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5-Fluorouracil causes alterations in the pharmacokinetic profile of tauromustine in NMRI mice

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Abstract. The pharmacokinetic profiles of 5-fluorouracil (5-FU) and tauromustine (TCNU) were investigated in NMRI mice following their administration either alone or as part of simultaneous or sequential combinations. The profile of 5-FU remained unaltered irrespective of the sequence of administration. However, following simultaneous administration of 5-FU and TCNU or pretreatment with 5-FU, plasma levels of TCNU were decreased by a factor of 2.5–3 as compared with those seen when TCNU was given alone. These decreased plasma levels were explained by an increase in the TCNU volume of distribution, although this was not due to alterations in TCNU plasma protein binding. These sequence-dependent alterations in the pharmacokinetic profile of TCNU correlate with and may explain previously reported differences in the anti-tumour activity and toxicity profile of this combination.

Introduction

Combination chemotherapy with 5-fluorouracil (5-FU) and chloroethyl nitrosoureas (CNUs) has typically proved to be successful in the treatment of colorectal cancer. In the mid-1970s it was shown that 5-FU given as a bolus injection in combination with methyl-CCNU produced a significant increase in response as compared with 5-FU given alone (31.8% vs 9.5%) [1]. More recent studies have employed continuous-infusion delivery of 5-FU in combination with CNUs and have also demonstrated therapeutic benefits against advanced colorectal cancers [14]. Consequently, combination chemotherapy with 5-FU and a CNU (MeCCNU) is now regarded as a standard regimen for the treatment of colorectal carcinomas [10].

In a recent review of treatment approaches to colorectal cancer the novel CNU tauromustine (TCNU) was considered to be a promising new agent [3]. However, when evaluated in combination with 5-FU in a clinical trial, this regimen produced unexpected negative activity, giving response rates lower than those achieved with either single agent [12]. Subsequent experimental studies with the combination of 5-FU plus TCNU have shown sequence-dependent activity. In vitro chemosensitivity studies against the human colon-cancer cell line HT 29 [4, 5] and a murine colon-tumour cell line, MAC 15A [5], demonstrated activity ranging from antagonism to synergy, depending upon the sequence of drug exposure. Extension of these in vitro studies against a well-differentiated murine colon-tumour model, MAC 29, in vivo again demonstrated sequence-dependent anti-tumour activity [8]. However, these effects were accompanied by sequence-dependent host toxicities [5, 6] such that the most effective combination sequence, 5-FU given 24 h after TCNU, was also the most toxic. Sequence-dependent toxicity profiles were especially apparent in the gastrointestinal tract and the liver, whereas acute bone marrow toxicity showed no sequence dependence and was high for all sequences. These experimental studies have suggested alterations in TCNU uptake [4], cell cycling [7] and DNA-repair enzyme (O^6-alkylguanine-DNA alkyltransferase) activity [8] as possible explanations for the sequence-dependent effects of this combination. As a consequence of these experimental studies, alterations in the combination sequencing were made, and by delaying the administration of 5-FU by 24 h, improvements in the response rates were seen [13]. No report of accompanying toxicities has been made.

The aim of this study was to investigate the pharmacokinetic profiles of both 5-FU and TCNU, given to NMRI mice as part of sequential combinations, to determine whether "whole-body factors" such as alterations in pharmacokinetics or metabolism may contribute to the previously reported sequence-dependent effects.

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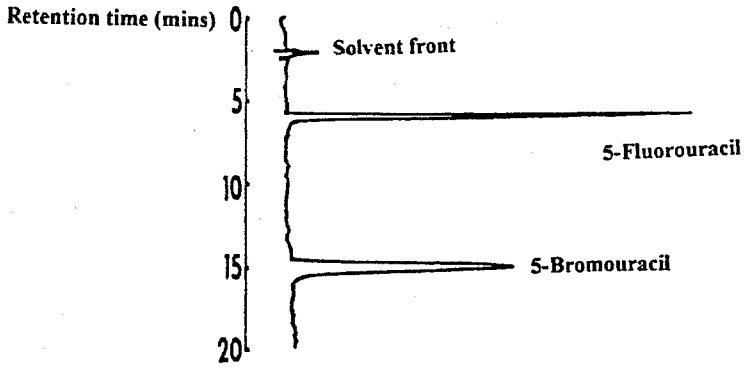

Fig. 1. A typical trace obtained from the reverse-phase HPLC analysis of plasma from NMRI mice treated with 125 mg kg^{-1} 5-FU

Materials and methods

Animals. Pure-strain male NMRI mice, aged 6–8 weeks, from our own inbred colony were used. They were given access to food (CRM pellet diet, Labsure, UK) and water ad libitum.

Treatments and sample collection. All treatment and sample-collection procedures were performed under the appropriate conditions and licences issued by the Home Office, London. 5-Fluorouracil (5-FU), a gift from Roche Pharmaceuticals (UK), and tauromustine (TCNU), a gift from Kabi Pharmacia (Sweden), were given via intraperitoneal injection at previously optimized doses [5] of 125 mg kg^{-1} 5-FU and 20 mg kg^{-1} TCNU using 0.9% saline as the vehicle. Each agent was given either alone (representing both the single-agent profile and that seen 24 h before administration of the second agent), simultaneously with, or 24 h after the second agent.

Blood samples were taken via cardiac puncture at appropriate time points following treatment and were placed in heparinized tubes to prevent clotting. They were then centrifuged (1500 *g* at 4° C for 10 min) and the plasma layer was removed and stored at –20° C until analysis. For simultaneous treatments the plasma was aliquotted into two samples for the analysis of each agent, thus reducing the number of animals used. All plasma for TCNU analysis was acidified with 0.1 *M* hydrochloric acid (10 µl ml^{-1} plasma) to reduce decomposition during storage.

Sample extraction and chromatography. All solvents used in sample extraction and analysis were of high-performance liquid chromatographic (HPLC) grade (Rhône Poulenc, UK) and all other reagents were of analytical grade. Triple-distilled water was used throughout the preparation of aqueous buffers.

The procedure used for extraction of 5-FU from plasma and subsequent reverse-phase HPLC separation was that described by Loadman et al. [9]. The extraction and reverse-phase HPLC of TCNU were performed using the methodology of Polacek et al. [11] with some modifications. Briefly, after the addition of 0.5 µg ml^{-1} 4′-bromoacetanilide (BAA; Sigma, UK) as an internal standard, plasma samples were extracted using phenyl solid-phase extraction cartridges (100 mg; Bond Elut, UK) and eluted in 400-µl volumes of 0.1% glacial acetic acid in methanol. Separation was carried out on a LiChrosorb CN column (250×4 mm, 7-µm particle diameter; Merck, UK) using an isocratic mobile phase of acetonitrile (2.2%), methanol (1.7%) in 5 m*M* phosphate buffer (pH 4.0) pumped at a flow rate of 1 ml min^{-1}. This method allowed for good separation and identification of TCNU and its mono- and di-demethylated metabolites with no plasma interference.

Calibration curves and extraction efficiency. A range of calibration standards (0–1000 µg ml^{-1}) were prepared by spiking untreated "blank" mouse plasma with appropriate concentrations of test compound and internal standard. Plasma for TCNU and metabolite calibration standards was acidified in a similar manner to that used for the samples. Mono- (dm-TCNU) and di-demethyl TCNU (ddm-TCNU) were kindly provided by Kabi Pharmacia (Sweden). After extraction the area ratios of drug to internal standard were calculated and plotted against the concentration. The extraction efficiency was calculated by dividing the area ratio for the compound extracted from plasma by the area ratio at the same concentration in buffer, and this was expressed as a percentage. Six samples were used for each of these calculations.

Calculation of pharmacokinetic parameters. C_{max} and T_{max} values were determined from the observed plasma concentration versus time curve as the maximal plasma concentration of drug and the corresponding time. The elimination rate constant (K_{el}) was calculated by log-linear regression analysis of the points on the terminal log-linear part of the plasma concentration versus time curve. The terminal half-life ($t_{1/2}$) was determined from the equation $t_{1/2} = \ln 2/K_{el}$. The area under the plasma concentration versus time curve (AUC) was calculated by trapezoidal integration from time 0 to the final time point t_z and extrapolated using the equation $AUC_{tz-t\infty} = C$ at t_z/K_{el}. Finally, the volume of distribution (V_d) was calculated by back-extrapolation of the terminal slope of the plasma concentration versus time curve to give a theoretical value of the instantaneous concentration at time 0 (C_0), which was used in the equation V_d = dose given/C_0.

TCNU plasma protein-binding studies. TCNU-spiked "blank" plasma samples (10 µg ml^{-1}) were also extracted via "centrifree micropartition system" cartridges (Amicon, UK). This involved the ultrafiltration of samples through narrow pore membranes (10-kDa cutoff) under centrifugal force and allowed for the quantification of free drug and, thus, the extent of plasma-protein binding as compared with the previous method, which could quantify only total drug plasma concentrations. TCNU-containing samples were also spiked with either 5-FU (100 µg ml^{-1}) or a 5-FU catabolite, α-fluoro-β-alanine (100 µg ml^{-1}; kindly provided by Dr. Ross Maxwell, Biochemistry Department, St. George's Hospital, London). The volume of drug/buffer added to plasma samples was maintained at <5% to insure that protein binding was affected as little as possible. All extracted samples were analysed by reverse-phase HPLC as described above.

Results

Chromatography

The HPLC methodologies used for detection of 5-FU, TCNU and the two demethylated TCNU metabolites produced good resolution and sensitivity (Figs. 1, 2). Extraction from a 100-µl plasma sample gave limits of detection of 50 ng ml^{-1} for 5-FU and 100 ng ml^{-1} for TCNU and the metabolites (representing 0.5 and 1 ng on column injections, respectively). Extraction efficiencies for the test compounds as determined at 10 µg ml^{-1} were: 5-FU, 90.01%±2.07%; TCNU, 90.91%±3.96%; dm-TCNU, 84.72%±5.57; and ddm-TCNU, 84.30%±2.73%. Cali-

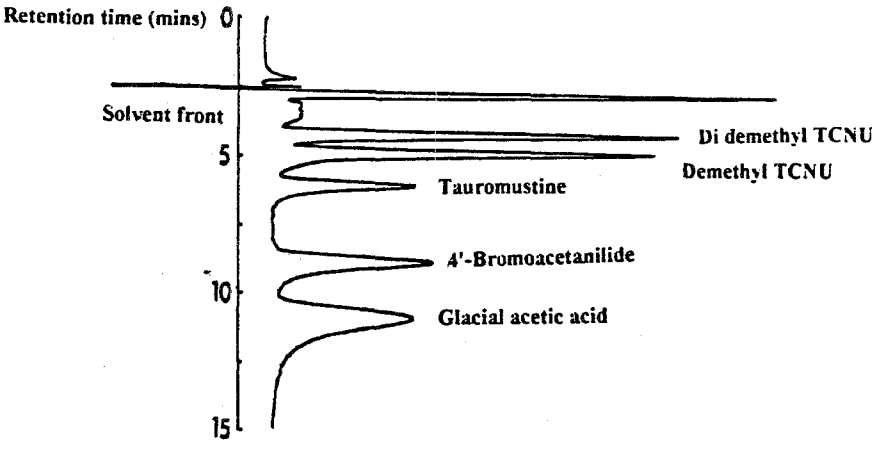

Fig. 2. A typical trace obtained from the reverse-phase HPLC analysis of plasma from NMRI mice treated with 20 mg kg^{-1} TCNU

bration curves for each agent were linear over the range tested (0–1000 μg ml^{-1}).

Pharmacokinetic profiles

The mean plasma clearance curves generated for 125 mg kg^{-1} 5-FU given as a single agent or as part of a simultaneous or sequential combination with TCNU were essentially the same (curves not shown). The pharmacokinetic parameters derived from these curves are shown in Table 1 and reflect the similar profiles of the three sequences of 5-FU administration. The slight differences observed in T_{max} and C_{max} values yielded no significance when tested by analysis of variance (ANOVA, $P > 0.05$) and reflect the individual variability between mice at these short time points.

The mean plasma clearance curves generated for 20 mg kg^{-1} TCNU given as a single agent or as part of a simultaneous or sequential combination with 5-FU are shown in Fig. 3. These curves clearly demonstrate that the pharmacokinetic profile of TCNU following simultaneous administration of 5-FU and TCNU or pretreatment with 5-FU is different from that of TCNU given alone. These differences are reflected in the derived pharmacokinetic parameters shown in Table 2. Peak TCNU plasma levels (C_{max}) and, indeed, all TCNU plasma clearance curves were significantly lowered following simultaneous administration of 5-FU and TCNU or pretreatment with 5-FU ($P < 0.05$, ANOVA). This was mirrored by significant decreases in AUC values and significant increases in calculated V_d values ($P < 0.05$, ANOVA), although no change was seen in T_{max}, K_{el} or $t_{1/2}$ values.

The plasma profiles obtained for the demethylated metabolites following each sequence of treatment with TCNU are shown in Fig. 4. The appearance of the metabolites in plasma follows the expected time course, with parent TCNU preceding the mono-demethylated metabolite, which in turn precedes the di-demethylated metabolites. Despite the sequence-dependent alterations observed in the TCNU plasma clearance profiles (Fig. 3), no significant difference was seen in the pharmacokinetic profile of either metabolite.

Protein-binding studies

The plasma protein binding of TCNU in spiked plasma (10 μg ml^{-1}) is shown in Fig. 5. As expected, no TCNU binding was seen in phosphate buffer; however, in plasma

Table 1. Pharmacokinetic parameters of 5-FU (n = 3)

	T_{max} (min)	C_{max} (μg ml^{-1})	AUC (μg h ml^{-1})	$T_{1/2}$ (h)	K_{el} (h^{-1})	V_d (ml)
5-FU alone	5	124.72±20.95	50.04±9.49	0.17±0.04	4.42±1.16	13.63±6.01
5-FU with TCNU	15	78.51±18.25	39.92±5.69	0.12±0.02	5.79±0.58	7.47±2.97
5-FU after TCNU	15	107.25±23.50	48.41±8.12	0.13±0.04	5.82±1.89	8.03±5.71

Table 2. Pharmacokinetic parameters of TCNU (n = 3)

	T_{max} (min)	C_{max} (μg ml^{-1})	AUC (μg h ml^{-1})	$T_{1/2}$ (h)	K_{el} (h^{-1})	V_d (ml)
TCNU alone	2	50.91±13.33	9.59±2.78	0.10±0.00	7.05±0.17	9.10±2.00
TCNU with 5-FU	2	13.61± 1.25*	2.84±0.24*	0.10±0.01	6.79±0.55	27.20±3.70*
TCNU after 5-FU	2	18.41±3.32*	3.30±1.20*	0.09±0.03	7.90±2.28	21.10±2.70*

* $P < 0.05$, ANOVA

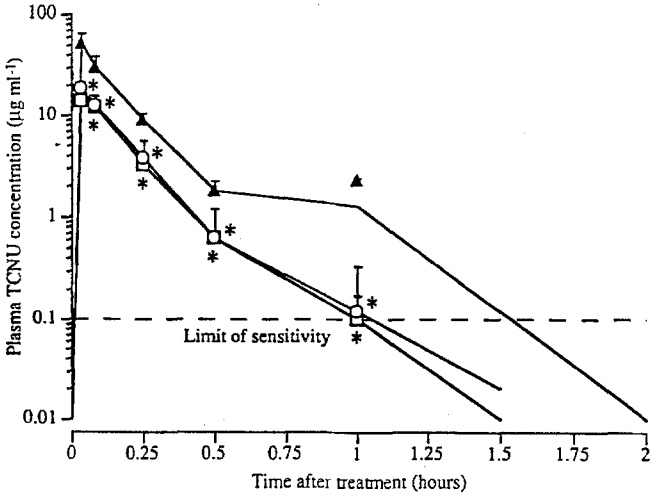

Fig. 3. TCNU plasma clearance curves in NMRI mice following administration of 20 mg kg^{-1} TCNU either alone (–▲–) or as part of a simultaneous (–□–) or sequential (–○–) combination with 5-FU. Plasma levels at 1.5-h time points fell below the limit of sensitivity; therefore, curves have been extrapolated using K_{el} and $t_{1/2}$ values. Each point represents the mean value + SD from 3 experiments. * $P < 0.05$, ANOVA

the percentage of free TCNU was only 46.96% ± 7.14%. The inclusion of 5-FU (100 μg ml^{-1}) or the 5-FU catabolite, α-fluoro-β-alanine (100 μg ml^{-1}), in the TCNU-spiked plasma produced no effect on the extent of TCNU protein binding. The free TCNU proportions were determined as 47.86% ± 3.08% and 46.83% ± 4.27%, respectively.

Discussion

Previous in vivo studies performed in this laboratory using NMRI mice have demonstrated that the combination of 5-FU plus TCNU produces sequence-dependent effects. These effects were manifested in terms of both anti-tumour activity [8] and host toxicity [6] such that increased activity was associated with increased toxicity. This study investigated the pharmacokinetic profiles of both 5-FU and TCNU given to NMRI mice as part of sequential combinations to determine whether "whole-body factors", such as pharmacokinetics and metabolism, may contribute to these sequence-dependent effects.

The pharmacokinetic profile of 5-FU was seen to be unaltered by the sequence of administration (Table 1); however, this was not the case for TCNU. Simultaneous administration of 5-FU and TCNU or pretreatment with 5-FU resulted in significant decreases in both the peak plasma levels of TCNU and the areas under the plasma clearance curves as compared with those of TCNU given alone (Fig. 3, Table 2). One possible explanation for the decreases in plasma levels of TCNU may be a reduction in uptake from the peritoneal cavity (the site of injection) into the bloodstream. This would corroborate the previous in vitro work of Hartley-Asp and Alenfall [4], who demonstrated decreases in the cellular and nuclear uptake of TCNU following pretreatment with 5-FU. However, the pharmacokinetic profiles of the mono- and di-demethylated metabolites of TCNU exhibited no sequence-dependent

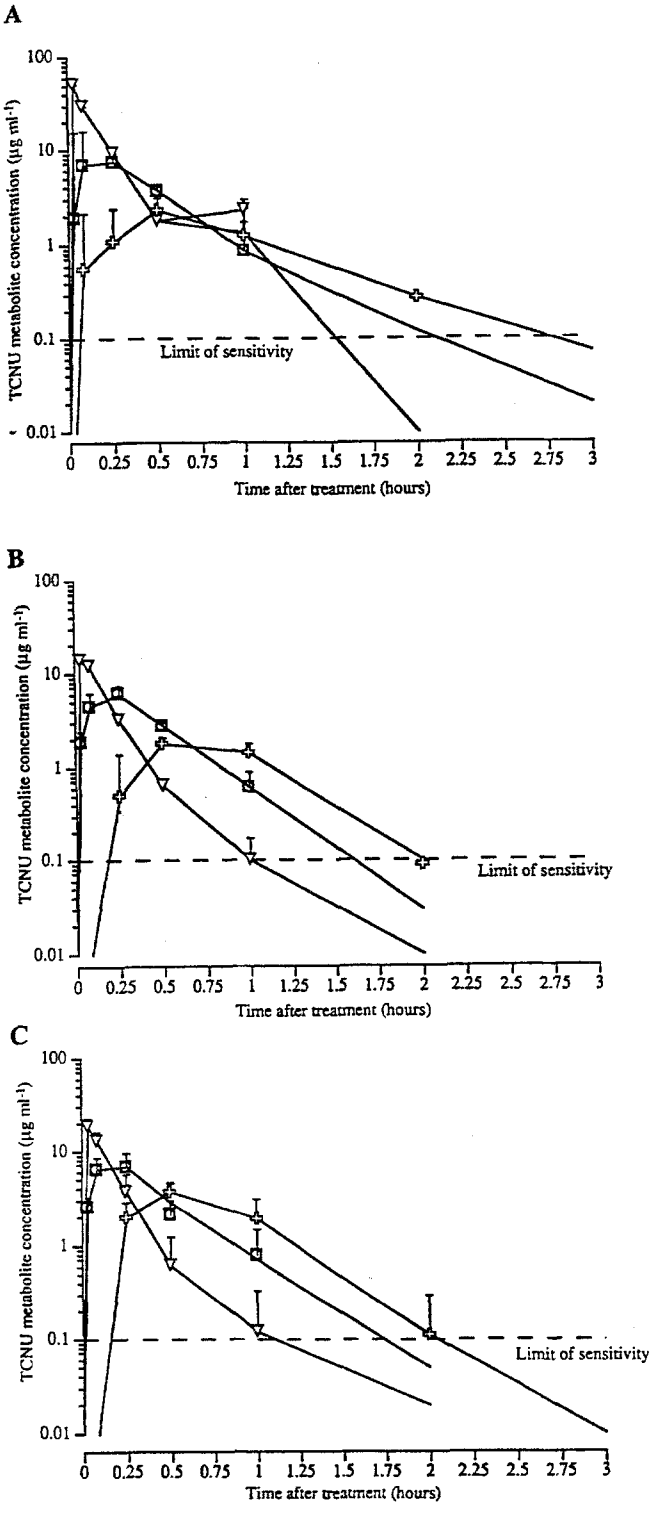

Fig. 4A–C. Plasma profiles of TCNU (–▽–) and its mono-demethylated (–⊟–) and di-demethylated (–✠–) metabolites in NMRI mice following administration of 20 mg kg^{-1} TCNU either alone (**A**) or as part of a simultaneous (**B**) or sequential (**C**) combination with 5-FU. Plasma levels at 2-h time points fell below the limit of sensitivity; therefore, curves have been extrapolated using K_{el} and $t_{1/2}$ values. Each point represents the mean value + SD from 3 experiments

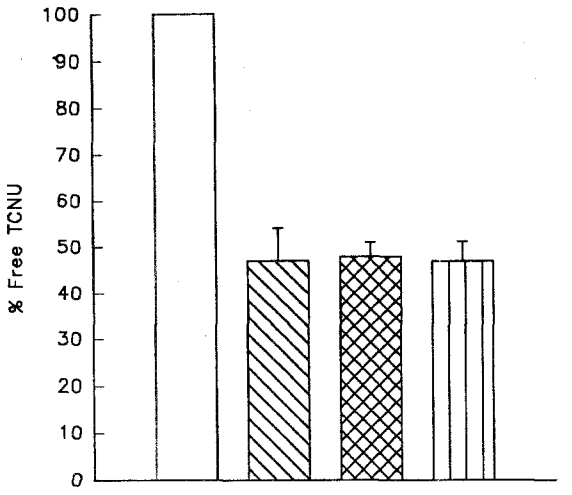

Fig. 5. Proportions of free TCNU in 5 m*M* phosphate buffer (▭) or spiked blank mouse plasma both alone (▨) and in the presence of 5-FU (▩) or the 5-FU catabolite α-fluoro-β-alanine (▥). Each bar represents the mean value + SD from 3 experiments

difference (Fig. 4). Such differences may have been expected if alterations in parent TCNU uptake were occurring. This lack of difference in the metabolite profiles also suggests that equal levels of TCNU are present in the liver, the primary site of metabolism, regardless of the sequence of administration.

Since the K_{el} and $t_{1/2}$ values obtained for TCNU following each sequence remain unaltered, the decreases in TCNU plasma levels might be associated with an increase in the volume of TCNU distribution (V_d). This possibility was confirmed by the calculation of the apparent V_d values for each sequence (Table 2). These increases in V_d, amounting to factors of approximately 2.5–3, correlate with decreases in both C_{max} and AUC values of a similar scale. One common cause of increases in the apparent volume of distribution is the displacement of plasma protein-bound drug. However, the protein-binding studies presented herein show that although TCNU is approximately 50% plasma protein-bound, the presence of 5-FU or its catabolite α-fluoro-β-alanine did not increase the percentage of free TCNU in the plasma (Fig. 5). Further possible explanations for the increase in TCNU distribution following simultaneous administration of 5-FU and TCNU or pretreatment with 5-FU are a displacement of tissue-bound drug or the distribution of TCNU into a normally inaccessible compartment. Extensive studies measuring the tissue levels of TCNU are needed to confirm or discount these possibilities. Such an increase in the TCNU V_d would also be accompanied by an increase in TCNU clearance (Cl), since $Cl = K_{el} \times V_d$ and K_{el} remains constant irrespective of the sequence of administration. Although this associated increase in Cl, following pretreatment or simultaneous treatment with 5-FU is evident from fitting of the collected data into the relevant pharmacokinetic equation, any drug-induced change in clearance may be measured by following the clearance profile of a marker compound such as inulin [2]. This would also determine whether combination with 5-FU is likely to affect the pharmacokinetics of other agents in addition to TCNU.

Although the reason for the alterations in the pharmacokinetic profile of TCNU described herein are not fully understood, they do offer a further explanation for the sequence-dependent anti-tumour activity and toxicities seen during our previous studies with this combination in NMRI mice. The pharmacokinetic profiles suggest that more TCNU is present in the plasma following the sequential combination of TCNU given 24 h before 5-FU (equivalent to TCNU alone in this study) as compared with the simultaneous or reverse sequential combinations. In our previous studies this sequence of administration was seen to be both the most active and the most toxic for this combination. This study also suggests that since no alteration was seen in the pharmacokinetic profile of 5-FU, TCNU is the primary determinant of the activity and toxicity in this combination.

It should be noted that although this study may provide an explanation for the sequence-dependent effects seen with the combination of 5-FU plus TCNU in NMRI mice, these results may not explain the negative results seen in the clinic with simultaneous combination of these agents [12] or the subsequent improvements seen with sequential administration [13]. There are obvious differences in the scheduling and routes of administration of the combination, 5-FU being given via infusion and TCNU being given orally in the clinic. Ongoing studies in this laboratory are utilising subcutaneous osmotic pumps so as to simulate 24 h infusion of 5-FU and determine whether the alterations in TCNU pharmacokinetics remain.

In conclusion, this study demonstrates that in NMRI mice, simultaneous administration of 5-FU and TCNU or pretreatment with 5-FU significantly alters the pharmacokinetic profile of TCNU. These alterations provide a plausible explanation for the sequence-dependent anti-tumour activity and toxicity profiles seen with this combination in previous studies. No alteration in TCNU metabolism or TCNU plasma protein binding was seen and, thus, no obvious explanation for the altered plasma levels of TCNU was apparent. The unexpected alterations found in the pharmacokinetic profile of TCNU when it was given in combination with 5-FU in this study demonstrate the role of pharmacokinetic factors as determinants for anti-tumour activity and host toxicity. No other report indicating that 5-FU interacts with other agents in such a way to alter their pharmacokinetics has been found. However, if such interactions were to occur, they would obviously have great implications as to the sequencing of 5-FU-containing combination regimens. Consequently this also demonstrates the importance of performing such studies both experimentally and in the clinic.

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