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Clinical pharmacokinetics of oral CCNU (Lomustine)

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Summary. *The plasma pharmacokinetics of orally administered CCNU (130 mg/m²) were studied in four patients using reversed-phase high-performance liquid chromatography (HPLC) analysis. Parent CCNU was not detected in the plasma of any of the patients, probably due to complete conversion to monohydroxylated metabolites during the 'first pass' through liver and gut. However, two monohydroxylated metabolites, trans-4-hydroxy CCNU and cis-4-hydroxy CCNU, were found at high concentrations, the relative amounts being about 6 : 4. Peak concentrations of the metabolites were reached 2–4 h after administration and were remarkably similar for all four patients, the total being 0.8–0.9 μg/ml. The metabolites were also detected in a tumour biopsy. Plasma clearance half-lives of the two metabolites were similar in each patient but showed a two-fold variation between patients, from 1.3 to 2.9 h. These results suggest that the antitumour activity and systemic toxicity of CCNU when given orally are due mainly to its monohydroxylated metabolites. Finally, comparison with data obtained in vitro and in mice showed that the nitrosourea exposures in these patients were at the lower limit of those required for significant antineoplastic activity.*

Introduction

CCNU (Lomustine; 1-(2-chloroethyl)-3-cyclohexyl-l-nitrosourea) was first evaluated in clinical trials in the late 1960s and has since been used in a broad spectrum of tumours, particularly those of the brain (for review see [30]). However, despite its wide use over a long period no detailed pharmacokinetics have yet been reported, particularly for the oral route (for reviews see [1, 8]). This appears to have been largely due to the lack of appropriate, sensitive analytical techniques for CCNU and its monohydroxylated metabolites (see Fig. 1 for chemical structures). We have used a modified version of the high-performance liquid chromatography (HPLC) procedures of Montgomery et al. [4, 11] for detailed pharmacokinetic studies in mice [5]. With minor further development we have found this was also suitable for investigations in man and we now describe the plasma pharmacokinetics of oral CCNU in four patients.

We are also interested in the possible pharmacokinetic interaction in patients between CCNU and the chemosensitizer benznidazole (*N*-benzyl-(2-nitroimidazolyl)-acetamide, Ro 07-1051; Radanil; Roche), a combination regimen which has shown particular promise in mice [22, 28, 33]. We have found that additional modification of the HPLC method was required to resolve the CCNU metabolites from benznidazole, and we also describe these procedures here.

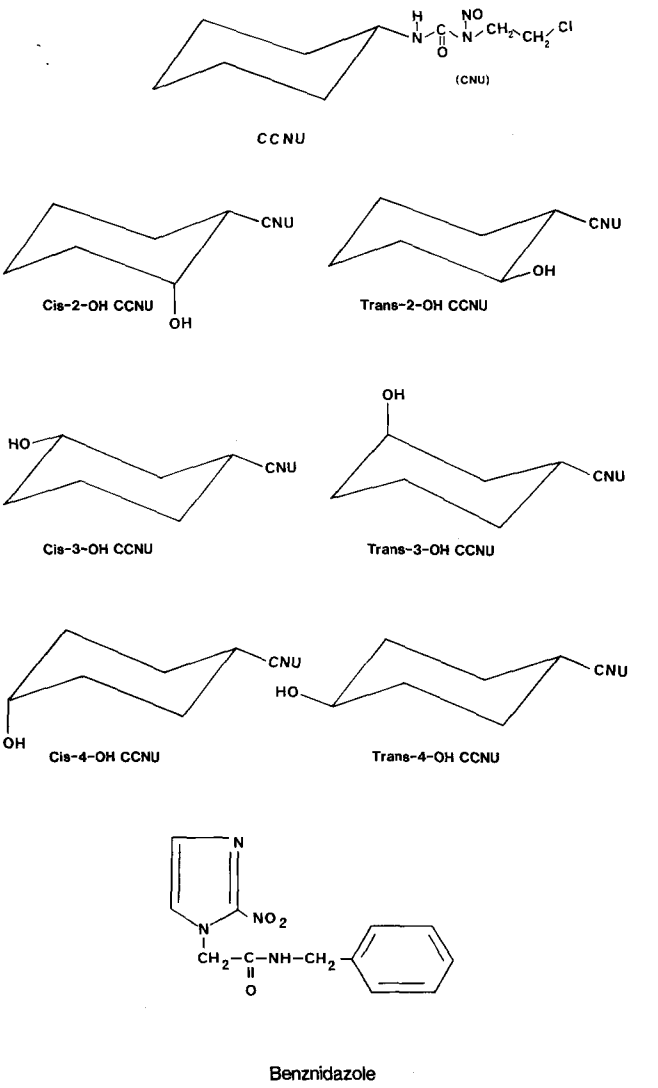

Fig. 1. Structures of CCNU and its six possible monohydroxylated metabolites. Also given is the structure of the chemosensitizer benznidazole

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Table 1. Details of patients

Patient	Sex	Weight (kg)	Body surface area (m^2)	Age (years)	Diagnosis	Other medication[a]
A	M	54.0	1.5	44	Metastatic malignant melanoma	None
B	M	68.8	1.85	31	Metastatic carcinoma of the caecum	None
C	M	75.0	1.91	59	Recurrent glioma	Dexamethasone 8 mg Phenobarbitone 300 mg Cimetidine 800 mg
D	M	82.0	2.0	57	Malignant melanoma	Dexamethasone 16 mg

[a] Dose per day

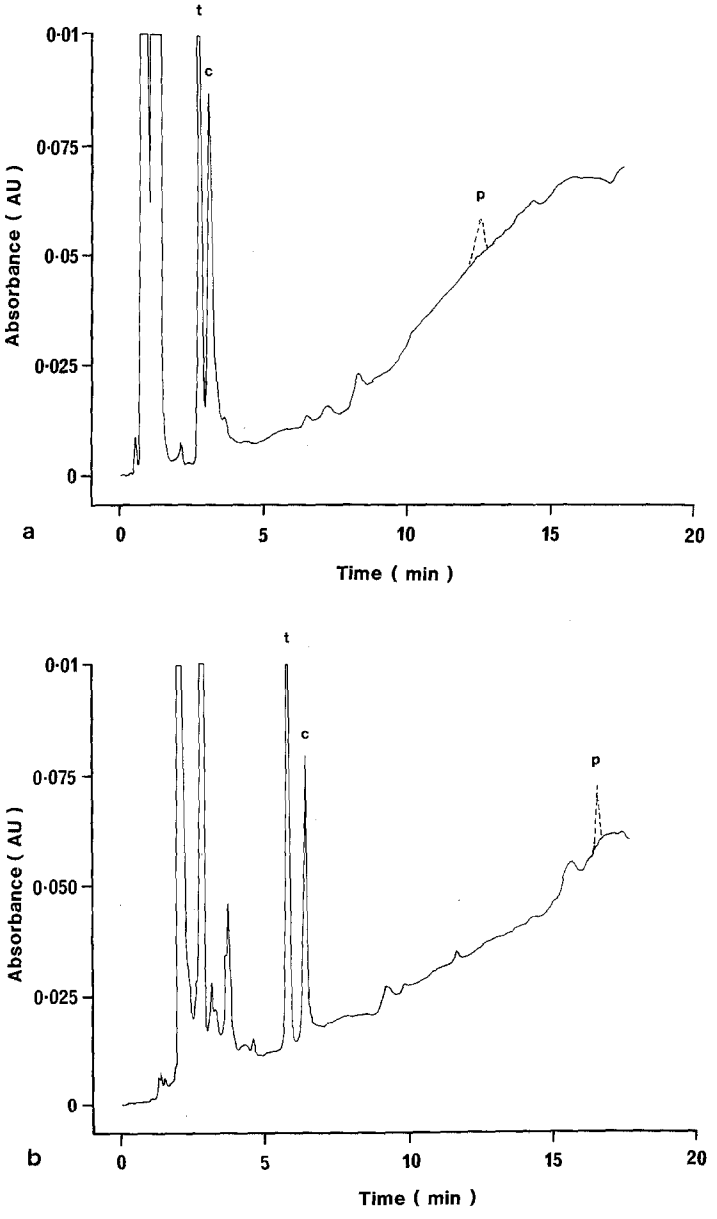

Fig. 2a, b. HPLC chromatograms of the ether extract of plasma obtained from patient A 4 h after an oral dose of 130 mg/m^2 CCNU. Parent CCNU (p, *broken line*) was not found in the plasma but was added to illustrate its retention characteristics. Peaks *t* and *c* represent the metabolites *trans*-4-hydroxy-CCNU and *cis*-4-hydroxy-CCNU, respectively. **a** chromatogram obtained using a single C18 column. **b,** chromatogram obtained using two C18 columns in series (see Methods for details)

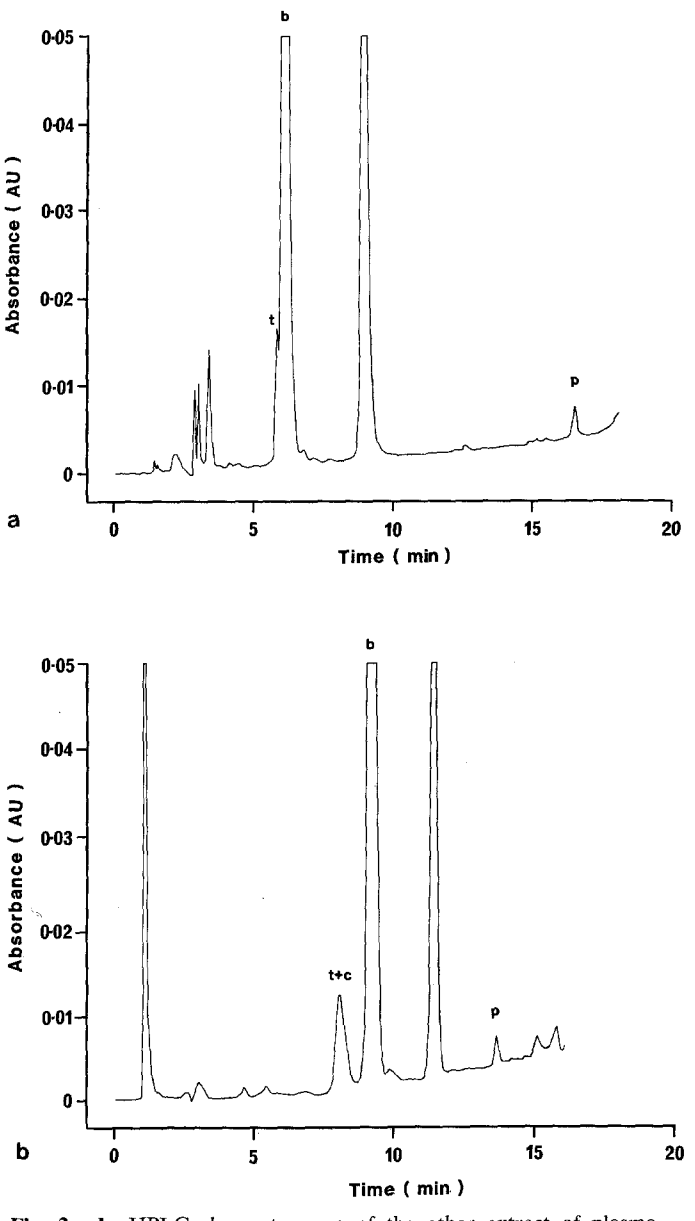

Fig. 3a, b. HPLC chromatograms of the ether extract of plasma obtained from a patient 3 h after an oral dose of 130 mg/m^2 CCNU and 7 h after an oral dose of benznidazole (20 mg/kg). P, parent CCNU; *t*, *trans*-4-hydroxy CCNU; *t* + *c*, mixture of *trans*-4-hydroxy CCNU and *cis*-4-hydroxy CCNU; b, benznidazole. **a** method using two C18 columns in series. **b** method using two CN columns in series (see Methods for details)

Table 2. Pharmacokinetic parameters of CCNU metabolites in four patients

Parameter Patient	$t\frac{1}{2}$ (h)[a]		Peak concentration (μg/ml)			$AUC_{0-\infty}$ (μg h/ml)		
	trans-4-OH CCNU	*cis*-4-OH CCNU	*trans*-4-OH CCNU	*cis*-4-OH CCNU	Total nitrosourea	*trans*-4-OH CCNU	*cis*-4-OH CCNU	Total nitrosourea
A	1.32 (1.22–1.44)	1.33 (1.22–1.45)	0.53	0.34	0.87	2.32	1.40	3.72
B	1.94 (1.84–2.04)	1.96 (1.75–2.20)	0.57	0.33	0.90	2.31	1.54	3.85
C	2.53 (2.33–2.78)	2.93 (2.70–3.19)	0.50	0.34	0.84	2.13	1.58	3.71
D	1.43 (1.11–2.02)	1.41 (1.14–1.86)	0.52	0.26	0.78	1.44	0.77	2.21

[a] Figures in parentheses show 95% confidence limits

Patients and methods

Four patients received an oral dose of 130 mg/m^2 CCNU (Lomustine capsules, Lundbeck) as part of their treatment. The dose was rounded up to the nearest 10 mg. Renal and hepatic function were normal. Patient details are given in Table 1.

Blood samples were taken into heparinised tubes and immediately cooled on ice. They were then immediately centrifuged at 4,000 *g* for 10 min at 4° C. Triplicate 3-ml aliquots of plasma were placed in 15-ml glass culture tubes and extracted with 5 ml cold diethyl ether (HPLC grade, Fisons). A 3-ml aliquot of the supernatant was then removed and evaporated to dryness in vacuo using a Savant Speed Vac Concentrator coupled to a Model 100A Refrigerated Condensation Trap (Uniscience, Cambridge, UK). The dry residue was redissolved in 50 μl ethanol for HPLC analysis. Preparation of tumour samples was as described previously for mice [5].

HPLC. The HPLC equipment used (Waters Associates) was as previously described [5] except that the RCM-100 Radial Compression Module was replaced by two Z-modules. Separation of CCNU and its metabolites was carried out on two Waters Radial-PAK reversed-phase bonded octadecylsilane (C 18, 8 mm ID) cartridge columns in series, the first containing spherical particles 5 μm in diameter, followed by a second with 10-μm-diameter particles. The mobile phase consisted of a two-step linear gradient with an initial condition of 34% acetonitrile in 0.1 *M* acetate buffer (pH 4), proceeding to 44% acetonitrile at 6 min and then to 72% at 16 min. The low pH does not affect the retention of CCNU or its metabolites, but removes an interfering peak which has a retention time similar to *trans*-4-hydroxy-CCNU.

For analysis of samples containing benznidazole and CCNU metabolites, separation was carried out on two cyanonitrile-bonded cartridge columns (CN, 8 mm ID, 10 μm) in series. Again a two-step linear gradient was used with an initial condition of 10% acetonitrile in 0.1 *M* acetate buffer (pH 4), proceeding to 30% at 6 min and then to 60% at 13 min. CCNU and its metabolites were detected by monitoring the effluent for absorbance at 254 nm. Quantiation was by peak height or area with reference to standard curves prepared for CCNU and *trans*-4-hydroxy CCNU. The latter was used to quantitate both *trans*-4- and *cis*-4-hydroxy metabolites. For both methods, linearity of the calibration curve was established over the concentration range of 0.005–5 μg/ml. The lower limits of detection were 0.005 and 0.01 μg/ml for methods with C18 columns and CN columns, respectively. Recoveries of CCNU and its metabolites by the ether extraction procedure were over 90%. The coefficients of variation for CCNU and *trans*-4-hydroxy-CCNU at 0.5 μg/ml were 7.7% and 5.9%, respectively, for the method using C18 columns and 4.8% and 5.4% for the method using CN columns.

Pharmacokinetic parameters. Elimination half-lives ($t_{1/2}$) with 95% confidence limits were estimated from post-peak concentration data using least squares linear regression analysis by the equation $t_{1/2} = \ln 2/k$, where k is the elimination rate constant. Area under the curve (AUC) from time 0 to the final time t was estimated by Simpson's rule. The remaining AUC from $t-\infty$ was given by Ct/k, where Ct is the concentration at t. Values of $AUC_{(0-\infty)}$ given are the sums of $AUC_{(0-t)}$ and $AUC_{(t-\infty)}$.

Results

Figure 2 shows typical chromatograms of the ether extracts of plasma obtained from a patient receiving 130 mg/m^2 CCNU orally. Note that much better resolution was achieved by using two columns in series (Fig. 2b) than with a single column (Fig. 2a). Of the six possible monohydroxylated metabolites of CCNU [3, 16, 31] only two were detected in plasma, namely *trans*-4-hydroxy-CCNU and *cis*-4-hydroxy-CCNU. This was true for each of the four patients studied. Further, the parent CCNU was not found in any of the patients and if present was below 5 ng/ml. The dotted lines in Fig. 2 show the position of CCNU spiked into the same sample for illustrative purposes.

Figure 3a shows that when C18 columns were used benznidazole was not resolved from the two metabolites of CCNU. However, when CN columns were used, they were well resolved (Fig. 3b). With this method the two isomeric metabolites of CCNU were eluted as a single peak (peak t + c, Fig. 3b). The area of the peak then gives an estimate of the concentration of the hydroxylated metabolites in the sample[1].

1 It will also be seen from Fig. 3 that parent CCNU was present in the plasma of this patient. Full details of the effect of benznidazole on CCNU pharmacokinetics will be given elsewhere (Roberts et al., in preparation).

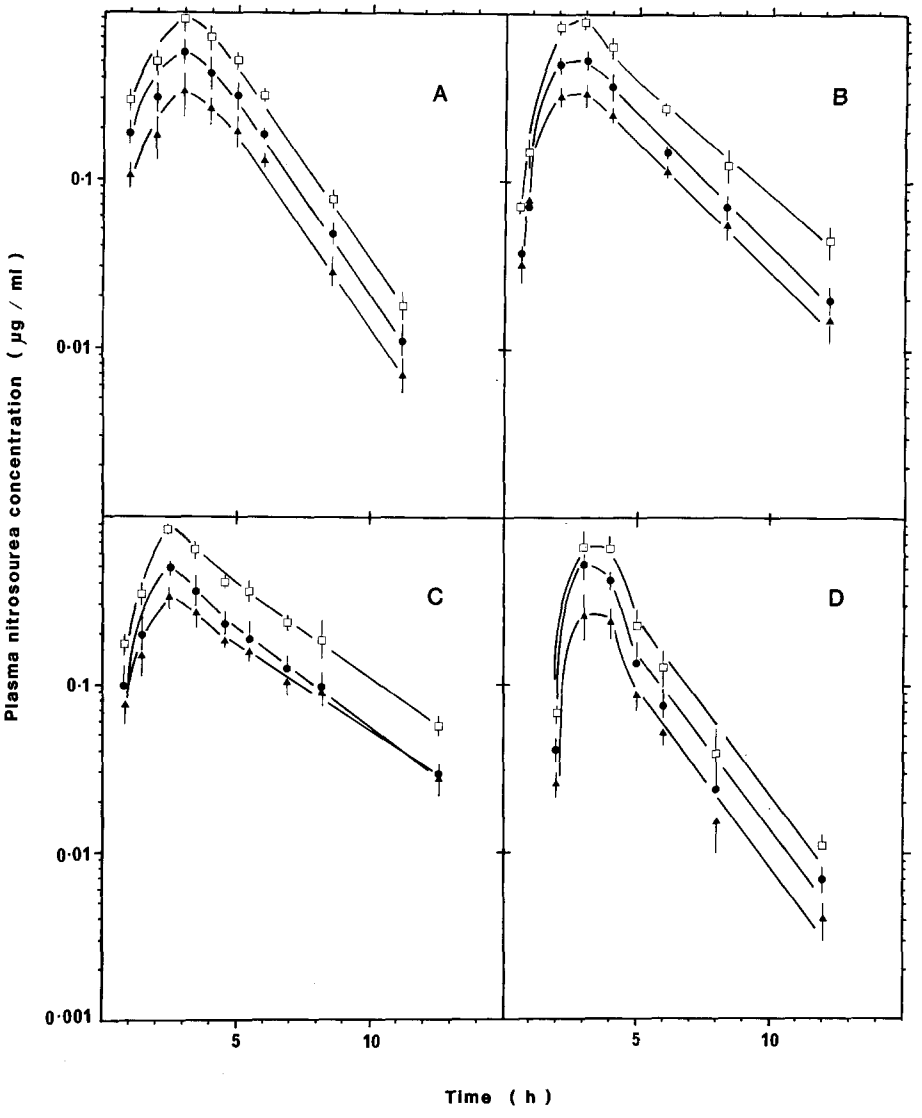

Fig. 4. Plasma pharmacokinetics of four patients receiving oral CCNU (130 mg/m^2). ▲ *cis*-4-hydroxy CCNU; ● *trans*-4-hydroxy CCNU; □ *cis*-4 and *trans*-4-hydroxy CCNU combined. Error bars represent 1 standard deviation

Figure 4 shows the plasma pharmacokinetics of the two isomeric CCNU metabolites individually and of their sum (total nitrosourea) in each of the four patients receiving CCNU alone. Peak concentrations of the metabolites were reached within 3–4 h. In all cases there was more *trans*-4-hydroxy-CCNU than *cis*-4-hydroxy-CCNU, the relative amounts being about 6 : 4. Post-peak plasma concentrations declined exponentially.

Table 2 gives the half-life, the peak concentration and the area under the concentration-time curve (AUC) of the two CCNU metabolites for all four patients. The half-lives of the two metabolites were very similar within each patient, but between patients the variation was up to two-fold. Peak concentrations were remarkably similar for all four patients. On the other hand the values of AUC in one patient (D) was much lower than the other three, due mainly to a relatively narrow peak.

For patient B, a biopsy of melanoma tissue was also taken at 4 h after treatment. The metabolites were readily detectable in the tumour, and tumour/plasma ratios were found to be 48% and 60% for *trans*-4-hydroxy-CCNU and *cis*-4-hydroxy-CCNU, respectively. As for plasma no CCNU was found in the tumour.

Discussion

Although CCNU has been used clinically for over 20 years in a number of human malignancies (for review see [30]), as yet there is scant data in the literature on its pharmacokinetics in either experimental animals or patients. This is due to a lack of sensitive and specific techniques operable at the nanogram level. Early studies used either radiolabelled drug [15, 25] or a modification of the Bratton-Marshall reaction for the nitroso moiety [4, 10]. These techniques, however, lack the specificity necessary to distinguish the parent CCNU from its various active metabolites, and the sensitivity needed for pharmacokinetic investigations in patients. In the case of studies using radiolabelled drug, interpretation of the results is further complicated by the covalent binding of CCNU or its breakdown products to biomolecules. The pharmacokinetic parameters, such as half-life, obtained from these studies usually bear no relation to those for the intact free drug.

The HPLC method reported here can individually quantitate CCNU and its metabolites and therefore gives precise, unambiguous results. Furthermore, the ability to quantitate the monohydroxylated metabolites makes it particularly suitable for studies of the metabolism of CCNU in patients, for

Table 3. Initial conditions and exposures required for CCNU cytotoxicity in several tumour cell lines in vitro

Tumour cell line	Details	End-point	Exposure time (h)	Initial concentration (μg/ml)	Exposure[a] (μg h/ml)	Reference (μg h/ml)
L1210 mouse leukaemia	Log phase cells	'Minimum cytotoxic concentration'	3	2	2.20	[24]
9L rat brain tumour	Log phase cells	90% cell kill	1	6	4.07	[32]
EMT6/Ca/VJAC mouse mammary tumour	Log phase cells	90% cell kill	1	10	6.78	[27]
EMT6/Ca/VJAC mouse mammary tumour	250 μm spheroids	90% cell kill	1	3[b]–6[c]	2.03[b]–4.07[c]	Twentyman et al. (personal commun.)
EMT6/Ro mouse mammary tumour	Log phase cells (oxic and hypoxic)	90% cell kill	4	12	13.8	[12]
RIF-1 mouse sarcoma, clones of different ploidy	Log phase cells	90% cell kill	1	10[d]–30[e]	6.78[d]–20.4[e]	[17]
L23 human large cell lung carcinoma	Log phase cells	90% cell kill	1	10	6.78	Twentyman et al. (personal commun.)
Normal and transformed human embryo cells	Log phase cells	90% cell kill	2	4.7[f]–7[g]	4.58–6.83	[2]

[a] Calculated on the basis of exponential decay with a $t_{1/2}$ of 50 min [5, 9, 13, 16]. For the data of Wheeler et al. [32] the value is within 5% of those authors' own estimate
[b] Immediate disaggregation
[c] 24-h delay
[d] Diploid clones
[e] Tetraploid and octoploid clones
[f] Normal (IMR-90)
[g] Virally transformed (VA-13)

which there is virtually no information in the literature. The only study reported is that by Walker and Hilton [29], who gave CCNU as a 1-h IV infusion to four patients. At the end of the infusion they identified three different CCNU metabolites, namely *trans*-4-hydroxy-CCNU, *cis*-4-hydroxy-CCNU and a much smaller amount of *cis*-3-hydroxy-CCNU. With the exception of radiolabelled studies, the present work represents the first description of CCNU pharmacokinetics after oral administration in man.

The most striking observation is the complete absence of parent CCNU. However, the *cis*- and *trans*-4-hydroxy metabolites were detected, thus indicating complete conversion of CCNU to its metabolites during the 'first pass' through the gut and the liver.

Wheeler et al. [31] investigated the physicochemical properties and antitumour activity of *cis*- and *trans*-4-hydroxy CCNU. They were found to be somewhat less lipophilic than CCNU, but similar in aqueous stability, alkylating activity, and carbamoylating activity. Toxicity in mice was similar to CCNU, but the administered metabolites were about twice as potent against both the intraperitoneal and intracerebral L1210 mouse leukaemia, resulting in therapeutic indices two to three times better than the parent drug.

The present results demonstrate that the antitumour activity and systemic toxicity of oral CCNU in man must be due solely to the hydroxylated metabolites. This behaviour is to some extent in contrast with the pharmacokinetics in mice given CCNU by the IP route, where a significant amount of CCNU does reach the systemic circulation and represents a considerable proportion of the total nitrosoureas at early times (though only a small proportion of the total AUC) [5]. This difference may be important since the IP route was used in almost all of the preclinical studies on which the development of CCNU for clinical application was largely based.

Following oral administration, peak metabolite concentrations were reached within 3–4 h. Although peak concentrations of total nitrosoureas were very similar in the four patients reported here (0.8–0.9 μg/ml), an on-going study involving a larger number of patients has revealed a wider range (0.4 μg/ml–1.1 μg/ml) (Roberts et al., unpublished results). The between-patient variability of the plasma clearance half-lives of the nitrosourea metabolites is also about two-fold, ranging from approx. 1.3 to 2.9 h.

It is interesting to note that the longest half-life was seen in patient C, who was also receiving both the hepatic enzyme inducer phenobarbitone and the inhibitor cimetidine. Phenobarbitone increases the rate of clearance of CCNU in mice and rats [9; Lee and Workman, unpublished] and reduces its toxicity and antitumour activity in these species [9, 13, 14, 22; Workman et al., unpublished], while cimetidine has been reported to enhance haematological toxicity in patients receiving BCNU and steroids [19]. It was not possible to relate haematological effects to pharmacokinetic parameters in this small preliminary series, but we are now attempting to do this in a larger study. All four patients experienced similar acute gastrointestinal toxicity.

It may be informative to compare the nitrosourea concentrations we have found in patients with those required for antitumour activity in vitro and in mice. The two parameters we would expect to have most importance are peak

concentration and area under the concentration × time curve (AUC) [4, 5–7, 9, 32]. For patients receiving the standard dose of 130 mg/m^2 the peak plasma concentrations of total nitrosoureas are about 1 μg/ml and the $AUC_{0-\infty}$ values between 2 and 4 μg h/ml. Table 3 summarizes, for given exposure times, the initial CCNU concentrations required for in vitro cytotoxicity against a number of cell lines, and the calculated exposures (AUC) under these conditions. It will be seen that the peak concentrations in patients are below the required initial concentrations in vitro, and that the exposures are at the lower limit of those needed for in vitro activity.

Since the hydroxylated metabolites of CCNU may be twice as active as the parent drug it could be argued that the above comparison underestimates the possible effects of the observed nitrosourea exposures in man. However, for mice receiving an IP dose of CCNU the underestimation can only be minimal, since the majority of the exposure results from the hydroxylated metabolites, particularly *cis*- and *trans*-4-hydroxy CCNU as in man [5]. We have shown that an IP dose of 20 mg/kg CCNU produces a peak plasma total nitrosourea concentration of approx. 6 μg/ml and an AUC of 8.4 μg h/ml; this gives a growth delay of about 15 days in the CCNU-sensitive KHT sarcoma [4], which represents a > 5-log cell kill (compare data in [21, 22]). On the other hand, a lower dose of 5 mg/kg, which gives a peak concentration of 1.5 μg/ml and an AUC of 2 μg h/ml, very similar to those found in our patients, gives essentially no growth delay (although there may be up to a 2-log cell kill, see [21, 22]). We are at present obtaining similar data for other mouse tumours and in particular attempting to relate response to actual tumour exposure.

Taken overall we suggest that these comparisons support the view that nitrosourea exposures in man are at the low end of those required for cytotoxicity, even in sensitive cells, and that this may be a major factor contributing to the relatively poor clinical performance of nitrosoureas as compared with their excellent activity in many murine tumours and human tumour xenografts (e.g., [18, 26]). This further illustrates the importance of including pharmacokinetic studies in the early stages of cytotoxic drug development, both in animals and in man. It also suggests that improved clinical results might be obtained by administering nitrosoureas by intravenous, intra-arterial or intracavitary routes to give higher tumour concentrations, or by giving higher doses with marrow rescue.

The reversed-phase HPLC method described here for the assay of CCNU and its metabolites is sensitive, precise, and convenient to use. With slight modification, this method is also suitable for the analysis of other nitrosoureas such as MeCCNU (Semustine), BCNU (Carmustine), and chlorozotocin [7]. In addition, the use of cyanonitrile columns has allowed us to resolve the hydroxylated CCNU metabolites from the chemosensitizer benznidazole. The combination of benznidazole plus CCNU has shown promising activity in mice [23, 28, 33] and altered pharmacokinetics has been implicated in the mechanism of chemosensitization [7]. The effects of benznidazole on CCNU pharmacokinetics are now under investigation in a phase-I study in man.

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