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Dissecting the reaction of Phase II metabolites of ibuprofen and other NSAIDs with human plasma protein†

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs on the market. Whilst they are considered safe, several NSAIDs have been withdrawn from the market as a result of adverse drug reactions. NSAIDs are extensively metabolised to their 1- β -*O*-acyl glucuronides (AGs), and the risk of NSAID AGs covalently modifying biomacromolecules such as proteins or DNA, leading to immune responses and cellular dysfunction constitutes a major concern in drug discovery and development. The assessment of the degree of protein modification and potential toxicity of individual NSAID AGs is therefore of importance in both drug monitoring and development. Herein, we report the covalent reaction of 1- β -*O*-acyl glucuronides of ibuprofen and several NSAID analogues with human serum albumin (HSA) protein *in vitro* under concentrations encountered in therapy. Stable transacylation and glycosylation adducts are formed; the observed protein product ratios can be rationalised by the degree of α -substitution in the acyl group. Structure-based protein reactivity correlations of AGs, such as these, may prove a useful tool in distinguishing between carboxylic acid-containing drugs of similar structure that ultimately prove beneficial (e.g., ibuprofen) from those that prove toxic (e.g., ibufenac).

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Introduction

Carboxylic acid containing drugs, most notably the NSAIDs are primarily metabolised to their 1- β -*O*-acyl glucuronides in the liver.¹ The glucuronidation of various drugs, which takes place during Phase II metabolism, is typically a detoxification process that also increases water solubility thereby enabling excretion (Fig. 1a); the resulting products are typically deemed to be pharmacologically generally unreactive.² However, in contrast to the unreactive glucuronides of alcohol and phenolic drugs, some of the resulting glucuronides of carboxylate drugs (acyl glucuronides, AGs) are highly reactive metabolites;³ as a consequence AGs have recently been deemed to be toxic by

regulatory authorities.² Detailed measurements of AG half-lives *in vitro* have sought to correlate reactivity with aglycon structure;⁴ however, there is no necessary or direct correlation of protein reactivity with measures of hydrolytic stability. Proper characterization of the extent and nature of the product adducts remains necessary. Although, the increased reactivity of AGs compared with their parent drugs was originally attributed to transacylation reactions with biological nucleophiles⁵ (Fig. 1b, e.g., formation of an amide bond with the amine group found at the terminus of the side-chain of lysine), alternative reaction pathways are potentially accessible to AGs (Fig. 1c)^{5a} that might lead to alternative protein modification products. Surprisingly, direct detection of these modification products has not been previously reported. Here we report the use of AGs of several NSAIDs (Fig. 2a) coupled with direct detection by MS of intact modified proteins to show that not only do both pathways (Fig. 1b and c) have the potential to covalently modify endogenous human proteins, but that the predominant pathway varies according to drug metabolite identity.

Results and discussion

Analysis of putative metabolic pathways

Once formed enzymatically during Phase II metabolism (Fig. 1b) AGs may react *via* several competing pathways. Hydrolysis to reform the parent drug and transacylation by

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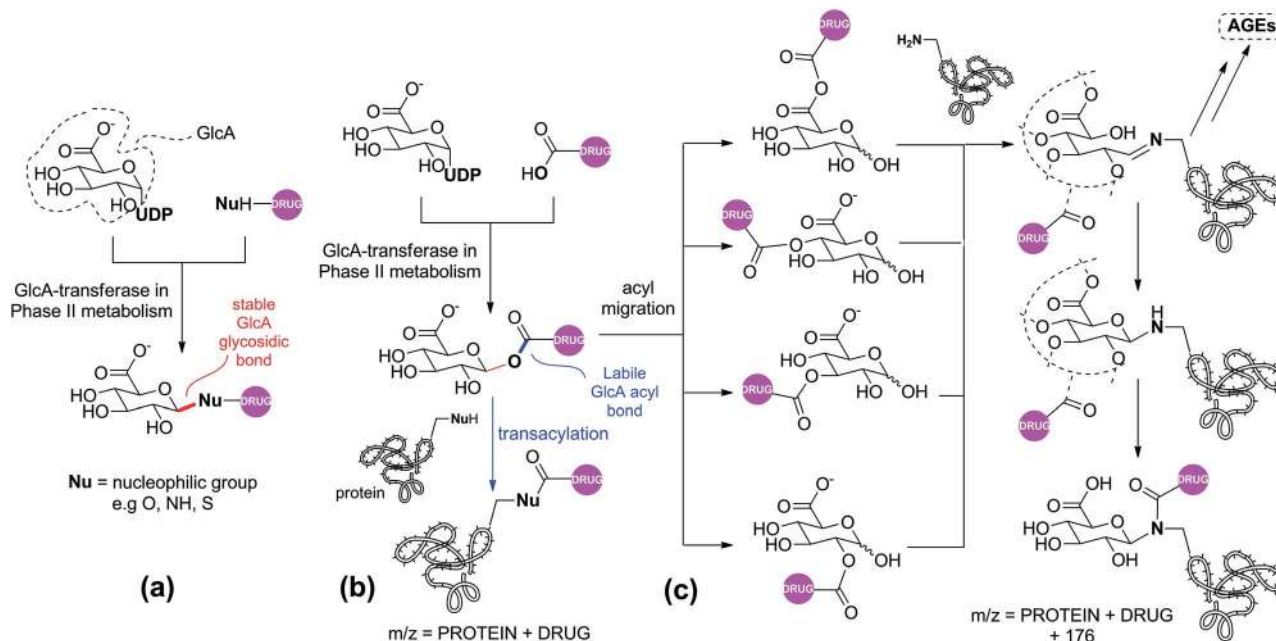


Fig. 1 The reactions and putative products of Phase II drug metabolism. (a) Typical glucuronidation of nucleophilic groups (Nu) in drugs during Phase II metabolism forms stable glucuronide glycosidic bonds and produces pharmacologically inactive metabolites (b) glucuronidation of carboxylic acid containing drugs forms acyl glucuronides (AGs) that can react with proteins through transacylation or (c) intramolecular acyl migration and subsequent reaction with proteins to form glycation and glycosylation products.

direct displacement by attack of nucleophiles (following Fig. 1b) competes with acyl migration to produce 2-, 3- or 4-O-AGs followed by *N*-glycosylation and possibly also the Amadori rearrangement⁶ (following Fig. 1c). 1- β -O-Acyl glucuronides are stable in acid, but under basic or neutral conditions migration occurs to produce 2-, 3- or 4-O-AGs.⁷ After migration, the acyl group is less prone to hydrolysis or direct displacement by transacylation, and stable 2-, 3- or 4-O-AGs can be isolated.⁸ Both the transacylation and the glycosylation pathways are theoretically capable of covalently modifying hepatic and plasma proteins *in vivo* however, the resulting modified proteins have not been fully characterised.^{5a}

Design and synthesis of representative metabolites

Around 25% of all drugs that are withdrawn due to severe toxicity contain carboxylic acid groups and acyl glucuronide activity has been implicated as a possible mechanism.⁹ Yet, the underlying chemical reactivity that might allow prediction of which drugs will show excellent efficacy leading to widespread use (*e.g.* ibuprofen) or toxicity in man leading to withdrawal (*e.g.* ibuprofen)¹⁰ is not well understood; the similarity in structures in the corresponding AGs, such as **1b/c** and **1a**, suggest that this is a subtle effect. It has been suggested that one of the potential mechanisms underlying this observed idiosyncrasy of toxicity of NSAIDs is AG-derived covalent modification of endogenous proteins.¹¹ Evidence for such an AG-induced toxicity mechanism is still lacking,^{1,5a,12} but, by way of circumstantial support, it has been shown that reversible binding of other compounds (like diazepam and warfarin) to plasma proteins, such as human serum albumin (HSA), is severely altered when plasma protein is modified by AGs.¹³

To investigate the nature and extent of AG binding to plasma proteins, a series of ibuprofen based 1- β -O-acyl glucuronides; ibuprofen **1a**, (*R*)- and (*S*)-ibuprofen **1b,c** and a dimethyl analogue **1d**, as well as AGs of *p*-bromobenzoic acid **2**, as a model NSAID, and the aldose reductase inhibitor ponalrestat¹⁴ **3** were incubated with HSA. AGs were synthesised *via* a 3-step protection-acylation-deprotection strategy (Fig. 2b) *via* allyl, benzyl or *para*-methoxybenzyl glucuronate esters.^{4b,15}

Reaction with human serum protein HSA

To obtain a representative single protein species suitable for precise reaction monitoring, abundant human serum protein HSA was reduced and re-purified. Commercially available and isolated HSA contains several post-translational modifications of which the disulfide oxidation of Cys34 by coupling to a single cysteine amino acid residue is the most abundant.¹⁶ HSA was treated with 13 mM DTT at pH 8 to reduce the Cys34–Cys disulfide bond, followed by removal of cysteine and excess DTT by size-exclusion chromatography. The protein was then refolded in 0.02 mM DTT under exposure to atmospheric air to reform the internal disulfide bridges; oxidative refolding of HSA to native secondary structure is known to occur dominantly.¹⁶ This novel protein purification strategy allowed ready access to pure HSA suitable for precise AG protein reactivity assessment.

AGs **1–3** were incubated with pure HSA at physiological levels (33 g L⁻¹).¹⁷ AGs are generally thought to be rapidly excreted, but substantial plasma concentrations, sometimes exceeding their parent drug, are often found.¹⁸ In order to mimic conditions encountered in therapy, an AG concentration of 0.5 mM in phosphate buffered saline (20 mM, pH 7.4) at 37 °C,

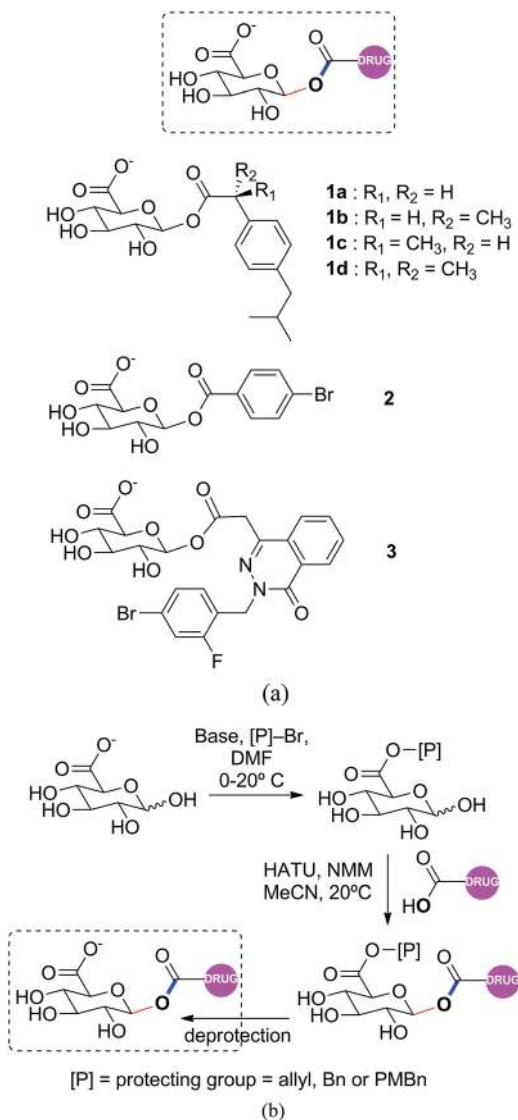


Fig. 2 AGs of NSAIDs and analogues. (a) Six representative, validated Phase II metabolites were used. These incorporated systematic variations in structure to probe variations in reactivity and reaction pathways (b) AGs were chemically synthesized through a novel partial protection strategy and made use of mild deprotection strategies that uniquely allowed access to these highly labile AG products (1–3); harsher conditions employed in other syntheses were incompatible.

corresponding to the peak plasma concentration of drug achieved in chronic cystic fibrosis patients treated with ibuprofen was used.¹⁹ In addition, high-concentration experiments using 5.0 mM AG were also performed. In all cases, identical conditions and incubation times were used for direct comparison of the reactivity of different AGs; this allows direct extrapolation of the levels of reactivity from the amount of reaction product. The extent of coupling to HSA was analysed by LC-coupled TOF MS (ESI+) (Table 1 and Fig. 3).

At concentrations of both 0.5 and 5.0 mM of *p*-bromobenzoic acid AG 2 (entries 1 and 2) glycosylation adducts (Fig. 1c) were observed following incubation for 16 h at a conversion of 58 and 59%, respectively. This is the first time a stable glycosylation

Table 1 Selectivity and conversion after incubation of HSA with AGs^a

Entry	AG	Conc. mM	Selectivity	
			TA : Glyc	Conversion %
1	2	0.5	<5 : 95	58
2	2	5.0	<5 : 95	59
3	3	0.5	55 : 45	4
4	3	5.0	72 : 28	24
5	1a	0.5	60 : 40	16
6	1a	5.0	61 : 39	44
7	1b	0.5	64 : 36	5
8	1b	5.0	63 : 37	23
9	1c	0.5	44 : 56	9
10	1c	5.0	55 : 45	24
11	1d	0.5	—	0
12	1d	5.0	34 : 66	8

^a Incubation of 33 g L⁻¹ HSA with AGs for 16 h at 37 °C and pH 7.4. Selectivities between the transacylation (TA) and glycosylation (Glyc) products and conversions (% modified HSA) were calculated based on peak heights in respective deconvoluted mass spectra.

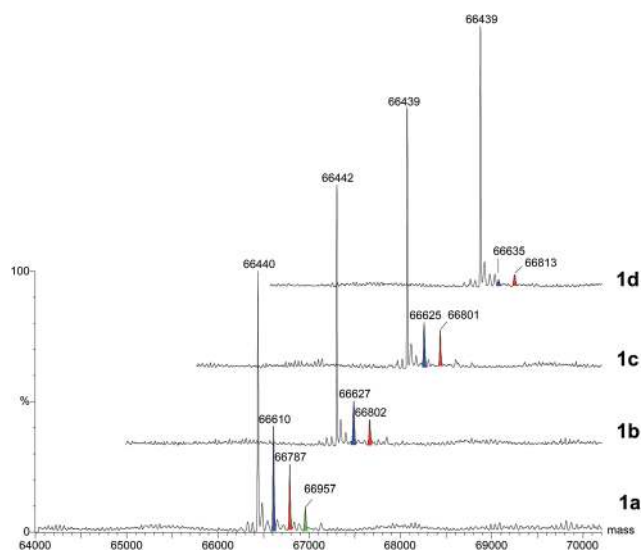


Fig. 3 Reaction of AGs with HSA. Mass spectra of 33 g L⁻¹ HSA incubated with ibuprofen analogues 1a–d at 5.0 mM for 16 h at 37 °C and pH 7.4. 1a: transacylation (blue), found: 66 610; expected: 66 612; glycosylation (red), found: 66 787; expected: 66 788; dual addition (both transacylation and glycosylation, green), found: 66 957; expected: 66 962. 1b: transacylation, found: 66 627; expected: 66 626; glycosylation, found: 66 802; expected: 66 802. 1c: transacylation, found: 66 625; expected: 66 626; glycosylation, found: 66 801; expected: 66 802. 1d: transacylation, found: 66 635; expected: 66 640; glycosylation, found: 66 813; expected: 66 816. Unmodified HSA is the major peak in all cases (found: 66 440, 66 442, 66 439 and 66 439 Da; expected: 66 438 Da).

adduct has been detected directly by MS of the full protein *in vitro* under relevant conditions. Previously, such *N*-glycosylation adducts of proteins have been found by forcing the reaction of imine intermediates with a reducing agent such as sodium cyanoborohydride^{7b,13,20} or at extreme high AG concentrations

as compared to HSA.^{7b,21} Furthermore, in most cases full characterisation of the formed adducts has not been performed.^{7b,13,22} Only a single observation of a glycosylation adduct with drug AGs with HSA under relevant conditions has been inferred from digests.²³ Notably our results show that stable glycosylation occurs on intact protein without addition of a 'chemically trapping' reducing agent and can be detected directly as intact protein adducts allowing quantification. These results are not only directly relevant to *in vivo* species but also now allow qualitative analysis of relevant reactivity. The high conversion observed even at 0.5 mM of **2** clearly indicates that irreversible glycosylation of endogenous proteins can occur during treatment with drugs that are metabolised to AGs.

p-Bromobenzoic acid AG **2** reacted exclusively *via* the glycosylation pathway, and no transacylation product could be observed (Fig. 1b). However, more subtle and intriguing results were observed following incubation with the AGs of ponalrestat **3** and the profens **1a–d** (entries 3–12, Table 1); both transacylation and glycosylation were unambiguously observed. These AGs were not as reactive as **2** with respect to protein modification. In most cases only trace modification occurred at 0.5 mM of AG, and significant amounts of coupling products could only be observed at relatively high AG concentrations (5.0 mM). In the case of ponalrestat AG **3** the transacylation and glycosylation adducts were formed in a 72 : 28 ratio at a combined yield of 24% at 5.0 mM **3** (entry 4). The ibuprofen analogues (entries 5–12) also gave a mixture of transacylation and glycosylation adducts, and the degree of modification can be visualised in the combined mass spectrum of **1a–d** (Fig. 3). Ibufenac (**1a**), (*R*)-(**1b**) and (*S*)-(**1c**) ibuprofen AGs showed clear reactivity with combined yields of 44, 23 and 24% respectively, whereas the α,α -disubstituted dimethyl analogue **1d** was significantly less reactive (entries 11 and 12, Table 1) showing no more than 8% reaction even at the higher concentrations and showing only trace products above background at lower concentrations. Interestingly, in addition to individual transacylation and glycosylation adducts, small amounts of dual adducts resulting from concomitant transacylation and glycosylation were also seen for **1a–c**.

These dual adducts highlighted that more than one reaction site was present on HSA. To map these sites and to

confirm the identities of the glycosylation and transacylation products modified proteins were characterized through 'peptide mapping' using tryptic digest followed by MS/MS analysis. The products of reaction of ibufenac-AG **1a** with HSA map to five different lysine residues and revealed that different sites were selectively modified by different reaction processes (Table 2 and Fig. 4); these sites only correlate in part with accessibility (see ESI†) and only Lys525 is reactive in both pathways.²⁵ As might be expected from the reactivity of α -unsubstituted ponalrestat AG **3**, both transacylation (lysines 195, 436 and 525) as well as glycosylation (lysines 137, 199 and 525) adducts could be observed for α -unsubstituted ibufenac AG **1a** (Table 2); in all cases the mapping results correlated strongly with the observed ratios determined by direct MS (Fig. 3 and Table 1).

Alkyl AGs displayed (Table 1) lower reactivities than aryl AGs; with *p*-bromobenzoic acid AG **2** a majority of HSA was modified even at 0.5 mM. Furthermore, both the nature and extent of protein modification with alkyl AGs was highly dependant on the degree of α -substitution; variation of α -substitution from unsubstituted (**1a** and **3**) to mono-substituted (**1b,c**) to disubstituted (**1d**) AGs, moves reaction preference from transacylation to a preference for glycosylation. This is a logical observation consistent with B_{Ac}2 nucleophilic substitution at the carbonyl group of an AG; a higher degree of α -substitution constitutes an increased steric hindrance disfavouring addition during such a transacylation pathway. In contrast, acyl migration is not affected to the same extent by increased α substitution,^{5a} and glycosylation adducts are therefore predominantly formed for highly α -substituted AGs as with the dimethyl analogue **1d**. In addition to different selectivity (transacylation *versus* glycosylation), alkyl AGs with no α -substitution are more reactive than mono- and disubstituted alkyl AGs, and AGs of drugs without α -substitution are therefore more likely to modify endogenous proteins *in vivo* than AGs of α -substituted drugs. The increased reactivity of ibufenac AG (**1a**) compared to (*R*)-(**1b**) and (*S*)-ibuprofen AG (**1c**) due to the lacking α methyl substituent is striking considering that ibufenac was withdrawn from the U.K. market in 1968 as a result of hepatotoxicity,^{10a,26} whereas (*R/S*)-ibuprofen is still extensively used.

Table 2 Peptide mapping of reaction sites in HSA when modified with ibufenac AG **1a**^a

Retention time (min)	<i>m/z</i> predicted	<i>m/z</i> observed	Peptide residues	Peptide sequence	Modified amino acid	Modification
33.49	704.1	704.1	137–144	K(+) YL YEIAR	K137	Glycosylation
24.36	921.8	921.4	191–197	ASSAK(+) QR	K195	Transacylation
27.80	621.6	621.6	198–205	LK(+) CASLQK ^b	K199	Glycosylation
24.16	450.5	451.2	433–439	VGSK(+) CCK	K436	Transacylation
22.07	652.7	651.4	525–534	K(+) QTALVELVK ^c	K525	Transacylation
18.86	740.7	741.1	525–534	K(+) QTALVELVK	K525	Glycosylation

^a Modified protein was hydrolyzed with trypsin followed by LC-coupled MS/MS analysis. The amino acid sequence of HSA was derived from the RCSB protein data bank: DOI: 10.2210/pdb1bm0/pdb.²⁴ Residues are numbered for this sequence of 585 amino acids. ^b Evidence for glycosylation of the C-terminal K205 of the 198–205 peptide was also obtained (see ESI for details). ^c Evidence for transacylation of the C-terminal K534 of the 525–534 peptide was also obtained (see ESI for details).

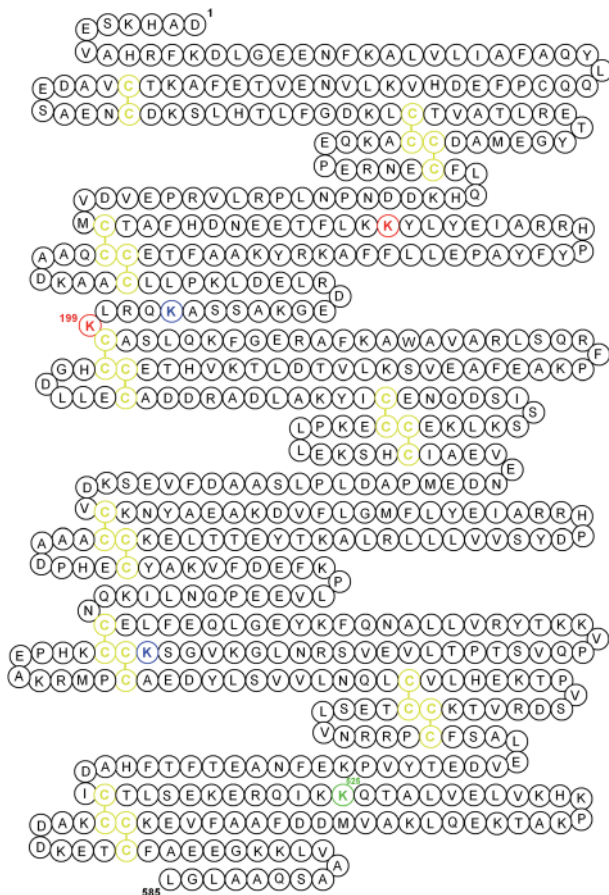


Fig. 4 Mapping the sites and types of reactivity of metabolites with human serum protein. Sequence map of HSA indicating primary amino acid sequence including disulfide bonds (yellow). Chemoselective reaction sites for glycosylation are coded red; sites for transacylation are coded blue, whereas Lys525 exhibiting both glycosylation and transacylation reactivity is coded green (see Table 2 for reactivity details).

Conclusions

In summary, we have shown that multiple modes of reactivity exist for 1- β -O-acyl glucuronides of widely-used pharmaceuticals with an abundant human protein under concentrations representative of those found *in vivo*. Importantly, we reveal here the first direct observations of stable AG-glycosylation adducts, which is highlighted by contrasting human protein reaction selectivities determined by both site and drug-AG identity; these range from near exclusive glycosylation (>95%) to majority acylation (>70%). These demonstrate that although glucuronic acid alone cannot glycosylate human protein (see ESI[†]), unlike glucose,²⁵ AGs of GlcA are sufficiently activated to cause extensive glycosylation. In this way we have established a rationalisation of AG reactivity based on structure, which we believe provides a useful tool in the informed development of new carboxylic acid containing drugs as well as in the monitoring, interpretation and assessment of the potential toxicity of existing drugs.² Our work also suggests that physiological levels of HSA glycosylation (~7–9%)²⁷ do not protect HSA from the

reactions described; this is also consistent with prior observations²⁸ that such glycosylation does not significantly alter interaction with drugs. This work strongly indicates that AG-induced modification of plasma proteins during treatment with carboxylic acid containing drugs can take place in a number of ways that lead to different, and in some cases previously unidentified covalent adducts. This seems of particular current relevance given recent decisions of certain drug development organizations to exclude all carboxylic acids, and in the light of regulatory opinions on AGs.² Prior observation²⁹ of enhanced hydrolysis rates in plasma over aqueous buffer seem likely to, in fact, be due also to reaction with components of plasma. Other proteins may also be substrates of the reactions shown here.³⁰ We are currently evaluating both the antigenic and immunogenic response³¹ of these unnatural protein motif structures as putative haptens, which may exist at high concentrations in patients chronically treated with *e.g.*, NSAIDs.

Representative methods

Purification of human serum albumin

To crude HSA (Lee BioSolutions, Inc., 50 mg, 0.75 μ mol) and EDTA (1.5 mg, 5 μ mol) in aq. phosphate buffered saline (5 mL, 20 mM, pH 8.5) was added dithiothreitol (10 mg, 65 μ mol) in phosphate buffered saline (68 μ L) and the reaction mixture was stirred at room temperature. The conversion was followed by mass spectrometry and after 2 h, full reduction of the Cys34–Cys disulfide was achieved. Cysteine and excess reducing agent was removed by PD10 size-exclusion chromatography eluting with a solution of EDTA (1 mM) and dithiothreitol (0.02 mM) in phosphate buffered saline (20 mM, pH 8.0). The solution was oxidatively refolded for 48 h at 4 $^{\circ}$ C. The protein solution was purified by PD10 size-exclusion chromatography eluting with phosphate buffered saline (20 mM, pH 7.4) to give HSA in more than 95% yield. The protein solution was concentrated using a YM10 Amicon ultrafiltration membrane to a concentration of 42 g L⁻¹. The protein concentration was determined from $\epsilon_{280\text{nm}} = 28\,730\text{ M}^{-1}\text{ cm}^{-1}$. ESI + TOF MS: found: 66 437, expected 66 438.

General incubation of acyl glucuronides with HSA

To an aq. solution of HSA (33 g L⁻¹) in phosphate buffered saline (20 mM, pH 7.4) were added acyl glucuronides **1a–d**, **2** or **3** at a final concentration of 0.5 or 5.0 mM. The reaction mixture was mixed very vigorously for 30 s and then gently shaken at 37 $^{\circ}$ C for 16 h. The protein solution was then cooled to 4 $^{\circ}$ C and purified by PD10 size-exclusion chromatography eluting with water to remove salts and excess reagent. The product was analysed by mass spectrometry (ESI + TOF MS).

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Notes and references

- 1 H. Spahn-Langguth and L. Z. Benet, *Drug Metab. Rev.*, 1992, **24**, 5.
- 2 US FDA, *Guidance for Industry: Safety testing of Drug metabolites*, Center for Drug Evaluation and Research, Rockville, MD, USA, 2008.
- 3 (a) A. R. Asif, V. W. Armstrong, A. Voland, E. Wieland, M. Oellerich and M. Shipkova, *Biochimie*, 2007, **89**, 393; (b) S. L. Regan, J. L. Maggs, T. G. Hammond, C. Lambert, D. P. Williams and B. K. Park, *Biopharm. Drug Dispos.*, 2010, **31**, 367.
- 4 (a) C. H. Johnson, I. D. Wilson, J. R. Harding, A. V. Stachulski, L. Iddon, J. K. Nicholson and J. C. Lindon, *Anal. Chem.*, 2007, **79**, 8720; (b) N. G. Berry, L. Iddon, M. Iqbal, X. Meng, P. Jayapal, C. H. Johnson, J. K. Nicholson, J. C. Lindon, J. R. Harding, I. D. Wilson and A. V. Stachulski, *Org. Biomol. Chem.*, 2009, **7**, 2525; (c) G. S. Walker, J. Atherton, J. Bauman, C. Kohl, W. Lam, M. Reily, Z. Lou and A. Mutlib, *Chem. Res. Toxicol.*, 2007, **20**, 876; (d) R. Sawamura, N. Okudaira, K. Watanabe, T. Murai, Y. Kobayashi, M. Tachibana, T. Ohnuki, K. Masuda, H. Honma, A. Kurihara and O. Okazaki, *Drug Metab. Dispos.*, 2010, **38**, 1857; (e) S. J. Vanderhoeven, J. Troke, G. E. Tranter, I. D. Wilson, J. K. Nicholson and J. C. Lindon, *Xenobiotica*, 2004, **34**, 889; (f) T. Yoshioka and A. Baba, *Chem. Res. Toxicol.*, 2009, **22**, 1559.
- 5 (a) A. V. Stachulski, J. R. Harding, J. C. Lindon, J. L. Maggs, B. K. Park and I. D. Wilson, *J. Med. Chem.*, 2006, **49**, 6931; (b) D. Noort, A. vanZuylen, A. Fidler, B. vanOmmen and A. G. Hulst, *Chem. Res. Toxicol.*, 2008, **21**, 1396.
- 6 H. S. Isbell and H. L. Frush, *J. Org. Chem.*, 1958, **23**, 1309.
- 7 (a) K. Akira, T. Uchijima and T. Hashimoto, *Chem. Res. Toxicol.*, 2002, **15**, 765; (b) J. R. Kenny, J. L. Maggs, X. Meng, D. Sinnott, S. E. Clarke, B. K. Park and A. V. Stachulski, *J. Med. Chem.*, 2004, **47**, 2816.
- 8 R. G. Dickinson and A. R. King, *Biochem. Pharmacol.*, 1991, **42**, 2301.
- 9 (a) O. M. Bakke, M. Manocchia, F. de Abajo, K. I. Kaitin and L. Lasagna, *Clin. Pharmacol. Ther.*, 1995, **58**, 108; (b) P. Zia-Amirhosseini, H. Spahn-Langguth and L. Z. Benet, *Adv. Pharmacol.*, 1994, **27**, 385; (c) H. Spahn-Langguth, M. Dahms and A. Hermening, *Adv. Exp. Med. Biol.*, 1996, **387**, 313.
- 10 (a) A. Herxheimer, *Drug. Ther. Bull.*, 1968, **6**, 48; (b) M. Castillo and P. C. Smith, *Drug Metab. Dispos.*, 1995, **23**, 566.
- 11 M. J. Bailey and R. G. Dickinson, *Chem.-Biol. Interact.*, 2003, **145**, 117.
- 12 (a) M. Shipkova, V. W. Armstrong, M. Oellerich and E. Wieland, *Ther. Drug Monit.*, 2003, **25**, 1; (b) M. Shipkova and E. Wieland, *Clin. Chim. Acta*, 2005, **358**, 2.
- 13 Y. J. Chiou, K. B. Tomer and P. C. Smith, *Chem.-Biol. Interact.*, 1999, **121**, 141.
- 14 C. Chalk, T. J. Benstead and F. Moore, *The Cochrane Database of Systematic Reviews*, 2007, CD004572.
- 15 (a) E. R. Bowkett, J. R. Harding, J. L. Maggs, B. K. Park, J. A. Perrie and A. V. Stachulski, *Tetrahedron*, 2007, **63**, 7596; (b) For synthesis of Statil, ed. D. R. Brittain and R. Wood, EP0002895A1, 1979.
- 16 S. J. Burton, A. V. Quirk and P. C. Wood, *Eur. J. Biochem.*, 1989, **179**, 379.
- 17 A. A. Ouameur, R. Marty and H. A. Tajmir-Riahi, *Biopolymers*, 2005, **77**, 129.
- 18 (a) P. C. Smith, P. N. Langendijk, J. A. Bosso and L. Z. Benet, *Clin. Pharmacol. Ther.*, 1985, **38**, 121; (b) G. E. McKinnon and R. G. Dickinson, *Res. Commun. Chem. Pathol. Pharmacol.*, 1989, **66**, 339.
- 19 M. W. Konstan, P. J. Byard, C. L. Hoppel and P. B. Davis, *N. Engl. J. Med.*, 1995, **332**, 848.
- 20 A. Ding, J. C. Ojingwa, A. F. McDonagh, A. L. Burlingame and L. Z. Benet, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 3797.
- 21 Y. Qiu, A. L. Burlingame and L. Z. Benet, *Drug Metab. Dispos.*, 1998, **26**, 246–256.
- 22 (a) H. Georges, I. Jarecki, P. Netter, J. Magdalou and F. Lopicque, *Life Sci.*, 1999, **65**, PL151; (b) N. Grubb, A. Weil and J. Caldwell, *Biochem. Pharmacol.*, 1993, **46**, 357.
- 23 A. Ding, P. Zia-Amirhosseini, A. F. McDonagh, A. L. Burlingame and L. Z. Benet, *Drug Metab. Dispos.*, 1995, **23**, 369.
- 24 S. Sugio, A. Kashima, S. Mochizuki, M. Noda and K. Kobayashi, *Protein Eng.*, 1999, **12**, 439.
- 25 N. Iberg and R. Fluckiger, *J. Biol. Chem.*, 1986, **261**, 13542.
- 26 L. Goldkind and L. Laine, *Pharmacoepidemiol. Drug Saf.*, 2006, **15**, 213.
- 27 K. A. Mereish, H. Rosenberg and J. Cobby, *J. Pharm. Sci.*, 1982, **71**, 235.
- 28 J. Anguizola, R. Matsuda, O. S. Barnaby, K. S. Hoy, C. Wa, E. DeBolt, M. Koke and D. S. Hage, *Clin. Chim. Acta*, 2013, **425**, 64–76.
- 29 C. H. Johnson, E. Karlsson, S. Sarda, L. Iddon, M. Iqbal, X. Meng, J. R. Harding, A. V. Stachulski, J. K. Nicholson, I. D. Wilson and J. C. Lindon, *Xenobiotica*, 2010, **40**, 9.
- 30 C. Skonberg, J. Olsen, K. G. Madsen, S. H. Hansen and M. P. Grillo, *Expert Opin. Drug Metab. Toxicol.*, 2008, **4**, 425.
- 31 G. P. Aithal, L. Ramsay, A. K. Daly, N. Sonchit, J. B. S. Leathart, G. Alexander, J. G. Kenna, J. Caldwell and C. P. Day, *Hepatology*, 2004, **39**, 1430.