INVOLVEMENT OF GLUTATHIONE IN THE METABOLISM OF THE ANILINOACRIDINE ANTITUMOUR AGENTS CI-921 AND AMSACRINE

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SUMMARY

4'-(9-Acridinylamino)methanesulphon-m-anisidide (amsacrine) and CI-921, the 4-methyl-5-(N-methyl-carboxamide) derivative of amsacrine, are two anilinoacridine antitumour agents in clinical use or trial. The elimination of these agents has been investigated in male BDF1 mice. 74% and 86% of the dose of [acridinyl-G-^{3}H]-amsacrine and -CI-921, respectively, was excreted in the faeces of mice by 72 hr after i.v. injection. Administration of both compounds also resulted in significant depletion of glutathione (GSH) in mouse liver, although the effect of CI-921 was delayed and reduced compared with amsacrine. In mouse bile, radiolabelled products which cochromatographed with amsacrine GSH conjugates at both the 5'- and 6'-positions of the anilino ring were present in similar amounts and constituted approximately 70% of the excreted radioactivity, the balance being minor, more polar metabolites. With hepatic microsomal fractions, both conjugates of amsacrine were formed but only the 6'- and not the 5'- conjugate was increased in the presence of cytosol. Preliminary evidence indicates the presence in mouse bile of at least the 5'-GSH conjugate of CI-921, and several other GSH derived products not seen with amsacrine. It is concluded that the elimination of CI-921 occurs by a mechanism similar to that of amsacrine. Further, the possible involvement of GSH transferase in the conjugation of amsacrine may have consequences for the hepatotoxicity of this agent.

I. INTRODUCTION

Amsacrine [4'-(9-acridinylamino)methanesulphon-m-anisidide] is used for treatment of acute leukaemia /1/. CI-921, the 4-methyl-5-(N-methyl-carboxamide) derivative of amsacrine is currently in phase II clinical trials. CI-921 was selected for further development primarily because of greater antitumour activity compared to amsacrine against experimental solid tumours /2/. The structures of the two compounds are given in Fig. 1.

The elimination of amsacrine is thought to occur by oxidative metabolism to a reactive quinone diimine intermediate with subsequent conjugation with GSH at the 5'-position of the anilino ring and excretion via the bile /3-7/. Recently, an additional 6'-GSH conjugate of amsacrine has been synthesised /8/. It was thus of interest to see if this conjugate is formed *in vivo*. In addition, we wished to determine if conjugation with GSH was also a significant pathway of elimination for CI-921.

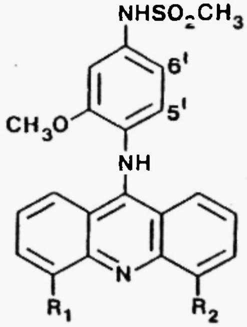

Fig. 1: Structures of amsacrine (R1 = R2 = H) and CI-921 (R1 = CH3; R2 = CONHCH3).

II. MATERIALS AND METHODS

Amsacrine, CI-921 and [acridinyl-G-^{3}H]-CI-921 (^{3}H-CI-921; 4.1 Ci/ mol; 97% radiochemical purity) were provided as the isethionate (2-hydroxyethanesulphonate) salts by Warner-Lambert Laboratories (Ann Arbor, U.S.A.). [Acridinyl-G-^{3}H]-amsacrine (^{3}H-amsacrine; hydrochloride salt; 406 Ci/μmol; 99% radiochemical purity) was provided by Dr W.A. Denny (Cancer Research Laboratory) and diluted to 12.9 Ci/μmol with unlabelled amsacrine. The standard GSH conjugates of amsacrine and CI-921 were prepared by Drs. W.A. Denny and B.D. Palmer according to the method of Shoemaker et al. /5/. Glucose-6-phosphate dehydrogenase, glucose-6-phosphate, NADP and reduced GSH were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). All other reagents were of analytical grade.

Microsomal and cytosolic fractions were prepared essentially as described /7/ from the livers of male BDF1 mice (20-25g). The cytosolic fraction was dialysed against 1.15% KCl, 2 mM sodium phosphate, pH 7.4. Microsomal incubations (0.5 ml) contained 20 M amsacrine (dissolved in dimethylacetamide, final concentration 0.04%), 0.2 mg microsomal protein, 0.2 mM GSH, 12 mM glucose-6-phosphate, 4 mM $MgCl_2$, 0.33 mM NADP, 1 unit glucose-6-phosphate dehydrogenase and 0 to 4 mg cytosolic protein in 50 mM sodium phosphate, pH 7.4. Incubations were for 5 min at 37° and the reaction was stopped by the addition of an equal volume of cold acetone. The samples were extracted with water-saturated ethyl acetate (2 x 2.5 ml) and hexane:carbon tetrachloride (9:1, v/v; 1 x 2.0 ml). The aqueous phase was centrifuged (10,000 g/4 min) to remove particulate material and aliquots analysed directly by reversed-phase HPLC. The Waters HPLC system consisted of a WISP 710 automatic sample injector, 6000A pump, RCM-100 compression module fitted with a C_{18} 4 µNovapak cartridge (8mm x 10cm) and a 440 absorbance detector. The mobile phase was 19% acetonitrile, 10 mM triethylammonium phosphate (TEAP) pH 3, at a flow rate of 1.8 ml/min and detection was by UV at 254 nm.

For *in vivo* treatments for determination of excretion of radiolabel and for the analysis of mouse bile, ^3H-amsacrine (approximately 20µCi; 57.7 mol/kg; constituted in dimethylacetamide/water) was injected into the tail vein of male BDF_1 mice. Mice were held in glass Metabowl cages (Jenkins, U.K.) allowing the separate collection of urine and faeces. At 72 hours the mice were killed by cervical dislocation. The carcasses and faeces were homogenised in water (4:1, v/g), and aliquots digested with Protosol (NEN, U.S.A.) and decolourised with hydrogen peroxide, before determination of radioactivity by liquid scintillation counting. For the analysis of mouse bile, the mice were killed by cervical dislocation at 3.5 to 4 hours after injection and the gall bladders removed. The bile samples were pooled and diluted in water, absorbed to 3 ml C_{18} Bond-Elut cartridges (Analyticalchem International, U.S.A.; which had been solvated with methanol and water), washed with water, eluted with 95% methanol 5% 1 M ammonium acetate, pH 4, and reduced to dryness. Under these conditions, approximately 85% of the radiolabel was recovered from the C_{18} cartridge. The samples were reconstituted in mobile phase (16% acetonitrile, 10 mM TEAP, pH 3) and analysed by HPLC as above. Fractions (1 ml) were collected and radioactivity determined by liquid scintillation counting. With CI-921, the same dose of unlabelled compound was used and the gall bladders removed at 2 hours. The samples were diluted in mobile phase (23% acetonitrile, 10 mM TEAP, pH3), centrifuged and assayed directly by HPLC.

For purification of the 5'-GSH conjugate of CI-921, the major peak fraction of the synthetic standard (Fig. 4, panel C) was collected after HPLC. Multiple samples were pooled and concentrated on a C_{18} cartridge as described above. The purified sample was dissolved in d_6-dimethylsulphoxide for NMR analysis. Fourier ^{1}H-NMR spectra were obtained on a Bruker AH400 spectrometer at 400 MHz and 294K using a 5mm proton probe and tetramethylsilane as a reference. Absorption spectra were obtained during HPLC with a Hewlett-Packard diode array detector.

For determination of reduction in total hepatic GSH, mice were injected i.p. with amsacrine or CI-921 (190 and 170μmol/kg, respectively). The mice were sacrificed by cervical dislocation and total GSH concentrations were determined as described /9/.

TABLE 1

Excretion of ^{3}H-radioactivity after i.v. administration of ^{3}H-amsacrine or ^{3}H-CI-921 (57.7 μmol/kg) to BDF1 mice

		^{3}H-amsacrine	^{3}H-CI-921
URINE	0 - 24 hr	13.5 ± 1.1[1]	7.7 ± 0.1
	24 - 48 hr	2.9 ± 0.6	0.7 ± 0.1
	48 - 72 hr	0.5 ± 0.1	0.4 ± 0.2
		16.9 ± 0.7	8.8 ± 0.3
FAECES	0 - 24 hr	62.3 ± 0.1	74.3 ± 3.9
	24 - 48 hr	7.9 ± 2.0	11.0 ± 0.7
	48 - 72 hr	3.9 ± 2.2	0.5 ± 0.4
		74.1 ± 0.1	85.8 ± 4.2
CARCASS	(72 hr)	4.1 ± 0.6	6.2 ± 1.2
TOTAL		95.1 ± 0.1	100.6 ± 3.6

[1] Percentage of dose administered, x ± S.D. (n = 3)

III. RESULTS

3.1 Excretion of ^{3}H-radioactivity

The excretion of ^{3}H-radioactivity after i.v. treatment of mice with ^{3}H-amsacrine or ^{3}H-CI-921 is shown in Table 1. The majority of the radiolabel in both cases was eliminated via the faeces within 24 hr.

3.2 Depletion of total hepatic glutathione

Treatment of mice with either amsacrine or CI-921 resulted in a decrease of total hepatic GSH concentrations up to 85% and 43%, respectively (Fig. 2). However, the reduction with CI-921 was less and delayed compared with amsacrine.

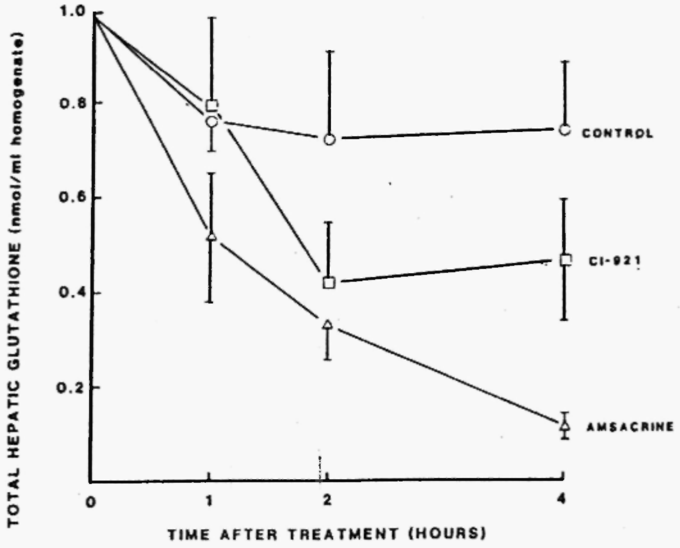

Fig. 2: Reduction in hepatic GSH concentrations after i.p. treatment of male BDF1 mice with amsacrine or CI-921. Values are the mean and S.D. (n = 3). Untreated control (○), amsacrine (△) and CI-921 (□), 190 and 170µmol/kg, respectively.

3.3 Detection of GSH products in mouse bile

The HPLC-UV and radiolabel profiles of mouse bile obtained after the treatment of mice with ^{3}H-amsacrine are shown in Fig. 3.

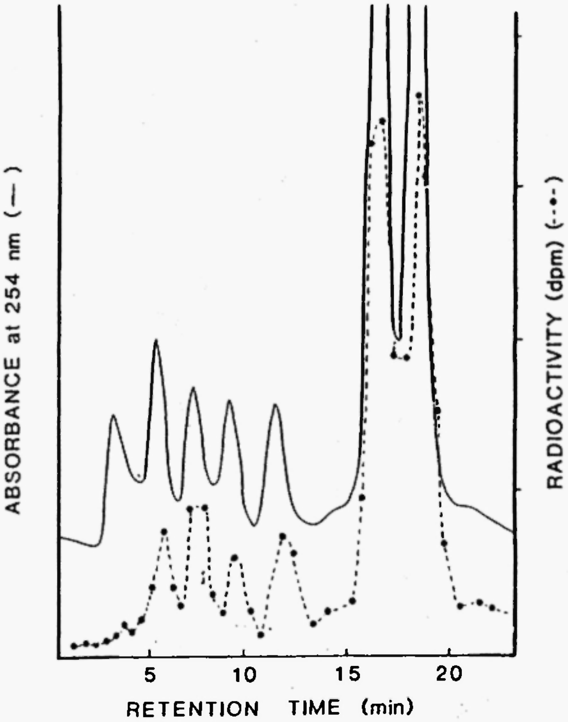

Fig. 3: HPLC-UV chromatogram and associated radiolabel profile of sample obtained from mouse bile after i.v. treatment with ^{3}H-amsacrine (57.7μmol/kg). Fractions eluting at 16.5 and 18.4 min correspond to the 5'- and 6'-GSH conjugates of amsacrine, respectively.

Two major peaks were obtained which cochromatographed with the authentic 5'- and 6'-GS-amsacrine conjugates with retention times of 16.5 and 18.4 min, respectively. These two components were detected in approximately equal quantities and comprised 70% of the recovered radiolabel. The remaining radiolabel was associated with four more polar, and as yet unidentified, products. A similar experiment was done with CI-921. A pattern of products similar to that of a synthetic standard, prepared by the reaction of the quinone diimine derivative of CI-921 and GSH, was obtained in mouse bile after treatment with CI-921 (Fig. 4). The major peak fraction in the synthetic standard, eluting at approximately 6 min, appears to contain two major components. However, attempts to separate these components have not been successful and identical UV/visible absorbance spectra are obtained during HPLC (diode array detector; maxima at 227, 265 and 449 nm). This peak fraction has been purified and, on ^{1}H-NMR analysis, doublets are apparent at chemical shifts of $\delta = 6.74$ and 6.93 ppm ($J_{2',6'} = 1.71$), resulting from the 2'- and 6'-phenyl aromatic protons. Computer assisted analysis of the UV/visible absorbance spectra obtained during HPLC also indicate the presence of a minor component with maxima at 265 and and 451 nm and a pronounced minimum at 237 nm.

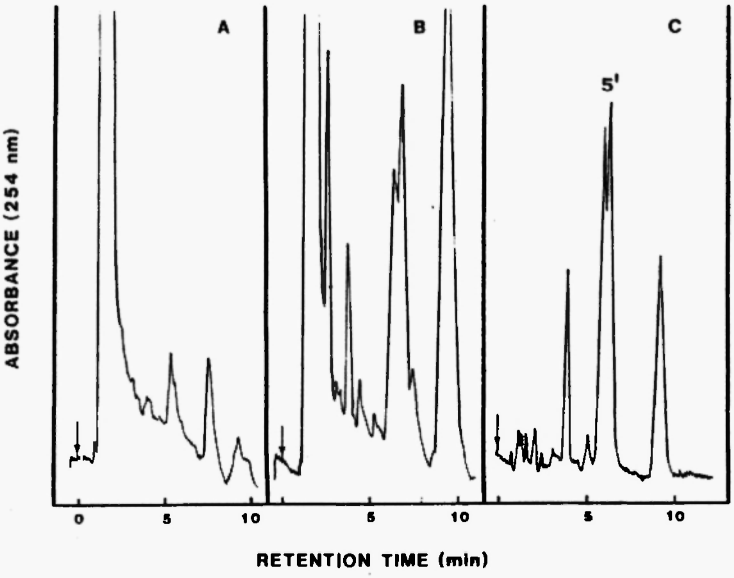

Fig. 4: HPLC-UV chromatogram of mouse bile after treatment with CI-921. A: untreated control; B: CI-921. 57.7μmol/kg; C: synthetic standard prepared by the reaction of the quinone diimine derivative of CI-921 and GSH.

3.4 In vitro formation of 5'-and 6'-GS-amsacrine conjugates

The *in vitro* formation of the 5'- and 6'-GS-amsacrine conjugates in incubations with mouse liver microsomal and cytosolic fractions is shown in Fig. 5. In the absence of the cytosolic fraction similar amounts of both isomers were formed. However, when the cytosolic fraction was included, the formation of the 6'-conjugate was increased, and that of the 5'-conjugate decreased such that the ratio of 6'- to 5'-conjugates was approximately 5.5. No effect was observed with cytosolic fraction which had been heated (90°, 10 min).

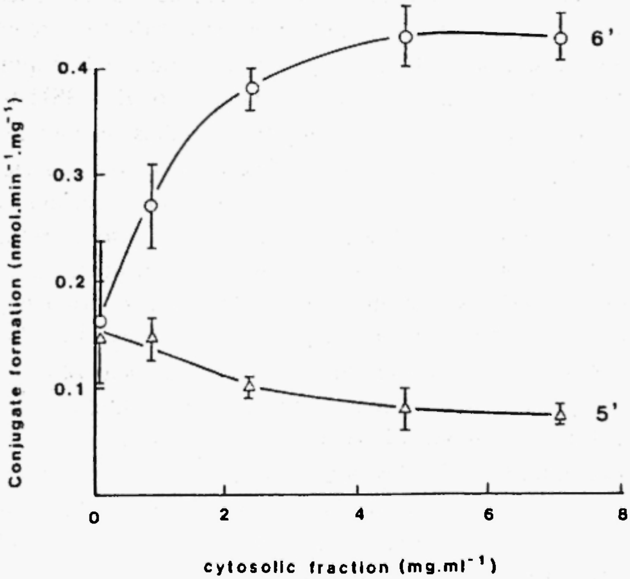

Fig. 5: Effect of addition of cytosolic fraction on the mouse hepatic microsomal mediated formation of 6'- (○) and 5'- (△) GS-amsacrine.

IV. DISCUSSION

Considerable work on the elimination of amsacrine has been done /3-7/. However, these workers failed to observe the formation of the GSH conjugate at the 6'-position of the anilino ring of amsacrine. This product has been recently synthesised by reaction of the quinone diimine derivative of amsacrine with GSH /8/. A possible reason for the lack of prior detection is the relative instability of the 6'-isomer /8,10/.

Radiolabelled components corresponding to both the 5'- and 6'-GSH conjugates were detected in approximately equal amounts in bile after treatment of mice with amsacrine. The main interest in the 6'-GSH conjugate, however, comes from the observation that the formation of this isomer, but not the 5'-conjugate, was increased in hepatic microsomal incubations by the addition of the cytosolic fraction (Fig. 5). This indicates that the formation of this isomer may be catalysed by GSH transferase. Consequently the toxicity of amsacrine might be increased by other clinical agents such as ethacrynic acid /11/ which might interfere with this reaction, resulting in a decreased rate of conjugation of the reactive quinone diimine intermediate. The possible involvement of the GSH transferases is thus being investigated further in studies on metabolism dependent changes in the elimination and hepatotoxicity of amsacrine.

The second question was the main route and mechanism of elimination of CI-921. The majority of CI-921 was also excreted via the faeces, to a greater extent than that observed for amsacrine. The involvement of GSH is indicated by the depletion of total hepatic glutathione on treatment of mice with CI-921. although the effect is not as pronounced as for amsacrine. Further, the toxicity of CI-921 is increased by pretreatment of mice with buthionine sulphoximine (J. Hardy, personal communication). Preliminary results also indicate the presence in mouse bile of products of the reaction of CI-921 with GSH (Fig. 4). The pattern of products obtained with CI-921 is more complex than with amsacrine, both in the synthetic standard, prepared from reaction of the quinone diimine derivative of CI-921 and GSH, and in bile. One of the products in bile cochromatographs with a synthetic product which has been identified as the 5'-GSH conjugate of CI-921. The UV/vis absorbance spectrum of a minor component in the standard is also very similar to that of the 6'-GSH conjugate of amsacrine. We are continuing with work to purify and identify the remaining components. However, it appears that the elimination of CI-921 follows a similar path to that of amsacrine.

V. ACKNOWLEDGMENTS

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