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THE CLINICAL PHARMACOLOGY OF A HEPATOMA-SPECIFIC ALKYLATING AGENT

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Abstract—Pharmacological studies have been made in hepatoma patients taking CB10-252, a latent enzyme-activated alkylating agent. Following oral administration, the drug was absorbed rapidly, and peak levels of unmetabolised drug were seen at 1 hr. The parent drug is cleaved by azoreductase to yield an alkylating agent with a very short half-life and stable metabolites. Peak plasma levels of these metabolites were seen at 2 hr with persistence of the compounds for 12 hr. Eight hr after drug administration, 80 per cent of the dose had been eliminated in the urine, the majority in the form of metabolites with only 2 per cent being recovered as unmetabolised drug. Azoreductase activity was demonstrated in normal human liver and in human bone marrow and lymphoid cells. This latter finding may explain the occurrence of myelosuppression observed in several patients. These data indicate that CB10-252 is not a hepatoma-selective alkylating agent, but that the drug is well absorbed following oral administration.

N,*N*-Di (2-bromo-*n*-propyl) amino-3-methyl-2′-carboxy-azobenzene, or CB10-252, is a latent enzyme-activated alkylating agent. Metabolism by a non-specific NADPH requiring cytosol azo-reductase yields 4-di-(2-bromo-*n*-propyl) amino-2-methylaniline, an active alkylating radical which hydrolyses with a half life of 45 sec [1, 2], and *o*-aminobenzoic acid (anthranilic acid) which is non-toxic (Fig. 1). The enzyme which metabolises CB10-252 has been found in liver and hepatoma tissue, and since the half-life of the active metabolite is so short, CB10-252 activated in liver would not be expected to cause myelosuppression. The properties of the compound and the demonstration of the enzyme in primary hepatomas were the basis for a clinical trial of CB10-252 in patients with in-operable hepatoma. The purpose of this study was to investigate the clinical and biochemical pharmacology of CB10-252. In this study, levels of the parent compound (I) and of anthranilic acid (III) were measured and activation of CB10-252 has been inferred from demonstration of anthranilic acid in the blood of patients taking CB10-252 (Fig. 1). In addition azoreductase assays were performed on bone marrow, spleen and lymphoid tissues.

MATERIALS AND METHODS

Plasma levels: unmetabolized drug, free and conjugated amines

Serial plasma samples (Heparinised blood specimens) were obtained from patients after an oral dose of 30 mg CB10-252. Drug absorption and metabolism was followed by measuring levels of *o*-aminobenzoic (anthranilic acid) (Fig. 1) using a modified Bratton–Marshall reaction [3]. The assay is quantitatively specific for anthranilic acid, and no reaction takes place with the parent drug or the alkylating radical [2].

Anthranilic acid assay. To 2 ml of plasma, 2 ml of 20% trichloroacetic acid (BDH Chemicals Ltd.) was added and the precipitate spun at 4,000 *g* for 10 min. 1 ml of 0.05% sodium nitrite (May and Baker Ltd.) was added to 3 ml of the supernatant, and the mixture allowed to stand for 3 min. Excess nitrite was removed with 1 ml of 0.5% ammonium sulphamate and the tube left for a further 3 min. Six ml of 0.1% *N*-naphylethylene-diaminedihrochloride (N.E.D.) (Kodak Chemicals Ltd.) was added, and the contents left for 30 min. Coupled N.E.D. was extracted with 4 ml of *n*-butanol. (B.D.H. Chemicals, Ltd.) and 1 gm of sodium chloride (May and Baker Ltd.). Optical density at 550 mμ was read on a Unicam SP 800 spectrophotometer and compared with a standard curve of anthranilic acid prepared in human plasma.

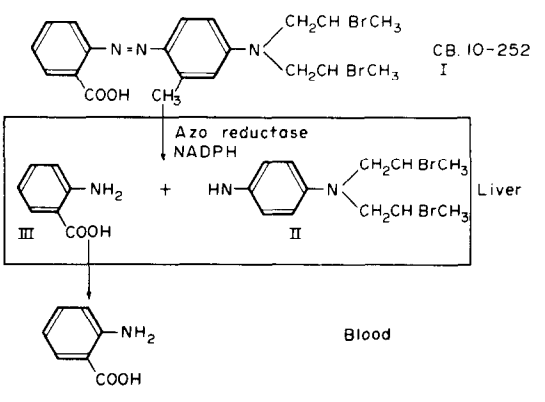

Fig. 1. The metabolism of CB10-252 (I). CB10-252 is reduced to anthranilic acid (III) and an active alkylating agent (II) by a non-specific azoreductase.

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Anthranilic acid: conjugation products. Anthranilic acid is metabolised to conjugation products undetectable by the above assay. To measure the conjugation products it was necessary to hydrolyse aliquots of plasma and then assay total anthranilic acid. 0.5 ml 1 N sodium hydroxide was incubated with 2 ml of plasma at 37° for 1 hr. The mixture was neutralised with 0.5 ml of 1 N hydrochloric acid, and anthranilic acid was then assayed as above with correction for dilution.

Unmetabolised drug. Unmetabolised CB10-252 was assayed by chemically reducing the compound to alkylating radical and anthranilic acid with sodium dithionite. 0.75 mg of sodium dithionite reduced 10 nmol of CB10-252 with stoichiometric amounts of anthranilic acid being released in the medium after reduction and the reaction was immediate at room temperature. 0.75 mg of sodium dithionite was added to 2 ml plasma samples, and the reaction products assayed for anthranilic acid using the assay described above.

Urine was assayed for CB10-252, anthranilic acid and conjugation products using the assays described above with comparison to a standard solution of anthranilic acid in saline.

Tissue azoreductase levels

Liver azoreductase proved to be unstable with a half-life of approximately 50 hr when stored at −20°. All enzyme analyses were therefore performed on fresh tissue. Human liver and spleen biopsies were obtained at staging laparotomy on a 63-year-old female with Hodgkin's Disease. Human bone marrow was harvested from a 32-year-old with testicular carcinoma, and a 12-year-old female leukaemia patient. Aliquots of the tissues were examined histologically and found to be normal. Liver specimens were also obtained from Charles River rats.

The liver specimens were ground in a glass tissue homogenizer (Uniform) to make an approximate 25% solution in ice cold 0.1 M phosphate buffer (pH 7.4). Free cells were expressed from splenic tissue, and red cells were lysed in this as well as the bone marrow aspirates by suspension in 0.3% saline with restoration of isotonicity after 30 sec. Cells were collected by low-speed centrifugation, suspended in phosphate buffer (pH 7.4) and sonicated (MSE sonicator) in an ice water bath for 60 sec.

The resulting suspensions were centrifuged for 20 min at 9000 *g*. 1 ml of the supernatant was added to 3 ml of phosphate buffer containing 300 μg CB10-252 and an NADPH generating system to catalyse reduction (15 μm glucose-6-phosphate 0.7 μm NADP, 50 μm nicotinamide, Sigma), and 17.5 μg of magnesium chloride (Analar) [4]. The mixture was incubated under nitrogen for 15 min at 37° and excess substrate was extracted with 10 ml of chloroform (BDH Chemicals). To 2 ml of the aqueous phase was added 3 ml of 20% TCA, and precipitate removed by centrifugation at 3000 *g* for 10 min. Three ml of the supernatant was assayed for anthranilic acid. Protein concentrations were determined by the Lowry reaction [5], and the enzyme specific activity was calculated.

RESULTS

Plasma levels

Total conjugated and free anthanilic acid were assayed and used as an index of drug activation, while levels of CB10-252 were estimated by measurement of total anthranilic acid levels following chemical hydrolysis with dithionite (combined free amine + CB10-252). Figure 2 shows the plasma levels of CB10-252 following oral administration of 30 mg to three patients. Peak values are reached at 1 hr and the drug persists for some hr in the blood and in one patient was still present at 10 hr. This particular patient's urine contained 28 nmol/ml of unmetabolised drug and 23% of the drug was excreted unmetabolised. Figure 3 shows the plasma levels of conju-

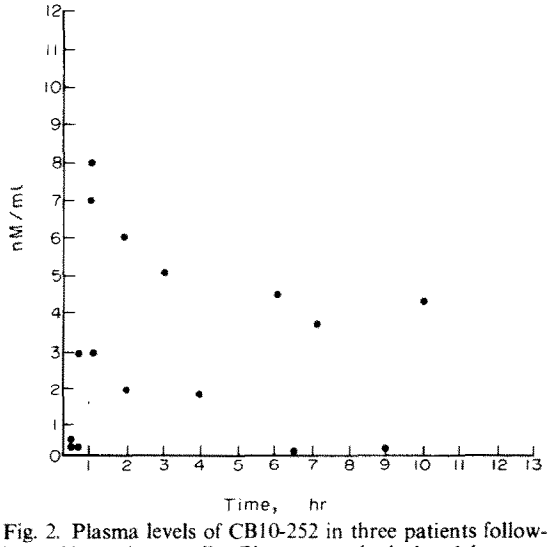

Fig. 2. Plasma levels of CB10-252 in three patients following a 30 mg dose orally. Plasma was hydrolysed by reaction with dithionite, and anthranilic acid levels were assayed (see text). Results were of conjugated and free anthranilic acid anlysis subtracted to give the levels of unmetabolised drug.

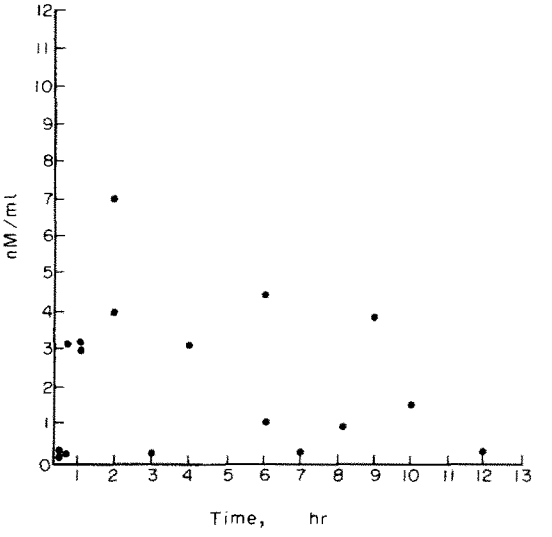

Fig. 3. Plasma levels of conjugated and free anthranilic acid in three patients following a 30 mg dose CB10-252 orally (see text).

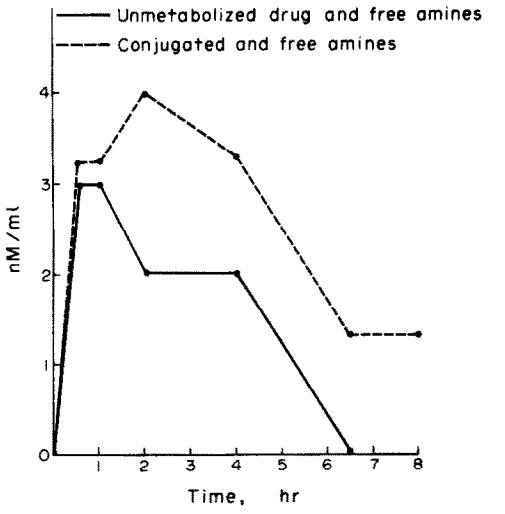

Fig. 4. Plasma levels of CB10-252 and conjugated anthranilic acid in one patient following a 30 mg oral dose of CB10-252.

gated and free anthranilic acid in the same three patients. Peak values occurred at 2 hr with clearance of the compounds by 12 hr. The patients who metabolised and cleared the drug most rapidly showed few side effects following drug treatment, but the patient in whom drug was still present in plasma 10 hr after administration developed myelosuppression after a few days treatment. A typical normal plasma drug profile is seen in Fig. 4; 30 min after treatment, drug + free amines and conjugated + free amines had

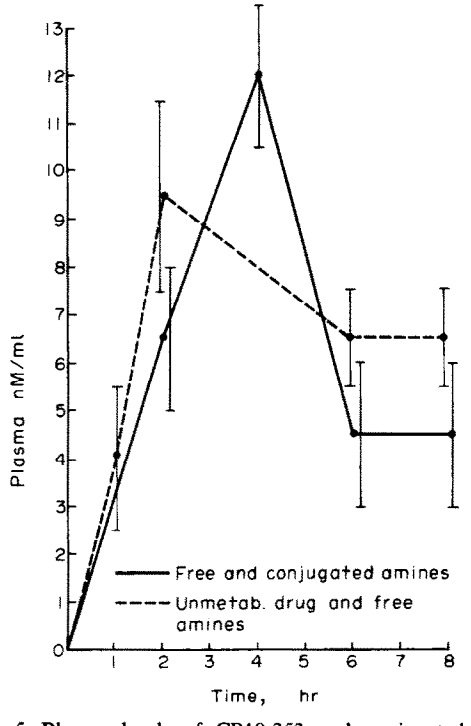

Fig. 5. Plasma levels of CB10-252 and conjugated anthranilic acid in one patient following a 50 mg oral dose of CB10-252. Bars indicate range of triplicate assays.

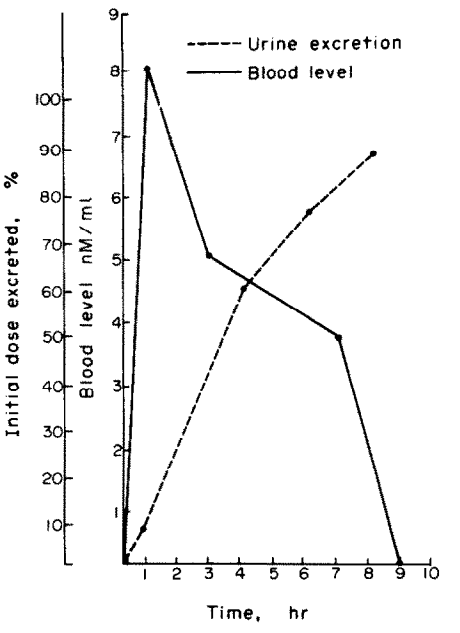

Fig. 6. Plasma levels of CB10-252 (unmetabolised drug) and excretory pattern in one patient following a 30 mg oral dose.

reached 3.0 and 3.25 nmol/ml respectively. At 2 hr, drug metabolism is evident, with unmetabolised drug + free amines falling to 2 nmol/ml and products of metabolism rising to 9 nmol/ml. The drug is not detectable by 6.5 hours, and no unmetabolised CB10-252 was found in this patient's urine, the drug being eliminated as free and conjugated amines.

The plasma profile in another patient after a 50 mg dose is seen in Fig. 5. Peak drug levels are reached after 2 hr and peak drug metabolites (12 nmol/ml) at 4 hr. With this dose both parent compound and metabolites are detectable throughout the 8 hr of study.

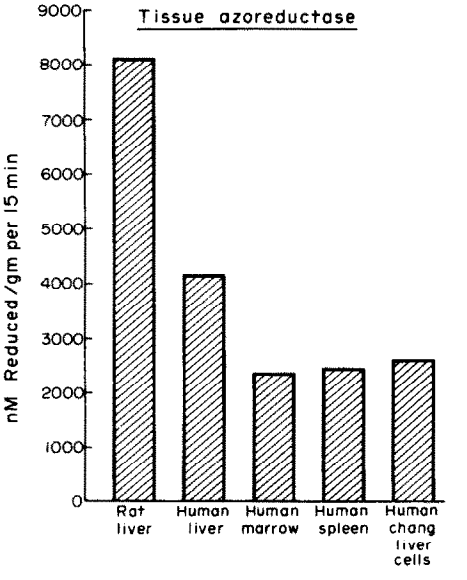

Fig. 7. Azoreductase activity of different tissues (see text for details).

Excretory patterns

Figure 6 shows the absorption and excretory pattern of CB10-252 in one patient. Eight hr after drug administration, 87 per cent of the dose had been eliminated in the urine, 85 per cent as metabolites (anthranilic acid) and 2 per cent as unmetabolised CB10-252. In another patient 23 per cent of the drug excreted in the urine was unmetabolised CB10-252.

Tissue azoreductase levels

Human liver has 51 per cent of azoreductase activity present in rat liver, and human Chang liver cells considerably lower levels (Fig. 7). Human bone marrow and spleen cells showed substantial azoreductase activity, approximately 30 per cent of that found in rat liver.

DISCUSSION

Our results indicate that CB10-252 is well absorbed following oral administration and 90 per cent of the administered dose is recoverable in the urine at 8 hr in the form of parent drug or metabolites. The drug caused no gastro-intestinal toxic effects suggesting that the drug was absorbed intact and not following activation by intestinal bacteria. In support of this hypothesis is the observation that CB10-252 is extracted from an acid medium by oil*, and it is thus possible that CB10-252 is absorbed from the stomach.

Azoreductase has been reported in normal liver and primary hepatoma, with greater activity in the normal tissue [4]. Nevertheless it has been suggested that CB10-252 might be selectively cytotoxic to hepatoma tissue because of the higher mitotic rate and consequent greater sensitivity to alkylating agents. Moreover, a preliminary observation that the Walker carcinoma (very low azoreductase activity) is curable by CB10-252 when the tumour is implanted in the liver and virtually completely refractory to this agent when the tumour is implanted in the flank, suggests that secondary tumour in the liver may be drug susceptible even if the tumour itself is unable to activate the drug†.

Our results indicate that azoreductase activity is present in normal human bone marrow and lymphoid cells. This may explain the occurrence of myelosuppression observed in several patients in the clinical trial. Thus if CB10-252 is to have true hepatoma selectivity, the drug must be administered directly into the liver at a rate not exceeding that which can be metabolised in the liver.

* G. P. Warwick, personal communication.
† T. A. Connors, unpublished results.

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