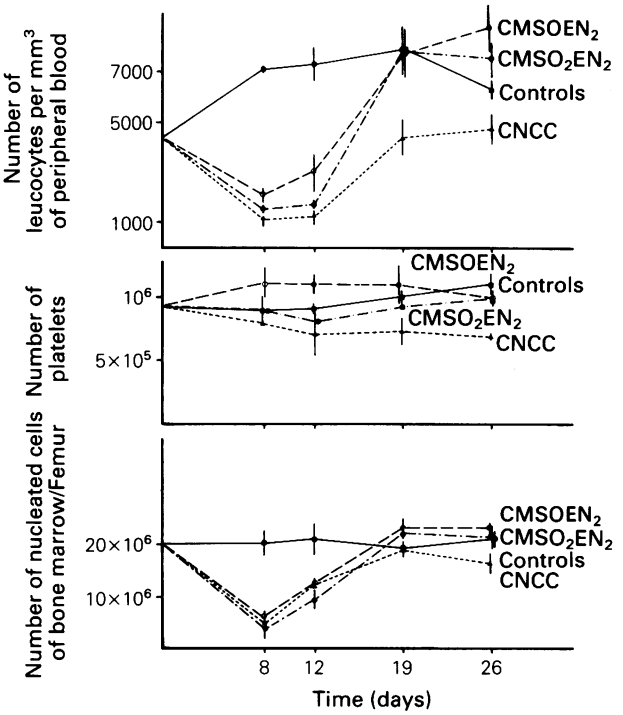

Figure 4 Effect of $CMSOEN_2$ (7.5 mg kg^{-1} i.v.) $CMSO_2EN_2$ (15 mg kg^{-1} i.v.) and CNCC (90 mg kg^{-1} p.o.) on leukocytes and platelets of peripheral blood and nucleated cells of bone marrow. Counts were done with an adjusted ZM Coulter counter, Each point is the mean for 5 mice. The vertical lines represent ± s.e.mean.

Bone marrow The curves of bone marrow nucleated cells presented a nadir at day 8 in the three treated groups. Recovery was complete on day 19 in the mice treated with the two metabolites.

Thus, the haematological toxicity of $CMSOEN_2$ and $CMSO_2EN_2$ appeared to be lower than that of CNCC. As with CNCC, a nadir was obtained with both metabolites in leukocyte and bone marrow nucleated cell counts, but complete recovery was more rapid for leukocytes. In addition, platelet numbers were always identical to that of the controls, which was not the case with CNCC.

Discussion

The nitrosoureas of first generation, BCNU, CCNU and MeCCNU, had a moderate activity on L1210 leukaemia. No MEDR was observed (Imbach *et al.*, 1979). Only one dose led to I = ∞ for CCNU and MeCCNU. The optimal dose of BCNU yielded I = 170. The maximum efficiency of CNCC (I = ∞)

was observed against L1210 leukaemia, for doses between 20 and 50 mg kg^{-1}. Thus, it appeared to be a more interesting compound than the first series of nitrosoureas.

The broader MEDR of CNCC as compared to its active metabolites $CMSOEN_2$ and $CMSO_2EN_2$ is only apparent. The present data show that the metabolites generated *in vivo* by the S-isomer $CMSOEN_1$ and $CMSO_2EN_1$ are inactive. So if the MEDR on L1210 leukaemia is expressed as μmol kg^{-1} and if we consider that CNCC is an isomeric mixture of 50% active isomer (M) and 50% inactive isomer (S), $CMSO_2EN_2$ (39–77 μmol kg^{-1}) seems to be more favourable than CNCC (23–58 μmol kg^{-1}) and $CMSOEN_2$ (20–40 μmol kg^{-1}).

Compared with CNCC, the two nitrosourea analogues studied are as strongly active on L1210 leukaemia, as moderately active on TLX lymphoma, as active on L40 lymphoma, much more active on glioma 26 and B16 melanoma, no more active on the other solid tumours studied, especially the MA-16C mammary neoplasia and the ICIG Ci4 fibrosarcoma. They are also as active on intracerebrally grafted L1210 leukaemia and their highest activity on glioma, as observed above, is encouraging for the development of the treatment of central nervous system-borne or metastasizing tumours. Unlike CNCC, they appeared to be not toxic for platelets and less toxic for white blood cells.

Compared to each other, they differ substantially only in the dose-effect relationship but, interestingly enough, not by a qualitative all-or-none cytostatic efficiency on a given murine neoplasia.

The two new analogues, the active metabolites of CNCC, open a new series of nitrosourea compounds which are: (a) pure compounds and not an isomeric mixture like the parent compound CNCC; (b) water-soluble; (c) very active on intracranial L1210 leukaemia, and on two solid tumours, glioma and melanoma rarely sensitive to cytostatics; (d) non toxic for platelets unlike other nitrosoureas.

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