# Identification of a novel carbamoyl glucuronide as a metabolism-dependent inhibitor of CYP2C8.

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## ABSTRACT

Glucuronidation is a major route of drug biotransformation and detoxification, whereby a drug is conjugated with glucuronic acid in a reaction catalyzed by UDP glucuronosyltransferases (UGTs). However, previous reports suggest glucuronide conjugates can be inhibitors of Phase I metabolism. Of particular note is gemfibrozil glucuronide, which is oxidized by CYP2C8, causing clinically relevant irreversible inactivation of this enzyme (Ogilvie *et al.*, 2006). In the present study, we evaluated the ability of Lu AA34893 and its carbamoyl glucuronide to inhibit *in vitro* the major drug metabolizing cytochrome P450 (CYP) enzymes. In NADPH-fortified human liver microsomes (HLM), Lu AA34893 was found to directly inhibit CYP2C19 ($IC_{50}$ = 0.20 µM), CYP2D6 ($IC_{50}$ = 6.9 µM), CYP3A4/5 (as measured by testosterone 6β-hydroxylation, $IC_{50}$ = 9.7 µM; midazolam 1´-hydroxylation, $IC_{50}$ = 15 µM; and nifedipine oxidation, $IC_{50}$ = 15 µM), CYP2A6 ($IC_{50}$ = 17 µM), CYP2B6 ($IC_{50}$ = 17 µM), CYP2C9 ($IC_{50}$ = 20 µM), CYP2C8 ($IC_{50}$ = 24 µM), and CYP1A2 ($IC_{50}$ = 54 µM). Furthermore, Lu AA34893 was found to competitively inhibit CYP2C19 and CYP3A4/5 (testosterone) with $K_i$ values of 0.08 µM and 6.7 µM, respectively, and to act as a mixed inhibitor of CYP2D6 ($K_i$ = 3.0 µM), and CYP3A4/5 (midazolam, $K_i$ = 6.1 µM; nifedipine, $K_i$ = 7.8 µM). Minimal metabolism-dependent inhibition (MDI) of several CYP enzymes by Lu AA34893 was observed. Assessment of the carbamoyl glucuronide of Lu AA34893, showed that, of the CYP enzymes evaluated, only CYP2C8 was inhibited directly ($IC_{50}$ = 71 µM). Of particular interest, when pre-incubated with NADPH-fortified human liver microsomes for 30 minutes prior to measurement of CYP activity, the carbamoyl glucuronide was found to be a MDI of CYP2C8, with over an 8-fold shift in $IC_{50}$ ($IC_{50}$ = 8.5 µM), unlike its aglycone. Additionally, there was some evidence of MDI of CYP2C19 ($IC_{50}$ shift from >100 µM to 66 µM). These findings demonstrate another example (*i.e.*, in addition to gemfibrozil) in which glucuronidation of a drug candidate converts the parent to a potent MDI of CYP2C8.

Reference:
Ogilvie BW, Zhang D, Li W, Rodrigues AD, Gipson AE, Holsapple J, Toren P, Parkinson A (2006) Glucuronidation converts gemfibrozil to a potent, metabolism-dependent inhibitor of CYP2C8: Implications for drug-drug interactions. *Drug Metab Dispos* **34**:191-197.

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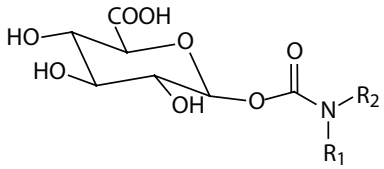
## INTRODUCTION

Glucuronidation is a major pathway of Phase 2 biotransformation that expedites the elimination of drugs or their Phase 1 metabolites. The conjugation of drugs with glucuronic acid is catalyzed by multiple microsomal UDP-glucuronosyltransferase (UGT) enzymes. Although Phase 1 reactions (oxidation, reduction and hydrolysis) generally precede glucuronidation and other Phase 2 reactions, there is a growing list of drugs (such as gemfibrozil, diclofenac, 17β-estradiol, naproxen, licofelone and the PPAR-a/γ agonist MRL-C) that undergo Phase 1 metabolism by CYP2C8 after they have undergone Phase 2 metabolism involving glucuronidation. In the case of gemfibrozil, CYP2C8 not only hydroxylates gemfibrozil glucuronide, but the enzyme becomes irreversibly inactivated due to heme alkylation (Ogilvie *et al.*, 2006; Baer *et al.*, 2009). The metabolism-dependent inactivation of CYP2C8 by gemfibrozil glucuronide is responsible, at least in part, for the pharmacokinetic and sometimes lethal interaction between gemfibrozil and cerivastatin (Baycol®), the cholesterol-lowering drug that was withdrawn from American, European and Japanese markets in 2001 (Backman *et al.*, 2002; Shitara *et al.*, 2004; Ogilvie *et al.*, 2006).

In the present study we describe a novel drug candidate conjugate, the carbamoyl glucuronide of Lu AA34893 that, like gemfibrozil glucuronide, functions as an irreversible metabolism-dependent inhibitor of CYP2C8. Lu AA34893 is a secondary amine (proprietary structure). Its conjugation with glucuronic acid likely involves the incorporation of carbon dioxide to form a carbamate, which is conjugated with glucuronic acid to form a carbamoyl glucuronide, as has been speculated for other amine-containing drugs like sertraline (Obach *et al.*, 2005):

$R_1R_2N\text{-}H + CO_2 \rightarrow R_1R_2N\text{-}COOH$

$R_1R_2N\text{-}COOH + \text{UDP-glucuronic acid} \rightarrow R_1R_2N\text{-}COO\text{-glucuronide} + \text{UDP}$




The basic structure of Lu AA34893 carbamoyl glucuronide

## MATERIALS & METHODS

### Chemicals

Pooled human liver microsomes (n=16, mixed gender) were prepared and characterized at XenoTech, LLC (Lenexa, Kansas) as described previously (Pearce *et al.*, 1996; Parkinson *et al.*, 2004). Lu AA34893 and its carbamoyl glucuronide were provided by H. Lundbeck A/S (Copenhagen, Denmark). Potassium ferricyanide was purchased from Sigma-Aldrich (St. Louis, Missouri). The sources of the other reagents used in this study have been described elsewhere (Robertson *et al.*, 2000; Ogilvie *et al.*, 2006; Paris *et al.*, 2009).

### *In vitro* CYP inhibition $IC_{50}$ determinations

The ability of Lu AA34893 and its carbamoyl glucuronide to inhibit the major CYP enzymes in a direct and metabolism-dependent manner was investigated with pooled HLMs, as described by Ogilvie *et al.*, 2006, 2008 and Paris *et al.*, 2009. To assess direct inhibition, incubations were conducted at 37°C in incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM, pH 7.4), an NADPH-generating system (consisting of 1 mM NADP, 5 mM glucose-6-phosphate and 1 Unit/ml glucose-6-phosphate dehydrogenase) and CYP marker substrate (*i.e.*, phenacetin, 40 μM; coumarin, 0.75 μM; bupropion, 50 μM; paclitaxel, 10 μM; diclofenac, 6 μM; *S*-mephenytoin 40 μM; dextromethorphan, 7.5 μM; midazolam, 4 μM; testosterone, 100 μM; or nifedipine, 10 μM), at the final concentrations indicated. Reactions were initiated by the addition of the NADPH-generating system and terminated after 5 min with an equal volume of acetonitrile (v/v) containing an appropriate internal standard.

To assess metabolism-dependent inhibition, Lu AA34893 or its carbamoyl glucuronide (0.1, 0.3, 1, 3, 10, 30 and 100 μM) were preincubated at 37°C with NADPH-fortified pooled HLMs for 30 minutes. After the preincubation period, the marker substrate (at a concentration approximately equal to its $K_m$) was added, and the incubation continued for 5 min to measure residual CYP activity. Reactions were terminated with an equal volume of acetonitrile containing an appropriate internal standard. Metabolite formation was determined by LC/MS/MS as described previously (Ogilvie *et al.*, 2008; Paris *et al.*, 2009).

### Reversibility of CYP2C8 inhibition by Lu AA34893 carbamoyl glucuronide

The reversibility of the observed metabolism-dependent inhibition of CYP2C8 by Lu AA34893 carbamoyl glucuronide was assessed based on an ultracentrifugation method as described by Buckley *et al.*, 2008. Briefly, Lu AA34893 carbamoyl glucuronide was preincubated with HLM for 30 min in the presence and absence of NADPH. After the preincubation, HLMs were **(a)** re-isolated by ultracentrifugation and then assessed for residual CYP2C8 activity, or **(b)** treated with potassium ferricyanide, re-isolated by ultracentrifugation and then assayed for residual CYP2C8 activity.

### The kinetics of CYP2C8 inactivation ($K_I/k_{inact}$)

To determine the $K_I$ and $k_{inact}$ values for the inactivation of CYP2C8 by the carbamoyl glucuronide of Lu AA34893, preincubations were performed with NADPH-fortified pooled HLM at approximately 25 times the normal protein concentration (*i.e.*, 1.25 mg/mL for paclitaxel 6α-hydroxylation) for zero, 3, 6, 9, 15 and 30 minutes. After the preincubation, an aliquot of the preincubation mixtures was transferred to a second tube containing the marker substrate, at approximately 2 times its $K_m$ and an NADPH-generating system resulting in a 25-fold dilution. The incubation with marker substrate was continued for 5 min, after which residual CYP2C8 activity was measured as described above.

## RESULTS

**Table 1** summarizes $IC_{50}$ values of Lu AA34893 and its carbamoyl glucuronide for direct and metabolism-dependent inhibition of CYP activity in HLM. The parent compound, Lu AA34893, caused direct inhibition of all CYP enzymes evaluated. It was an especially potent inhibitor of CYP2C19 ($IC_{50}$ 0.2 μM), although even the least potently inhibited enzyme (CYP1A2) was inhibited with an $IC_{50}$ value of 54 μM. Further evaluation of the most potently inhibited enzymes revealed that, as a direct inhibitor, Lu AA34893 competitively inhibits CYP2C19 and CYP3A4/5 (testosterone) with $K_i$ values of 0.08 μM and 6.7 μM, respectively, and functioned as a mixed inhibitor of CYP2D6 ($K_i$ = 3.0 μM), and CYP3A4/5 (midazolam, $K_i$ = 6.1 μM; nifedipine, $K_i$ = 7.8 μM) (data not shown).

In contrast to the parent compound, the carbamoyl glucuronide metabolite, when evaluated as a direct inhibitor, failed to inhibit any of the CYP enzymes evaluated with an $IC_{50}$ value less than 100 μM, with the exception of CYP2C8, which was inhibited with an $IC_{50}$ value of 71 μM. Furthermore, the carbamoyl glucuronide of Lu AA34893 caused metabolism-dependent inhibition of CYP2C8 with a greater than 8-fold shift in $IC_{50}$ following a 30-min preincubation with NADPH-fortified HLM (from $IC_{50}$ 71 μM to 8.5 μM), as shown in **Figure 1**. Furthermore, there was some evidence of modest metabolism-dependent inhibition of CYP2C19 by the carbamoyl metabolites ($IC_{50}$ shift from >100 μM to 66 μM). However, there is some evidence that this inhibition of CYP2C19 may not be NADPH-dependent (data are not shown).

**Figure 2** shows the results of an ultracentrifugation experiment designed to assess the reversibility of the metabolism-dependent inhibition of CYP2C8 by Lu AA34893 carbamoyl glucuronide. The metabolism-dependent inhibition of CYP2C8 by Lu AA34893 carbamoyl glucuronide was not reversed by isolating the HLM (which otherwise reverses the inhibition associated with the formation of a more potent, direct-acting inhibitor), nor was it reversed by treatment with potassium ferricyanide (which otherwise reverses the inhibition associated with formation of a metabolite inhibitor complex [MIC] due to coordination of a metabolite with the ferric heme iron). The results in **Figure 2** suggest that Lu AA34893 carbamoyl glucuronide is an irreversible inactivator of CYP2C8, as previously demonstrated for gemfibrozil glucuronide.

The kinetics of CYP2C8 inactivation by the carbamoyl glucuronide of Lu AA34893 were determined as described in Materials & Methods, and the results are shown in **Figure 3**. The carbamoyl glucuronide of Lu AA34893 inactivated CYP2C8 with a $k_{inact}$ of 0.038 $min^{-1}$ and a $K_I$ of 48 μM ($k_{inact}/K_I$ = 0.79 $min^{-1}$ $mM^{-1}$).

## Table 1.

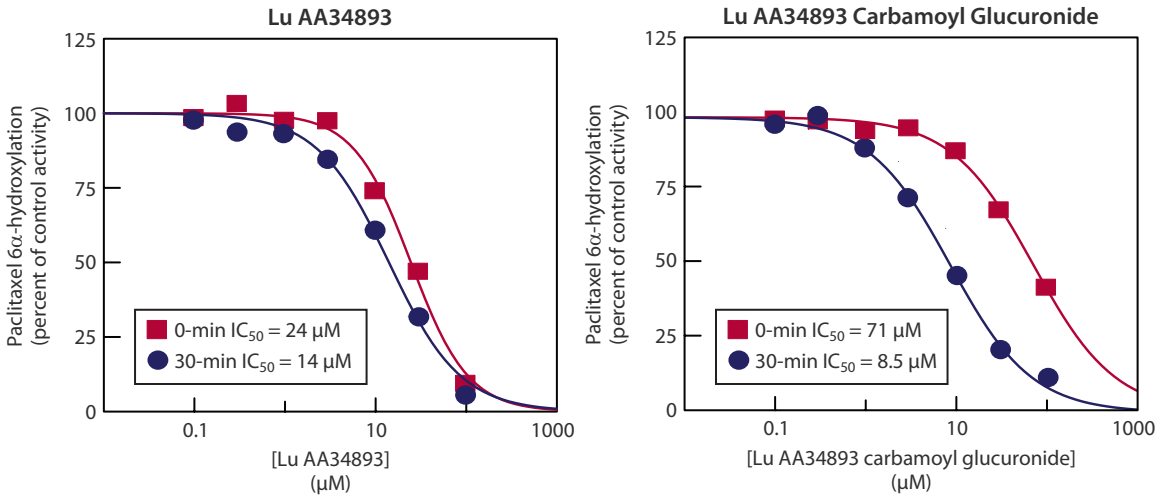
***In vitro* evaluation of Lu AA34893 and its carbamoyl glucuronide as inhibitors of CYP enzymes ($IC_{50}$ values)**

| | Lu AA34893 | | Carbamoyl glucuronide of Lu AA34893 | |
|---|---|---|---|---|
| Enzyme | 0-min Preincubation | 30-min Preincubation | 0-min Preincubation | 30-min Preincubation |
| CYP1A2 | 54 | 56 | >100 | >100 |
| CYP2A6 | 17 | 34 | >100 | >100 |
| CYP2B6 | 17 | 13 | >100 | >100 |
| CYP2C8 | 24 | 14 | 71 | 8.5 |
| CYP2C9 | 20 | 15 | >100 | >100 |
| CYP2C19 | 0.20 | 0.12 | >100 | 66 |
| CYP2D6 | 6.9 | 3.9 | >100 | >100 |
| CYP3A4/5 (M) | 15 | 8.8 | >100 | >100 |
| CYP3A4/5 (T) | 9.7 | 6.0 | >100 | >100 |
| CYP3A4/5 (N) | 15 | 8.9 | >100 | >100 |

M, T, N denote midazolam, testosterone and nifedipine, respectively
Note: $IC_{50}$ values in μM

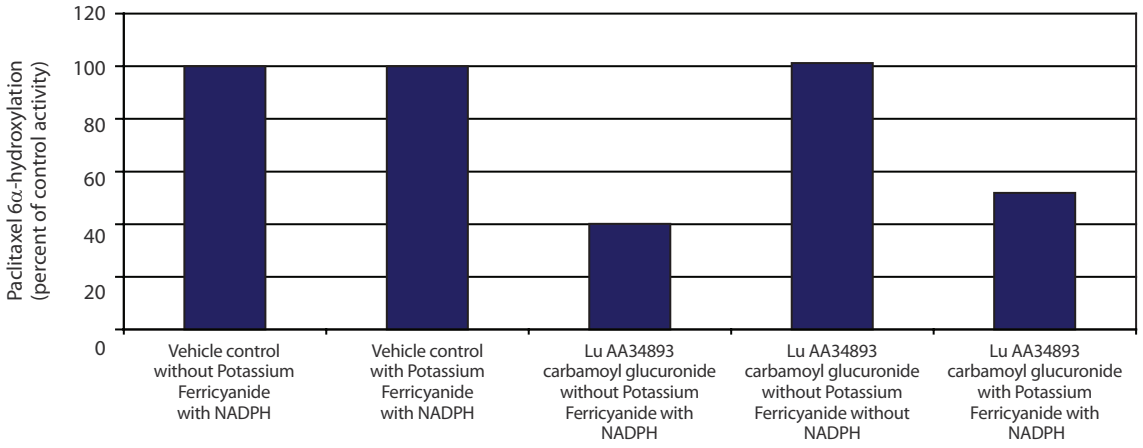
## Figure 1.

**Comparison of CYP2C8 $IC_{50}$ plots between Lu AA34893 and its carbamoyl glucuronide**




## Figure 2.

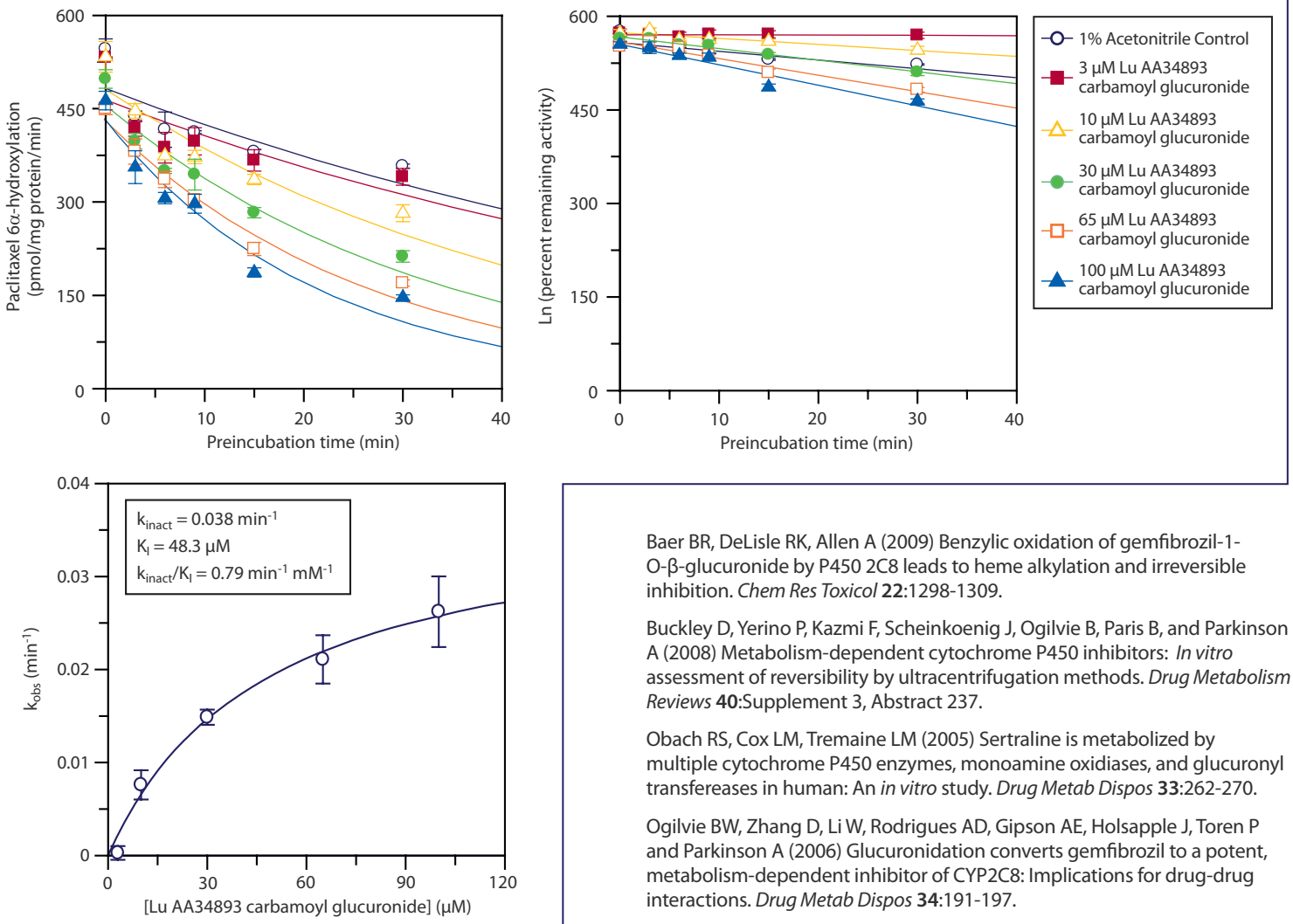
**Assessment of the reversibility of CYP2C8 inhibition by Lu AA34893 carbamoyl glucuronide**




| | | | | | |
|---|---|---|---|---|---|
| 100 μM Lu AA34893 carbamoyl glucuronide | − | − | + | + | + |
| Solvent | + | + | + | + | + |
| NADPH | + | + | + | − | + |
| $K_3Fe(CN)_6$ | − | + | − | − | + |
| Re-isolated | + | + | + | + | + |

**Figure 3.**

**Determination of the kinetic parameters of inactivation for the carbamoyl glucuronide of Lu AA34893 with CYP2C8**




## CONCLUSIONS

- The secondary amine Lu AA34893 was a direct-acting inhibitor of all the CYP enzymes examined and was a potent inhibitor of several of them ($IC_{50}$ < 10 μM). However, the parent compound caused little or no metabolism-dependent inhibition.
- In contrast to the parent compound, its carbamoyl glucuronide metabolite was a weak inhibitor of all the CYP enzymes examined (with $IC_{50}$ values exceeding 100 μM in all but one case).
- In contrast to the parent compound, its carbamoyl glucuronide metabolite functioned as an irreversible metabolism-dependent inhibitor of CYP2C8 ($k_{inact}$ = 0.038 min$^{-1}$; $K_I$ = 48 μM; $k_{inact}/K_I$ = 0.79 min$^{-1}$ mM$^{-1}$).

Lu AA34893 serves as another example, along with gemfibrozil, of a compound that is converted by glucuronidation to a conjugated metabolite that functions as an irreversible metabolism-dependent inhibitor of CYP2C8.

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