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SHORT COMMUNICATIONS

Biliary excretion and enterohepatic circulation of aniline mustard metabolites in the rat and the rabbit

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Aniline mustard [*N*,*N*-di-(2-chloroethyl) aniline (AM)] (Fig. 1) has been used successfully by Whisson and Connors to cure a mouse plasma cell tumour (Adj PC5), which has certain features in common with human multiple myeloma [1, 2]. Double and Workman [3] have recently reported another mouse tumour (HT 67), which is cured by this cytotoxic agent. These achievements have been attributed to a latent activity of the drug, which is metabolized to the glucuronic acid conjugate (AMGL) (Fig. 1) of 4-hydroxyaniline mustard in the liver, and subsequently deconjugated to yield the more toxic 4-hydroxyaniline mustard (AMOH) (Fig. 1) in the high β-glucuronidase environment of the tumour cells [4, 5]. Only a limited response was observed when AM was used in clinical trials for the treatment of multiple myeloma [6]. However, tumour regression did occur in conjunction with high levels of β-glucuronidase, in certain human prostate and kidney lesions [7].

The relatively non-toxic glucuronide (AMGL) is a major metabolite in rats and is excreted via the bile [5]. Hydrolysis of the conjugate by the β-glucuronidase of the intestinal microflora may release the more toxic AMOH into the intestine where it may be reabsorbed into the body to undergo an enterohepatic circulation. The aim of the present work is to assess the extent of reabsorption of reactive metabolites from the intestine following biliary excretion in the rat. The rabbit appears to be more similar to man than is the rat in terms of the extent to which it excretes foreign compounds in the bile [8]. The route of excretion of aniline mustard in the rabbit therefore, has also been studied in order to consider the likelihood of enterohepatic circulation of AM metabolites in man.

The animals used were adult male Wistar rats (250–300 g) obtained from Lions Lab., Ringwood, Hants., U.K. and New Zealand White Rabbits (2.8–3.0 kg) obtained from Brock Rabbits, Sholden, Kent, U.K. Water and Labsure diets 41B-modified (rats) and R14 (rabbits) were provided *ad lib.*

Sodium pentobarbitone (Sagatal; May & Baker Ltd., Dagenham, Essex, U.K.) was administered to rats (50 mg/kg; i.p.) and rabbits (30 mg/kg; i.v.) to allow acute bile duct cannulations to be performed. Anaesthesia was maintained by the administration of further pentobarbitone as required. The body temperature of the animals (rectal thermometer) was maintained at 37° using a heated platform. Bile duct cannulations were made with clear vinyl tubing (0.5 mm i.d. 0.8 mm o.d.; obtained from Dural Plastics, Dural, Australia). For the collection of bile from rats which were not under anaesthesia, a re-entrant cannulation technique [9] was employed. Rats were given a postoperative recovery period of 10 days prior to administration of AM. The urine was taken from the bladder at the end of the experiments.

AM, [3, 5-^{3}H]-AM and authentic AMOH were gifts from the Chester Beatty Research Institute, London, U.K. Unlabelled and [^{3}H]-labelled AM were mixed to give a final specific activity of either 1.1 or 10.9 μCi/μmole (for rats) and 2.2 μCi/μmole (for rabbits). The dose solutions of [^{3}H]-AM were purified, prior to injection, by application in benzene to a column (10 × 1 cm) of silica gel (May & Baker Ltd.). The compound was eluted with benzene (10 ml) and applied to t.l.c. plates (see below). The u.v.-absorbing material with an R_f value of 0.37 (solvent system A) was scraped from the plates and eluted with methanol (20 ml). Following evaporation of the methanol under a stream of nitrogen, the [^{3}H]-AM was taken up in benzene. The t.l.c. procedure was then repeated to yield [^{3}H]-AM with a radiochemical purity of > 95 per cent. The dose solutions of radioactive AM were prepared, after evaporation of the benzene, in dimethylsulphoxide at a concentration of 20 mg/ml (rats) and 120 mg/ml (rabbits). These were administered to animals by i.p. injection (10 mg/kg).

For enterohepatic recycling experiments, bile samples (1 ml) obtained from rats injected with [^{3}H]-AM, containing radioactive AM metabolites, were directly infused (1 ml/hr) into the duodena of anaesthetized bile duct cannulated rats using a slow infusion pump (Scientific and Research Instruments Ltd., Kent U.K.). For this procedure a cannula was inserted, at the time of bile fistula surgery, into the duodenum via the bile duct.

Biliary metabolites were analysed by the method of Epstein *et al.* [10] to determine the presence of alkylating agents. Bile samples, containing approximately 0.1 μmole of AM metabolites or authentic AMOH, were taken from storage at −20° and incubated at 80° for 30 min with 1 ml of 2%w/v Epstein reagent [4-(4-nitrobenzyl) pyridine; British Drug Houses, Poole, Dorset, U.K.) in 90% ethylene glycol/10% acetate buffer, 0.5 M, pH 4.6. Samples were then immediately cooled on ice and 3 ml of triethylamine: acetone (1:1) added. A positive reaction was indicated by a purple colouration of the sample, which gave an extinction reading at 570 nm when measured against blank samples where bile from untreated animals was used.

T.l.c. was carried out using DC-Alufolien or DC-plastikfolien Kieselgel 60 F_{254} plates (Merck, Darmstadt, F.R.G.). For the purification of AM, glass plates (20 × 20 cm) were coated with 0.4 mm Kieselgel GF_{254} (Merck) and activated at 110° for 30 min prior to use. Kontes preparative plates PLKF (Uniscience, Cambridge, U.K.) were employed for the preparation of methylated AMGL for mass spectroscopy. The following solvent systems were used: A, chloroform–dichloromethane, 4:1; B, chloroform–methanol, 1:1; C, chloroform–methanol, 9:1; D, chloroform–benzene, 1:1. All proportions are v/v. The developed plates were scanned for radioactivity using a Packard radiochromatogram scanner (Model 7201), and viewed under u.v. light. Where samples contained very low amounts of radioactivity, the thin layer plates were cut into 5 mm segments and then eluted with methanol (0.5 ml) and the ^{3}H present in this methanol measured by scintillation counting, using a Packard Tri-Carb 3255 instrument. The scintillant used for determination of radioactivity consisted of 2, 5-diphenyloxazole (5% w/v) and 1, 4-di-2-(5-phenyloxazolyl)-benzene (0.3% w/v) in toluene (scintillation grade; BDH) mixed with Triton X-100 at a ratio of 2:1. Bile or urine (0.02 ml) samples were also counted using this scintillation fluid.

AMGL (Fig. 1) was isolated from rat bile using the method of Connors *et al.* [5]. Bile samples (0.3–2.0 ml), collected on ice, were adjusted to pH 2 using 0.1 M HCl

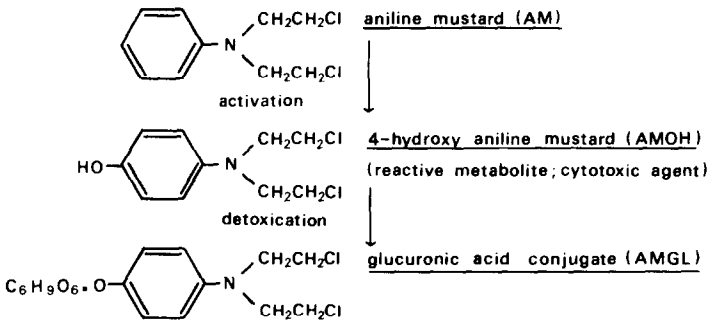

Fig. 1. Metabolism of aniline mustard.

and extracted with ethyl acetate 3 × 5 ml). Following t.l.c. (solvent system B) of the ethyl acetate extract, the radiolabelled material having a mobility appropriate for AMGL (R_f 0.24) was eluted with methanol (10 ml). AMGL was methylated using an ethereal solution of diazomethane in excess (method supplied by BDH technical services). The mass spectrum of the methylated glucuronide was investigated with an AE1 MS12 spectrometer at the Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey, U.K., using the conditions previously employed [5].

In anaesthetized rats, 28.4 ± 2.2 (S.E.M.) and 8.2 ± 0.7 per cent of the dose was excreted within 8 hr into bile and urine, respectively (N = 6). Anaesthetized rabbits excreted 5.1 ± 0.6 per cent of the dose in bile and 22.5 ± 2.1 per cent in urine within 5 hr (N = 2). The excretion data for rats which were not under anaesthesia, having previously been equipped with re-entrant bile duct cannulae, were similar to those for anaesthetized rats: 22.3 ± 2.8 per cent dose in bile; 4.7 ± 1.5 per cent dose in urine in 5 hr (N = 3).

Rat bile samples containing radioactive AM metabolites gave a positive reaction with the Epstein test for alkylating agents, confirming the presence of metabolites containing reactive chloroethyl moieties. AMGL was hydrolysed when bile samples (0.5 ml) were incubated with β-glucuronidase (Ketodase from W. R. Warner and Co. Ltd., Eastleigh,

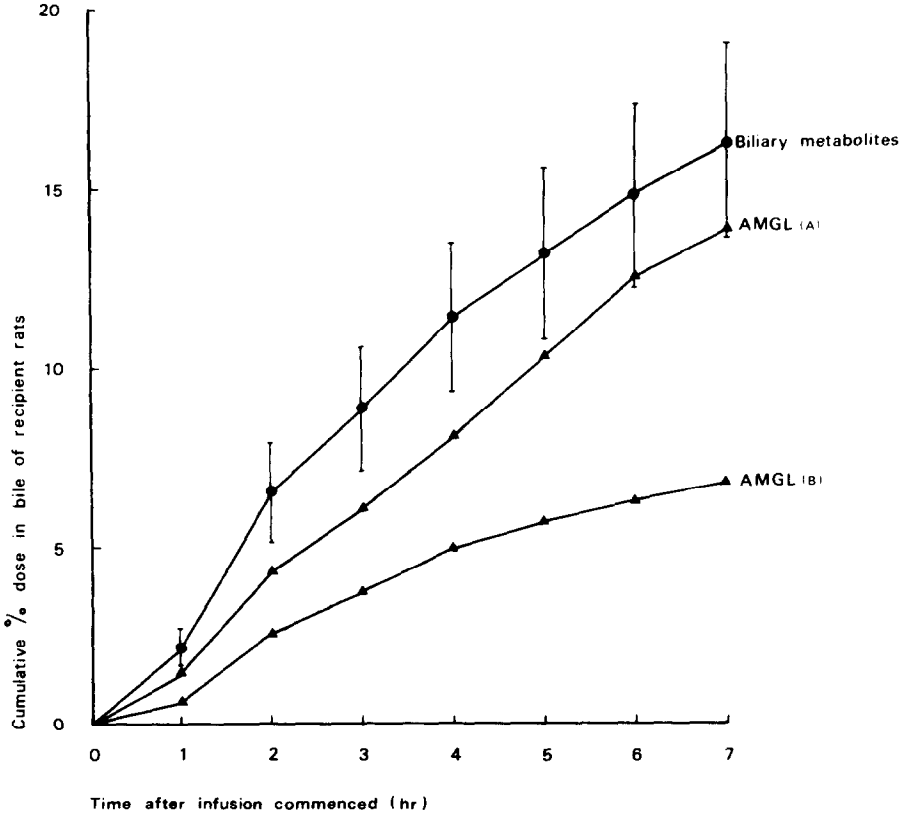

Fig. 2. Enterohepatic circulation of AM metabolites in the rat. Biliary excretion of radioactivity after intraduodenal infusion of biliary metabolites of [^{3}H]-AM (approx. 6 μmoles/kg; mean ± S.E.M. N = 5) or [^{3}H]-AMGL in rat bile [2.0 μmoles/kg (A) and 4.6 μmoles/kg (B)].

Hants., U.K., 0.5 ml; 25,000 units) in a shaking water bath at 37° for 18 hr, yielding products which, following precipitation of protein with methanol, co-chromatographed with authentic AMOH and degradative products of this mustard (solvent systems B and D). AMGL was isolated from bile as described above. When the glucuronide was treated with diazomethane, a derivative was formed which was isolated by t.l.c. (R_f 0.22, solvent system C) and investigated by mass spectroscopy. The most abundant fragments were m/e 233/235 and 184/186, in accordance with the mass spectrum of authentic methylated AMGL and the aglycone AMOH [5].

The metabolite AMGL accounted for approximately 40 per cent of the total metabolites in rat bile collected up to 2 hr after injection. This agrees with results from a previous study [5] where AMGL was shown to be a major metabolite in the bile of rats, which had been pretreated with phenobarbitone and given a higher dose of aniline mustard (ca. 200 mg/kg). AMGL constituted a lower proportion (approximately 30 per cent) of total metabolites in pooled bile collected between 2 and 8 hr after injection. The metabolites remaining in rat bile following extraction of AMGL were not characterized but might be expected to include products resulting from hydrolysis of chloroethyl groups. The mean half lives for the mustard hydrolysis reactions at physiological pH and temperature are approximately 60, 12 and 21 min for AM, AMOH and AMGL, respectively [11]. A continual hydrolysis of the mustards could explain the fall in the relative abundance of AMGL with respect to time after administration of AM.

When rat biliary metabolites of [^{3}H]-AM were directly infused (approximately 6 μmoles/kg) into the duodena of another group of bile duct cannulated rats, recipient animals excreted 16.3 ± 2.7 (N = 5; see Fig. 2) and 8.9 ± 3.0 per cent (N = 3) of the dose into the bile and urine, respectively, within 7 hr. The values for 24 hr collections from restrained rats were 18.9 ± 2.2 per cent in bile 13.4 ± 1.3 per cent in urine (N = 2). The bile from these recipient rats did not contain a sufficient concentration of metabolites to be tested for alkylating ability by the Epstein test.

In a further experiment, the tritiated metabolite AMGL, isolated by t.l.c. (see above), was dissolved in 1 ml of rat bile and infused into the duodena of two rats (2.0 μmoles/kg, rat A, and 4.6 μmoles/kg, rat B). A total of 13.8 (rat A) and 6.8 per cent (rat B) of the administered radioactivity was re-excreted via the bile of the recipient animals within 7 hr (Fig. 2). Approximately 30 per cent of this recycled material could be partitioned into ethyl acetate at pH2 and the sole significant component of this extract co-chromatographed with AMGL (t.l.c. system B, R_f 0.24).

The data imply that, in the rat, a large proportion of the biliary metabolites absorbed from the intestine and re-excreted in the bile are derived from AMGL. The recycled material was both in the form of the bifunctional AMGL, which possesses anti-neoplastic activity, and also in the form of other unidentified metabolites which may be partially hydrolysed to monofunctional mustards which remain cytotoxic but do not possess anti-neoplastic activity [12].

The bile was only a minor excretory route for AM metabolites in the rabbit. If the rabbit is taken to be a better model for man than is the rat, as regards capacity for biliary excretion (see ref. 8), it seems unlikely that biliary excretion and enterohepatic circulation of AM metabolites will be significant in man. However, for other anti-neoplastic agents, which may be excreted in human bile, the enterohepatic recycling of reactive metabolites could well be of considerable importance with regard to their bioavailability.

In summary, the bile is a major excretory route for metabolites of aniline mustard (AM) in anaesthetized and non-anaesthetized rats. Metabolites, which are capable of reacting with DNA and which possess anti-neoplastic activity, undergo an enterohepatic circulation in the rat. With the rabbit, however, bile is only a minor excretory route for metabolites of AM. Since the biliary excretion of organic compounds in man appears to be similar to that in the rabbit, enterohepatic circulation of reactive metabolites of AM is expected to be negligible in man.

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