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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—IV

COVALENT BINDING OF DIFLUNISAL TO TISSUES OF THE RAT*

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Abstract—Acyl glucuronides have been shown to be reactive electrophilic metabolites capable of undergoing hydrolysis, rearrangement (isomerization via acyl migration) and covalent binding reactions to plasma protein. The present study was undertaken to explore the occurrence and extent of *in vivo* formation of covalent adducts of diflunisal (DF), a salicylate derivative which forms a reactive acyl glucuronide, with tissues and plasma protein of rats. Groups of rats were given 50 mg DF/kg i.v. twice daily for periods of up to 7 days. Steady state plasma concentrations of reversibly bound DF and its conjugates (as measured 6 hr after a dose) were achieved by the third day of dosing. $T_{\frac{1}{2}}$ values after cessation of dosing were about 5–10 hr. By contrast, covalent DF–tissue adducts steadily accumulated over the 7-day dosing period. Maximum concentrations, measured 6 hr after the last dose, were 4.8 (liver), 1.0 (kidney), 0.74 (plasma), 0.26 (small intestine minus contents), 0.27 (large intestine minus contents) and 0.20 (skeletal muscle) μg DF/g tissue or/mL plasma. $T_{\frac{1}{2}}$ values of about 50, 67, 18, 38 and 43 hr were obtained for liver, kidney, plasma and small and large intestine (respectively) after cessation of dosing. Thus, the study of acyl glucuronide reactivity and the question of any derived toxicity or immune responses should consider the formation of long-lived adducts in tissues as well as in plasma.

A major metabolic pathway for drugs and endobiotics bearing carboxylic acid groups is coupling with glucuronic acid to yield acyl glucuronide conjugates. These metabolites are reactive electrophilic species which have been shown to undergo three reactions *in vitro* and *in vivo*: hydrolysis (liberation of aglycone), intramolecular rearrangement (isomerization via acyl migration) and intermolecular covalent binding reactions with protein [1, 2]. The last reaction, whose mechanism has not been established, has attracted most attention, as covalent attachment of foreign compounds to endogenous macromolecules has usually been associated with, or at least raised the spectre of, ensuing toxicity [3, 4]. Thus, the list of carboxylic drugs known to form covalent adducts with proteins has been growing steadily [2]. However, *in vivo* studies have been limited to plasma protein, with the exception of one recent report [5] which documented formation of adducts of clofibric acid with rat liver proteins.

We have shown previously that diflunisal (DF,‡ Fig. 1), a salicylate derivative with analgesic and anti-inflammatory properties, is metabolized in humans and rats to a reactive acyl glucuronide (DAG) [6, 7], a stable phenolic glucuronide (DPG) [6, 8] and a stable sulphate conjugate (DS) [9]. The present study was undertaken to explore the *in vivo* occurrence and extent of covalent binding of DF to plasma protein and various tissues of the rat.

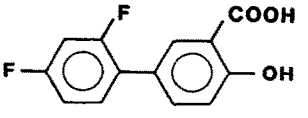

Fig. 1. Chemical structure of diflunisal.

MATERIALS AND METHODS

Materials and animals. DF was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Authentic samples of DAG, DPG and DS used as analytical standards were prepared as described previously [8, 9]. Clofibric acid was a gift from ICI Pharmaceuticals Division (Macclesfield, U.K.). Methanol and acetonitrile (HPLC grade), diethyl ether (AR grade) and hexane (nanograde) were purchased from Mallinckrodt (Melbourne, Australia). Reagents were AR grade. Methoxyflurane anaesthetic (Penthrane®) was purchased from Abbott Australasia (Sydney, Australia). Male Sprague–Dawley-derived rats (290–350 g) were obtained from The University of Queensland Medical Faculty Animal House, and were maintained on

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‡ Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; DPG, diflunisal phenolic glucuronide; DS, diflunisal sulphate; *iso*-DAG, mixture of 2-, 3- and 4-*O*-acyl isomers of diflunisal acyl glucuronide formed by acyl migration; $T_{\frac{1}{2}}$, half-life.

standard rat pellets (Norco Cooperative, Lismore, Australia) and water. Experiments were approved by the University's Animal Experimentation Ethics Committee.

Administration of DF. Rats were prepared, under methoxyflurane anaesthesia, with a catheter in the right external jugular vein as described previously [10], and then placed unrestrained in metabolism cages and allowed to recover for at least 2 hr before administration of the first dose. Food and water were available *ad lib.* Doses of 50 mg DF/kg (10 mg DF/mL 0.1 M $NaHCO_3$) were administered i.v. twice daily (about every 12 hr) for periods of up to 7 days. Blood samples (150 μL) were drawn (jugular catheter) at 6 hr after the 1st, 2nd, 4th, 6th, 10th and 14th (final) dose, and additionally at 1, 3 and 7 days after the 14th dose (4–8 rats sampled at each time). The samples were immediately centrifuged, and aliquots of plasma (50 μL) were snap frozen and stored in dry ice for later analysis (as soon as practicable) of reversibly bound DF and its metabolites. Groups of rats (N = 3 or 4) were killed by exsanguination (aorta) under methoxyflurane anaesthesia 6 hr after the 1st, 6th and 14th dose, and additionally 1, 3 and 7 days after the 14th dose. The plasma was separated and stored as above. Liver, kidneys, small and large intestine (minus contents) and samples of skeletal muscle were collected without delay, rinsed in saline and frozen (−20°) for later analysis of DF covalently bound to tissues. The contents of the intestines were removed before freezing by slitting open and gentle rinsing of the tissue in saline two times.

Another group of rats (N = 3) was dosed once daily at 100 mg DF/kg i.p. for 3 weeks and then killed 18 hr after the last dose. Plasma and tissues were collected and stored as described above.

Analysis of reversibly bound DF and metabolites in plasma. DF and its metabolites in 50 μL aliquots of plasma were analysed using the direct isocratic HPLC method described previously [11]. Isomers of DAG formed by intramolecular acyl migration were measured using the same molar extinction as DAG itself, an approach that has been verified experimentally [6].

Analysis of DF covalently bound to tissues. The tissues were thawed, chopped and homogenized (Ika Ultraturrax, Staufen, Germany) in *ca.* 2 vol. of 0.1 M phosphate buffer pH 4.5. To duplicate samples (2.0 g) of the homogenates were added 2 mL ice-cold methanol, 4 mL acetonitrile and 200 μL orthophosphoric acid, with thorough vortex mixing after each addition. After centrifugation, the supernatants were discarded and the pellets exhaustively washed eight times by resuspending in 10 mL portions of methanol–diethyl ether (3:1, v/v) followed by centrifugation [5]. Duplicate 0.2 mL samples of plasma were similarly precipitated with 0.2 mL methanol, 0.4 mL acetonitrile and 2 μL orthophosphoric acid, and then washed eight times with 1.2 mL portions of methanol–diethyl ether. The pellets were then separated, dried and digested in 2 M NaOH (1 mL) for 3 hr at 75°. After cooling and acidification (400 μL of 10 M HCl), 50 μL of internal standard solution (100 μg clofibric acid/mL water) was added and the mixture equilibrated with 10 mL hexane–diethyl ether (1:1, v/v). The organic layer was removed and evaporated at 50° under a stream of air. The residue was reconstituted in 200 μL of HPLC mobile phase, and a 60 μL sample analysed for DF as described previously [11].

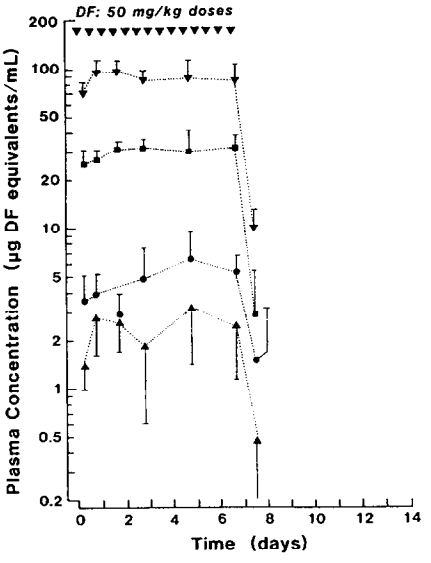

Fig. 2. Plasma concentration–time profiles for DF (▼) and its conjugates, DAG (▲) (measured here as the sum of DAG and its isomers), DPG (●) and DS (■) during and after dosage of rats at 50 mg DF/kg i.v. twice daily for 7 days. Results are means ± SD, N = 3–6.

Standard curves were prepared from tissues and plasma of untreated rats by spiking precipitated and washed pellets (prior to alkaline digestion) with the calculated amounts of DF. Calibration ranges appropriate to the adduct concentrations found in each tissue were determined by pilot experiments. Standard curves for each tissue were derived from at least five concentrations. Coefficients of determination (r^2) were about 0.99 in each case. The adequacy of the washing procedure to remove non-covalently bound DF was verified by analysis of tissue homogenates and plasma (from untreated rats) which had been spiked with DF at *ca.* 100 μg DF/g tissue or/mL plasma.

Data analysis. The apparent half-life ($T_{\frac{1}{2}}$) of DF covalently bound to tissue was calculated from the slope of the log concentration–time profile after the last dose, using linear regression analysis of the mean concentrations at each time point.

RESULTS

Steady state plasma concentrations of DF and its conjugates (as measured 6 hr after a dose) were achieved by the third day of twice daily i.v. dosing at 50 mg DF/kg (Fig. 2). After the last (14th) dose, the concentrations were DF, 83 ± 24; DAG + *iso*-DAG, 2.4 ± 1.1; DPG, 5.3 ± 1.4 and DS, 32 ± 6.3 μg DF equivalents/mL plasma (means ± SD, N = 6). Elimination thereafter was relatively rapid: these

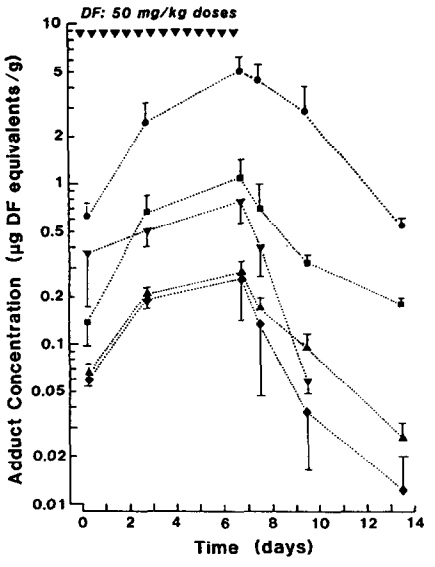

Fig. 3. Concentration–time profiles for DF covalently bound to liver (●), kidney (■), plasma (▼), small intestine minus contents (◆) and large intestine minus contents (▲) during and after dosage of rats at 50 mg DF/kg i.v. twice daily for 7 days. Results are means ± SD, N = 3 or 4.

(reversibly bound) DF species were unmeasurable 3 days after the last dose, and calculated $T_{\frac{1}{2}}$ values (using the two available points) were in the range 5–10 hr.

By contrast, concentrations of DF covalently bound to plasma and tissue proteins increased steadily over the 7 days of twice-daily dosing (Fig. 3). The peak concentrations measured 6 hr after the last dose were highest in the liver and lowest in the intestines and skeletal muscle (Table 1). Elimination of the adducts thereafter was relatively slow: calculated $T_{\frac{1}{2}}$ values were in the range 38–67 hr except for plasma 18 hr (Table 1). These $T_{\frac{1}{2}}$ values should be regarded as essentially illustrative: they are of necessity derived from the line of best fit through single points (each representing the mean of tissue "homogenates" from the individual rats examined at that time). Also, it cannot be assumed that the rates of elimination will remain unchanged at adduct concentrations lower than those presently measurable.

Plateau concentrations of tissue adducts had not been reached by 7 days of twice-daily i.v. dosing at 50 mg DF/kg. The concentrations achieved after 21 days of once-daily i.p. dosing at 100 mg DF/kg however were not appreciably different (Table 1). Comparison of the two data sets should be made bearing in mind that in the latter, tissues and plasma were sampled 18 hr after the last 100 mg/kg i.p. dose whereas in the former they were sampled 6 hr after the last 50 mg/kg i.v. dose. This is particularly relevant to levels of the plasma protein adducts, which were shown (Table 1) to be eliminated most rapidly.

DISCUSSION

This investigation documents the covalent binding of DF to plasma protein and tissue macromolecules after dosing rats with the drug. The covalent binding of drugs to plasma protein (notably serum albumin) via their acyl glucuronide conjugates has now been well established through *in vitro* incubation experiments [2]. Covalent adduct formation with plasma protein *in vivo* has also been reported for zomepirac [12], tolmetin [13], DF [14], probenecid [14], clofibric acid [5] and valproic acid [15]. Two mechanisms to explain the covalent binding have been advanced. The first involves direct transacylation with consequent loss of the glucuronic acid moiety [16, 17], whilst the second requires acyl migration prior to adduct formation via glycation, with consequent retention of the glucuronic acid moiety

Table 1. Pharmacokinetic data on DF covalently bound to plasma protein and tissues of rats after multiple dosing with DF

	Rats dosed i.v. at 50 mg DF/kg twice daily for 7 days			Rats dosed i.p. at 100 mg DF/kg once daily for 21 days
Tissue	Concentration* (μg DF/g tissue or /mL plasma)	$T_{\frac{1}{2}}$ (hr)	r^2	Concentration† (μg DF/g tissue or /mL plasma)
Plasma	0.74 ± 0.18 (3)	18	0.99	0.44 ± 0.14 (3)
Liver	4.8 ± 1.2 (4)	50	0.98	5.8 ± 2.5 (3)
Kidney	1.0 ± 0.2 (4)	67	0.92	1.3 ± 0.4 (3)
Small intestine (minus contents)	0.26 ± 0.13 (4)	38	0.94	0.12 ± 0.02 (3)
Large intestine (minus contents)	0.27 ± 0.05 (4)	43	0.94	0.19 ± 0.03 (3)
Skeletal muscle	0.20 ± 0.10 (2)	—	—	0.26 ± 0.10 (2)

* Sampled at 6 hr after the last dose, means ± SD (N).
† Sampled at 18 hr after the last dose, means ± SD (N).

in the adduct [12, 18]. However, neither the binding sites on albumin nor the structures of the adducts have yet been established.

We have shown previously that adducts of DF are formed with rat and human serum albumin *in vitro* [7, 19] and with plasma protein of humans *in vivo* after multiple oral dosing [14]. In the latter study, the adducts were very long-lived: terminal $T_{\frac{1}{2}}$ values were about 10 days. This compares to a $T_{\frac{1}{2}}$ value for human serum albumin itself of about 20 days [20]. In the present work, DF–plasma protein adducts were also formed *in vivo* in rats after multiple DF dosing and had $T_{\frac{1}{2}}$ values of about 18 hr (Table 1). Although this value is considerably shorter than that found in humans, it should be interpreted in the context of the 2-day $T_{\frac{1}{2}}$ for serum albumin in rats [21]. Interestingly, bilirubin covalently bound to serum albumin of rats (presumably via its acyl glucuronide) circulates with the same $T_{\frac{1}{2}}$ as albumin itself [21]. The slower rates of formation and elimination of DF adducts in plasma contrast with those of the reversibly bound DF conjugates (Fig. 2). In terms of quantitative importance in plasma at termination of the 7-day dosing regime, reversibly bound DF, reversibly bound DAG (plus its isomers) and covalently bound DF were in the approximate ratio 100:3:1. This compares to a ratio of about 100:1:1 achieved in humans after a 6-day oral course of DF [14]. In addition to being the ultimate or penultimate precursor of covalent DF adducts in plasma, DAG will be disposed along various competing pathways including renal excretion, uptake into tissues (including hepatocytes), systemic hydrolysis and rearrangement [6, 8].

Uptake of circulating DAG into tissues will be limited by its degree of reversible binding to plasma proteins (recently shown to be about 99% in human plasma*) and hydrophilicity. The tissues selected for analysis of covalent DF adducts in the present study were those expected to encounter high concentrations of DAG (i.e. liver, kidney and intestines) and skeletal muscle. Both the formation and elimination of DF adducts in tissues were slower than observed for plasma protein (Fig. 3 and Table 1). The highest concentrations of adducts were found in the liver, the principal site of DAG biosynthesis, and in the kidney, a major organ of DAG excretion. The intestines are of interest because DAG synthesized in the liver undergoes preferential excretion into bile in the rat [22]. Also, DAG reactivity is promoted in slightly alkaline milieu [11] such as found in the bile and some regions of the intestines. However, the concentrations of adducts found in the small and large intestines (excluding contents) were modest in comparison to those in liver, kidney and plasma protein (Table 1), probably due to the efficient hydrolysis of DAG by mammalian and bacterial enzymes in the gut [22]. Adducts were found also in skeletal muscle, and it thus seems likely that they will form to some extent in all tissues which come into contact with DAG and/or its isomers. In this context it should be noted that a contribution to adduct levels measured in the various tissues will be made by the blood (and plasma protein adducts therein) remaining in the tissues. Given the relative adduct levels found in plasma (Table 1), the vascularity of the tissues examined, and the exsanguination of animals prior to tissue removal, such contributions would seem likely to be modest. It should also be noted that the present work does not directly establish the intermediacy of DAG and/or its isomers in DF–macromolecule adduct formation after dosing rats with DF. However, this seems highly likely given the earlier *in vitro* work with serum albumin [7, 19] and the systemic stability in the rat of the other major metabolites of DF, i.e. DPG and DS [6, 8, 9].

Whether any toxicity, immune responses or alterations to cellular function will follow covalent binding of DF to tissues remains to be seen. DF use has occasionally been associated with cutaneous and fixed drug eruptions [23], and hypersensitivity responses [24]. As a general class, the non-steroidal anti-inflammatory drugs are usually acidic, frequently form acyl glucuronides as major metabolites and are associated with a relatively high incidence of immune or toxic responses [2]. Clofibric acid, an acidic hypolipidaemic agent forming a reactive acyl glucuronide, has been shown recently to bind covalently to rat liver proteins. Long-term use of clofibrate is thought to be associated with a higher incidence of liver, kidney and gastrointestinal disease [25]. We have shown recently [15] that adducts of the anti-epileptic agent valproic acid were found in the plasma of patients taking the drug chronically. Furthermore, anti-adduct antibodies were also found, albeit at very low titres, in the plasma of nine of 57 patients tested.

In conclusion, the present investigation shows that the study of acyl glucuronide reactivity in the context of any derived immune responses or toxicity should include consideration of the formation of long-lived adducts with various tissue macromolecules as well as plasma protein.

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* Dickinson RG, King AR, McKinnon GE, Hooper WD, Eadie MJ and Herkes GK, submitted.

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