UGT Inhibition Studies in the Presence or Absence of Alamethicin: Evaluation of UGT1A1 and UGT2B7 Inhibition in HLM and Recombinant Enzymes

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Introduction

The importance of evaluating new drug candidates for the potential to inhibit UDP-glucuronosyltransferase (UGT) enzymes has recently garnered scientific and regulatory interest. To date, several published studies have demonstrated the importance of various membrane disruptors (*e.g.,* alamethicin or CHAPS) and other exogenous protein sources (*e.g.,* BSA or FABP) for improvement of the *in vitro* to *in vivo* prediction of metabolic clearance due to glucuronidation (Fisher *et al.*, 2000, Rowland *et al.*, 2007, Walsky *et al.*, 2012). The active site of UGTs faces the lumen of the endoplasmic reticulum, and typically a detergent is used to disrupt the membrane allowing for maximal enzyme activity. However, there is little data available to evaluate the impact of these exogenous factors on evaluating inhibition of UGT enzymes *in vitro*.

In the present study, we examined the effect of alamethicin, a pore forming agent, on UGT1A1 and UGT2B7 inhibition (IC_{50}) in HLM and recombinant UGTs by a variety of commonly used UGT inhibitors with the goal of optimizing assays conditions.

Materials & Methods

Chemicals

Alamethicin, 3′-azido-3′-deoxythymidine (AZT), bilirubin, clomipramine, diazepam, diclofenac, ethynylestradiol, hyodeoxycholic acid and itraconazole were purchased from Sigma Aldrich (St. Louis, MO). Cyclosporin was purchased from Fluka (Buchs, Switzerland), and erlotinib from Toronto Research Chemicals (Toronto, Canada).

Test system

Pooled human liver microsomes (HLM, n=200, mixed gender) were prepared from non-transplantable livers and characterized at XenoTech, LLC (Lenexa, KS) as described previously (Pearce *et al.*, 1996; Parkinson *et al.*, 2004). Recombinant UGTs were purchased from BD Biosciences (Franklin Lakes, NJ).

In vitro chemical inhibition under initial rate conditions in HLM

In vitro inhibition experiments were conducted under initial rate conditions in either UDPGA-fortified pooled HLM (n=200) or recombinant UGTs (0.1 mg/mL) in the presence or absence of alamethicin (25 µg/mg). HLM and rUGTs were activated with the addition of alamethicin for 15 minutes prior to starting the reaction. In all cases, the concentration of marker substrate was chosen to approximately equal to its experimentally determined K_m values +/– alamethicin in HLM or rUGT (Table 1, Figures 1 and 2). Several inhibitors were evaluated, namely bilirubin, cyclosporin, ethynylestradiol, erlotinib, itraconazole, diazepam, diclofenac, AZT, hyodeoxycholic acid and clomipramine. Incubations were initiated by the addition of 10 mM UDPGA and marker substrate ([S] ≈ K_m) for 5 minutes to determine residual UGT1A1 (estradiol-3-*O*-glucuronidation) and UGT2B7 (morphine-3-*O*-glucuronidation) activities. Reactions were quenched with acetonitrile containing deuterated internal standards, followed by protein precipitation by way of centrifugation. Metabolite formation was then determined by LC-MS/MS analysis as described previously (Parkinson *et al.*, 2011).

Table 1. Experimentally determined K_m values +/– alamethicin in HLM or rUGT

		HLM		rUGT	
	Substrate	**K_m – Ala (µM)**	**K_m + Ala (µM)**	**K_m – Ala (µM)**	**K_m + Ala (µM)**
UGT1A1	Estradiol	19.1 ± 6.5	12.1 ± 0.8	11.8 ± 1.1	11.5 ± 0.5
UGT2B7	Morphine	401 ± 25	384 ± 56	339 ± 37	403 ± 21

Figure 1. Determination of S_{50} (K_m) for estradiol-3-*O*-glucuronidation in **(A)** HLM or **(B)** rUGT1A1 in the presence or absence of alamethicin

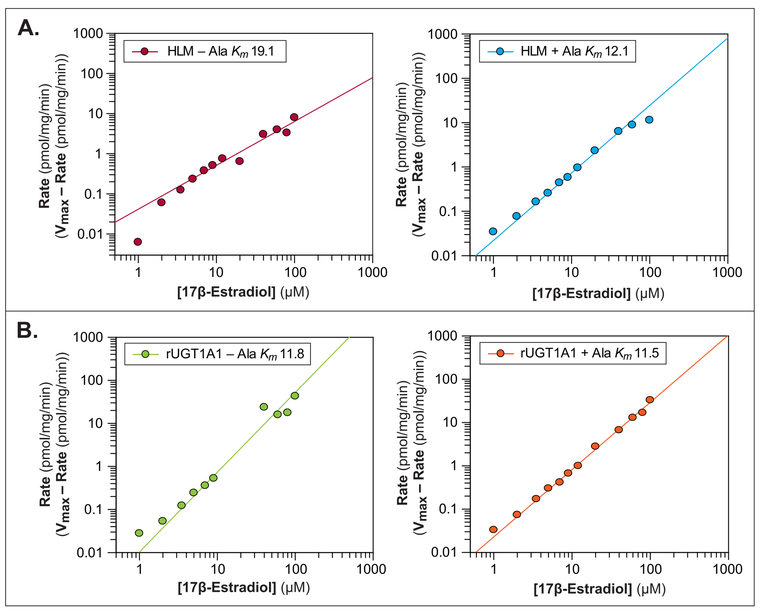
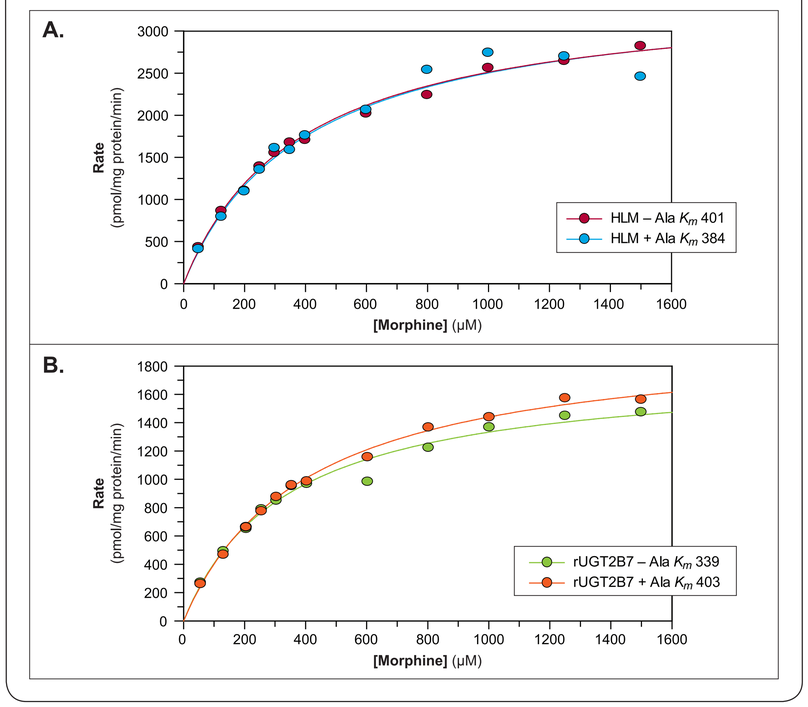


Figure 2. Determination of K_m for morphine-3-*O*-glucuronidation in **(A)** HLM or **(B)** rUGT2B7 in the presence or absence of alamethicin



Results

Results for UGT1A1 (Table 2, Figure 3) indicated that alamethicin activation had little to no effect on the inhibition (IC_{50} values) of estradiol glucuronidation by bilirubin, cyclosporin, ethynylestradiol, erlotinib and itraconazole in either HLM or rUGT1A1. Similarly, the inhibition results for UGT2B7 (Table 3, Figure 4) also suggest that the presence of alamethicin had little to no effect on the inhibitory potency of morphine glucuronidation by diazepam or diclofenac in either native or activated HLM and rUGT2B7.

Table 2. Inhibition of UGT1A1 (estradiol-3-*O*-glucuronidation) in HLM and rUGT1A1 in the presence or absence of alamethicin

	HLM		rUGT1A1	
Inhibitors	**IC_{50} – Ala (µM)**	**IC_{50} + Ala (µM)**	**IC_{50} – Ala (µM)**	**IC_{50} + Ala (µM)**
Bilirubin	2.75 ± 0.14	2.70 ± 0.47	2.78 ± 0.23	2.49 ± 0.20
Cyclosporin	43.7 ± 15.4	44.3 ± 11.9	46.4 ± 14.4	55.5 ± 14.7
Ethynylestradiol	45.2 ± 2.9	46.2 ± 3.8	47.9 ± 2.4	46.9 ± 3.0
Erlotinib	3.14 ± 1.58	3.22 ± 0.79	1.40 ± 0.20	1.63 ± 0.22
Itraconazole	10.0 ± 4.0	7.05 ± 2.75	4.46 ± 1.75	3.13 ± 1.14

Figure 3. Inhibition of UGT1A1 enzyme activity in **(A)** HLM or **(B)** rUGT1A1 by bilirubin in the presence or absence of alamethicin

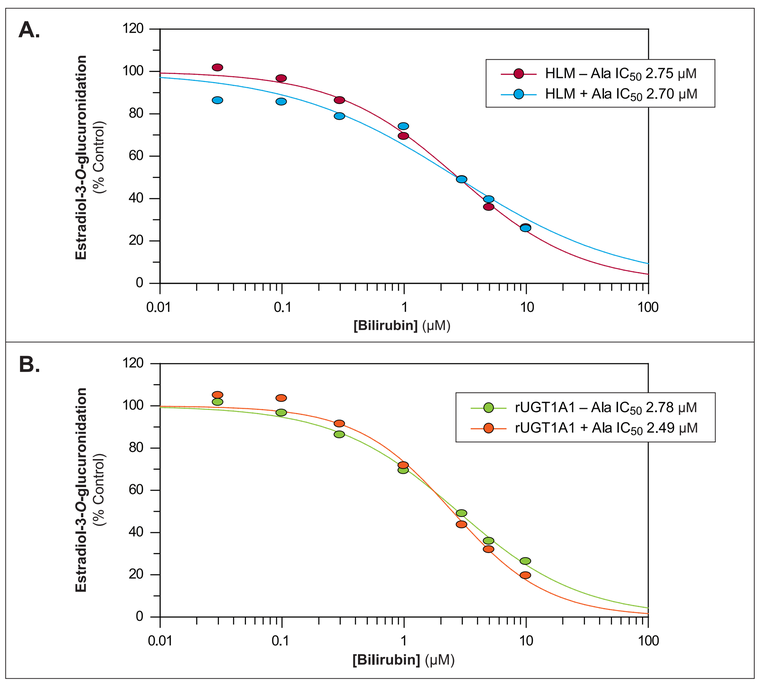
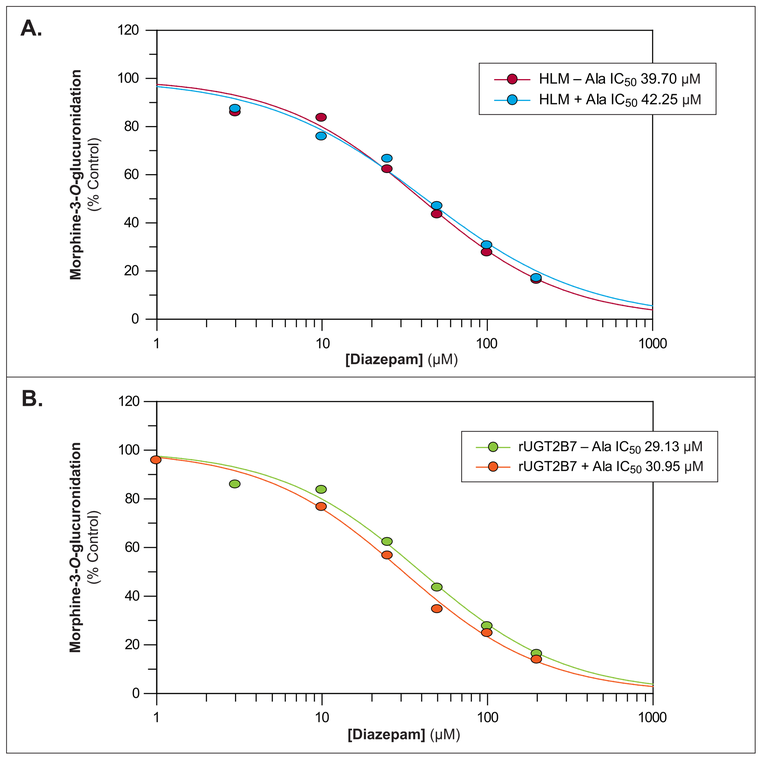


Table 3. Inhibition of UGT2B7 (morphine-3-*O*-glucuronidation) in HLM and rUGT2B7 in the presence or absence of alamethicin

	HLM		rUGT2B7	
Inhibitors	**IC_{50} – Ala (µM)**	**IC_{50} + Ala (µM)**	**IC_{50} – Ala (µM)**	**IC_{50} + Ala (µM)**
AZT	1030 ± 100	1420 ± 110	257 ± 113	401 ± 100
Clomipramine	19.9 ± 5.3	22.0 ± 2.2	19.8 ± 1.8	19.1 ± 0.9
Hyodeoxycholic acid	171 ± 37	157 ± 26	30.1 ± 7.6	44.5 ± 4.9
Diazepam	39.7 ± 3.2	42.3 ± 3.4	29.1 ± 3.2	31.0 ± 1.3
Diclofenac	49.2 ± 5.5	54.2 ± 6.2	64.1 ± 11.5	42.6 ± 6.2

Figure 4. Inhibition of UGT2B7 enzyme activity in **(A)** HLM or **(B)** rUGT2B7 by diazepam in the presence or absence of alamethicin



Conclusions

- These findings suggest that activation of UGT enzyme activity by alamethicin in either HLM or recombinant UGT enzyme sources does not affect the inhibitory potencies (IC_{50} values) across a range of compounds.
- Specifically, the inhibition potencies towards UGT1A1 and UGT2B7 enzyme activities were not affected if the *in vitro* experiments were conducted with marker substrate concentrations equal to their experimentally determined K_m values (determined with or without alamethicin).

References

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