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Short communication

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Inhibition of canalicular and sinusoidal taurocholate efflux by cholestatic drugs in human hepatoma HepaRG cells

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Running title: Inhibition of taurocholate efflux from human HepaRG cells

Abstract

HepaRG cells are highly-differentiated human hepatoma cells, which are increasingly recognized as a convenient cellular model for *in vitro* evaluation of hepatic metabolism, transport and/or toxicity of drugs. The present study was designed to evaluate whether HepaRG cells can also be useful for studying drug-mediated inhibition of canalicular and/or sinusoidal hepatic efflux of bile acids, which constitutes a major mechanism of drug-induced liver toxicity (DILI). For this purpose, HepaRG cells, initially loaded with the bile acid taurocholate (TC), were re-incubated in TC-free transport assay medium, in the presence or absence of calcium or drugs, before analysis of TC retention. This method allowed to objectivise and quantitatively measure biliary and sinusoidal efflux of TC from HepaRG cells, through distinguishing cellular and canalicular compartments. In particular, time-course analysis of the TC-free re-incubation period of HepaRG cells, *i.e*., the efflux period, indicated that a 20 min-efflux period allowed to reach biliary and sinusoidal excretion indexes for TC around 80 % and 60 %, respectively. Addition of the prototypical cholestatic drugs bosentan, cyclosporin A, glibenclamide or troglitazone during the TC-free efflux phase period was demonstrated to markedly inhibit canalicular and sinusoidal secretion of TC, whereas, by contrast, incubation with the non-cholestatic compounds salicylic acid or flumazenil was without effect. Such data therefore support the use of human HepaRG cells for *in vitro* predicting DILIs due to inhibition of hepatic bile acid secretion, using a biphasic TC loading/efflux assay.

Key-words

Bile salt export pump, cholestatic drug, efflux, HepaRG cells, taurocholate.

1. Introduction

Alteration of hepatic bile acid transport is recognized as a major mechanism of drug-induced liver injury (DILI) (Chatterjee & Annaert, 2018; Fernández-Murga et al., 2018; Mosedale & Watkins, 2017). It notably corresponds to the inhibition of the canalicular ATP-binding cassette (ABC) transporter bile salt export pump (BSEP/*ABCB11*) and results in bile acid retention into hepatocytes and cholestasis (Dawson et al., 2012; Izat & Sahin, 2021). Beside canalicular efflux, sinusoidal secretion of bile acids, mediated notably by the ABC transporters multidrug resistance-associated protein (MRP) 3 (*ABCC3*) and MRP4 (*ABCC4*), contributes to the regulation of bile acid accumulation into hepatocytes (Keppler, 2011; Köck & Brouwer, 2012). Inhibition of this sinusoidal bile acid efflux can consequently be considered as an additional cause of DILI (Köck et al., 2014). Potential interactions of new molecular entities with BSEP and MRP3/MRP4 have therefore likely to be considered during their pharmaceutical development, in order to identify and discard candidate drugs that may cause DILIs (Hillgren et al., 2013; Kenna et al., 2018; Morgan et al., 2010; Morgan et al., 2013). For such a purpose, various *in vitro* assays are available, such as sandwich-cultured hepatocytes-based efflux assays and BSEP-, MRP3- or MRP4-related vesicular assays (Cheng et al., 2016; Kenna et al., 2018; Kostrubsky et al., 2003; Pedersen et al., 2013; Van Brantegem et al., 2019; Wolf et al., 2010). The use of cultured hepatocytes, but not that of vesicles, allows an integrated approach, with determination of drug effects towards both canalicular and sinusoidal efflux of bile acids such as taurocholate (TC) (Susukida et al., 2015). In this context, owing to the differences between species with respect to hepatobiliary disposition of TC (Yang et al., 2015), primary human hepatocytes have likely to be preferentially considered. The poor and scarce availability of plateable human hepatocytes, together with the variability of their quality (depending on their production process and origin) and/or their drug detoxifying protein expression, may however limit their use, thus underlining the interest of surrogates for them, such as human hepatoma

HepaRG cells (Andersson et al., 2012; Lübberstedt et al., 2011). Indeed, HepaRG cells exhibit high levels of drug detoxifying proteins, including drug transporters (Hammer et al., 2021). In particular, they display expression of bile acid transporters, including BSEP, MRP3 and MRP4, as well as functional bile canaliculi (Bachour-El Azzi et al., 2015; Le Vee et al., 2013). They have consequently permitted to characterize the toxic effects of cholestatic drugs, especially towards bile canaliculi dynamics (Burbank et al., 2016). In the present study, we demonstrate using a biphasic TC loading/efflux assay that HepaRG cells can be used for simultaneously investigating the effects of cholestatic drugs on canalicular and sinusoidal efflux of TC. This illustrates thus the potential relevance of HepaRG cells for *in vitro* assays aimed at predicting DILIs due to impairment of hepatobiliary transport of bile acids.

2. Material and Methods

2.1 Chemicals

Bosentan, cyclosporin A, flumazenil, glibenclamide, ketoconazole, probenecid, rifampicin, salicylic acid and troglitazone were provided by MedChemExpress (Sollentuna, Sweden). The cholestatic or non-cholestatic character of these drugs as well as their known inhibitory effects towards BSEP and MRP3/MRP4 activities are reported in Table S1. Radiolabelled [^{3}H(G)]-TC (specific activity = 15.5 Ci/mmol) was from Perkin-Elmer (Villebon-sur-Yvette, France).

2.2 Cell culture

Highly differentiated human HepaRG cells from passages 13 to 17 were cultured as already described (Le Vee et al., 2013). Briefly, cells, plated at a density of 2×10^5 cells/cm^2, were first grown in Williams' E medium supplemented with 10% (vol/vol), 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine, and 5×10^{-5} M hydrocortisone hemisuccinate for 2 weeks. Cells were next cultured for an additional 2 weeks in the same medium supplemented with 2% (vol/vol) dimethyl sulfoxide (DMSO), to get a full

differentiation status of the cells (Gripon et al., 2002). This differentiated status of DMSO-treated HepaRG cell cultures was routinely checked by phase-contrast microscopy analysis, demonstrating the presence of hepatocyte-like islands and the formation of bile canaliculi, as previously reported (Le Vee et al., 2013).

2.3 Biphasic TC loading/efflux assay

HepaRG cells were first incubated with 12.9 nM [^{3}H]-TC in transport assay buffer comprising 136 mM NaCl, 5.3 mM KCl, 1.1mM $KHPO_4$, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 11 mM D-glucose and adjusted to pH=7.4 for 30 min at 37°C (TC loading phase). After washing twice with ice-cold phosphate-buffered saline, cells were re-incubated at 37°C for up to 60 min in the absence of TC (TC efflux phase), in the transport medium described above (Calcium-containing medium) or with the same buffer, except that $CaCl_2$ and $MgSO_4$ were withdrawn and 1 mM EGTA was added (Calcium-free medium). Incubation with this calcium-free buffer promotes disruption of tight junctions and opening of bile canaliculi networks (Annaert et al., 2001; Liu et al., 1999) and thus allows to distinguish between cellular and canalicular compartments (Kenna et al., 2018; Kostrubsky et al., 2003). Chemicals tested towards canalicular and sinusoidal TC transport were added in the assay buffer only during the efflux phase. Cells were next washing twice with ice-cold phosphate-buffered saline. Intracellular levels of TC were finally measured by scintillation counting and were normalised to protein content, determined by the Bradford's method (Bradford, 1976). They were expressed as pmol TC/mg protein or as % of initial TC loading. Biliary excretion index (BEI) and sinusoidal excretion index (SEI) were calculated using the equations (A) and (B):

$$BEI\ (\%) = \frac{\left[TC_{\ Efflux/+Ca^{2+}}\right] - \left[TC_{\ Efflux/-Ca^{2+}}\right]}{\left[TC_{\ Efflux/+Ca^{2+}}\right]} \times 100\ (A)$$

$$SEI\ (\%) = \frac{\left[TC_{\ Loading}\right] - \left[TC_{\ Efflux/+Ca^{2+}}\right]}{\left[TC_{\ Loading}\right]} \times 100\ (B)$$

with [TC $_{Efflux/+Ca^{2+}}$] = TC concentration after the efflux phase in the presence of Ca^{2+}, *i.e.*, in cells + bile canaliculi after efflux, [TC $_{Efflux/-Ca^{2+}}$] = TC concentration after the efflux phase in the absence of calcium, *i.e.*, in cells after efflux, and [TC $_{Loading}$] = TC accumulation after the loading phase in the presence of calcium, *i.e.*, in cells + bile canaliculi after loading.

Effects of drugs towards canalicular or sinusoidal TC efflux, corresponding to % of BEI or SEI inhibition, were finally determined according to the equations (C) and (D):

$$Canalicular\ TC\ efflux\ inhibition\ \% = BEI\ inhibition\ \% = \frac{BEI_{\ Control} - BEI_{\ Drug}}{BEI_{\ Control}} \times 100 \quad (C)$$

$$Sinusoidal\ TC\ efflux\ inhibition\ \% = \ SEI\ inhibition\ \% = \frac{SEI_{\ Control} - SEI_{\ Drug}}{SEI_{\ Control}} \times 100 \quad (D)$$

with BEI $_{Control}$ = BEI in the absence of drug, BEI $_{Drug}$ = BEI in the presence of drug during the efflux phase, SEI $_{Control}$ = SEI in the absence of drug and SEI $_{Drug}$ = SEI in the presence of drug during the efflux phase.

2.4 Statistical analysis

Experimental data were usually expressed as means ± SD. They were statistically analysed through analysis of variance (ANOVA) followed by the Dunnett's or the Tukey's post-hoc test, or by Spearman's rank correlation, using the Prism 8.4.3 software (GraphPad Software, San Diego, CA, USA). The criterion of significance was $p < 0.05$.

3. Results and Discussion

To specifically and simultaneously analyse the effects of drugs towards canalicular and sinusoidal efflux of TC in HepaRG cells, we developed a biphasic functional assay in the present study, with a first step of TC loading (without drug) and a second step of TC efflux (with or without drug). Indeed, our preliminary assays demonstrated that at least the two prototypical cholestatic drugs cyclosporin A and troglitazone (Table S1), used either at 10 or

100 µM, markedly reduced TC loading in HepaRG cells (Figure S1). This likely reflects the inhibitory effects of these compounds towards the sodium-taurocholate co-transporting polypeptide (NTCP/*SLC10A1*) (Grosser et al., 2021), responsible for the entry of bile acids at the sinusoidal pole of hepatocytes (Kullak-Ublick et al., 2004). Initial loading of cells without drugs was therefore a pre-requisite to reach high intracellular levels of TC before efflux and to avoid false or misleading outcomes that may occur if the tested compound interferes with TC uptake (Cheng et al., 2016).

In order to optimize the efflux time of TC from HepaRG cells maintained either in the presence or absence of calcium, *i.e.*, with or without functional bile canaliculi, we next determined TC retention kinetic over a 60-min efflux period in our functional assay. As shown in Figure 1A, cellular retention of TC was falling rapidly in the absence of calcium, to 11.4% of initial loading after a 20 min efflux period. Cellular retention of TC also decreased with efflux time in the presence of calcium, but less quickly; it thus corresponded to 40.2% of initial loading after a 20 min efflux period (Figure 1A). With respect to SEI and BEI values, reflecting sinusoidal and canalicular secretion of TC, respectively, they increased with efflux time, to reach the high values of 60%-80% after a 20 min efflux time (Figure 1B and Figure 1C). This 20-min efflux time was consequently retained for further experiments investigating the effects of drugs toward both sinusoidal and canalicular efflux of TC.

BEI and SEI values in the absence or the presence of various drugs, used at 10 µM and 100 µM for most of them, are shown in Figure S2A and Figure S2B and corresponding inhibitions of canalicular and sinusoidal TC efflux, *i.e.*, BEI and SEI inhibition %, are indicated in Table 1. The markedly increased retention of TC in response to the reference cholestatic agent troglitazone in HepaRG cells, in the presence or absence of calcium, as well as the lack of effect of the non-cholestatic compound salicylic acid, are additionally shown in Figure 1D. Most of the cholestatic compounds included in the study, *i.e.*, bosentan, cyclosporin A,

glibenclamide, ketoconazole and troglitazone, were found to inhibit both canalicular and sinusoidal efflux of TC when used at 100 µM, whereas, by contrast, the non-cholestatic compounds flumazenil and salicylic acid failed to impair TC secretion (Table 1). This absence of TC leakage in response to cholestatic drugs used at 100 µM likely discards any major unspecific toxic effect of these chemicals; the fact that treatment by drugs, in the absence or presence of calcium, failed to alter cellular protein concentrations (Figure S3) also argues in favour of this conclusion. The dual repression of canalicular and sinusoidal TC efflux by cholestatic compounds indicates that these chemicals may act through inhibiting both BSEP and MRP3/MRP4, as already suggested using vesicular assays (Köck et al., 2014; Morgan et al., 2013). The fact that the percentages of BEI inhibition by drugs used either at 10 or 100 µM were significantly correlated with those of SEI inhibition (Figure S4) fully supports this hypothesis. Nevertheless, accurate comparison of IC_{50} values of cholestatic compounds towards BSEP-, MRP3 and MRP4-mediated transport of TC remains to be determined and would deserve further studies. Moreover, the concentrations required to inhibit canalicular or sinusoidal efflux of TC appeared to differ for some cholestatic drugs; indeed, glibenclamide used at 10 µM inhibited sinusoidal efflux of TC, but not canalicular one, whereas troglitazone used at 10 µM blocked canalicular, but not sinusoidal, efflux of TC (Table 1). This confirms that canalicular and sinusoidal efflux of TC are mediated by different transporters, that may exhibit differential sensitivities to inhibitors. This assertion may also be supported by the fact that probenecid, used at a 2 mM concentration known to block BSEP activity (Table S1) (Dawson et al., 2012), inhibited only canalicular efflux of TC in HepaRG cells (Table 1). Rifampicin was also found to differently impair the two types of TC efflux in HepaRG cells; thus, this compound, used either at 10 or 100 µM, markedly inhibited (by at least 50%) sinusoidal efflux of TC from HepaRG cells, but exerted a less potent inhibitory effect towards canalicular efflux (around 25% of inhibition), which moreover failed to reach a significant level

(Table 1). Such a lack of major inhibition of canalicular TC efflux from HepaRG cells may be rather surprising, because rifampicin is usually considered as a potent inhibitor of BSEP, as notably demonstrated by vesicular assays (Dawson et al., 2012; Stieger et al., 2000). The nature of the assays used for investigating BSEP inhibition (vesicular assays versus cells-based assays) may be hypothesized to contribute to this discrepancy. In this context, it is noteworthy that our HepaRG cells-based assay is likely more physiological than vesicles-based counterparts, and notably allows an holistic approach, taking into account both sinusoidal and canalicular secretion of TC. By this way, interplay of BSEP and other bile acid efflux transporters such as MRP3/MRP4, and possibly also the canalicular transporter MRP2 (*ABCC2*) and the sinusoidal organic solute transporter (OST) α/β (*SLC51A/SLC51B*), which may additionally be implicated in cholestasis (Kenna et al., 2018; Malinen et al., 2018), are considered. Moreover, TC is used as substrate for sinusoidal efflux from HepaRG cells, whereas by contrast, the MRP3/MRP4 probe substrate in vesicular assays is usually an estradiol derivative (Morgan et al., 2013), therefore less relevant for bile acid transport issues. Besides, the possible role of drug metabolites in bile salt transporter inhibition can likely be explored in HepaRG cells, which display high expression of drug metabolizing enzymes (Andersson et al., 2012); this may allow to take into account the transporter-enzyme interplay, relevant for hepatic processing of drugs (Matsunaga et al., 2018). The use of calcium-free conditions, which corresponds to a non-physiological situation, may however impair the exposure of canalicular transporters to drugs in HepaRG cells, which may constitute a limitation of the experimental system.

The present study fully supports the use of HepaRG cells for predicting bile acid transport inhibition by drugs and, beyond DILIs, as already claimed (Burbank et al., 2016; de Bruijn et al., 2022). This also confirms that HepaRG cells may serve as surrogates for human hepatocytes, as proposed for drug metabolism, transport and toxicity studies (Andersson et al., 2012; Guillouzo et al., 2007; Le Vee et al., 2006; Lübberstedt et al., 2011), including high-

throughput ones (Ooka et al., 2020). It should however be kept in mind that *in vitro* prediction of DILIs due to transporter inhibition remains rather complex (Chan & Benet, 2018; Garzel et al., 2019; Kenna & Uetrecht, 2018; Kotsampasakou et al., 2017). In particular, cutoff values for BSEP IC_{50} values with respect to "concerning levels" of BSEP inhibition have to be harmonized (Hafey et al., 2020). In addition, the unbound drug concentration in hepatocytes at the site of interaction with BSEP or MRP3/MRP4 remains a key-parameter to consider for predicting *in vivo* inhibition of hepatic bile acid efflux from *in vitro* data (Kenna et al., 2018; Riede et al., 2017). Hepatic partition coefficient (Kp) is also a notable point to take into account for improving hepatic exposure to cholestatic drugs (Martin et al., 2022). In this context, direct measurement and modeling of intracellular unbound drug concentrations in HepaRG cells may help to evaluate *in vivo* inhibition of canalicular and/or sinusoidal secretion of bile acids, as recently described for assessment of acetaminophen toxicity through integration of *in vitro* data from cultured HepaRG cells and physiologically-based pharmacokinetic modeling (Zhang et al., 2020). Finally, it is noteworthy that regulation of hepatic transporter expression, including that of BSEP, can be analysed in HepaRG cells (Le Vee et al., 2006), which are therefore also suitable for studying drug-induced repression of BSEP, a possible cause of cholestasis recently reported for metformin (Garzel et al., 2020).

4. Conclusion

Human hepatoma HepaRG cells were demonstrated to exhibit canalicular and sinusoidal efflux of TC through a biphasic functional assay consisting of a first step of TC loading and a second step of TC efflux, in the presence or absence of functional bile canaliculi. These canalicular and sinusoidal secretions of TC were found to be inhibitable by cholestatic compounds, thus underlining the interest of HepaRG cell cultures for the *in vitro* prediction of bile acid secretion inhibition by drugs and, beyond, of DILIs.

Conflict of interest

The authors have no conflict of interest to disclose.

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Legend to figure

Figure 1: Canalicular and sinusoidal secretion of taurocholate (TC) from HepaRG cells.
(A) HepaRG, initially loaded with 12.9 nM [^{3}H] TC for 30 min, were re-incubated in TC-free medium in the presence or absence of calcium for various times (from 5 min to 60 min). TC retention was next determined by scintillation counting. Data are expressed as percentages of initial TC loading and are the means ± SD of at least 3 independent experiments. (B) TC sinusoidal excretion index (SEI) and (C) biliary excretion index (BEI) values were determined for various times of TC efflux (from 5 to 60 min), as described in Materials and Methods; they are the means ± SD of at least 3 independent experiments. (D) HepaRG cells, initially loaded with 12.9 nM [^{3}H] TC for 30 min, were re-incubated for 20 min in TC-free medium in the presence or absence of calcium, and without (control) or with troglitazone or salicylic acid, used at 10 µM or 100 µM. TC retention was finally determined by scintillation counting. Data are expressed as percentages of initial TC loading. They are the means ± SD of 3 independent experiments and have been analysed through ANOVA followed by the Tukey's post-hoc test (***, $p<0.001$ when compared to corresponding controls, *i.e.*, control in the presence of calcium or control in the absence of calcium).

Table 1. Inhibitory effects of drugs towards taurocholate BEI and SEI in HepaRG cells.

Drug	Concentration	BEI inhibition %[a]	SEI inhibition %[a]
Bosentan	10 µM	10.7 ± 2.7	<0
	100 µM	51.4 ± 3.2***	46.7 ± 5.9**
Cyclosporin A	10 µM	46.5 ± 19.6***	49.6 ± 18.5***
	100 µM	60.5 ± 13.0***	65.6 ± 18.0***
Flumazenil	10 µM	1.3 ± 1.7	4.0 ± 4.1
	100 µM	13.6 ± 4.0	16.0 ± 24.2
Glibenclamide	10 µM	10.7 ± 10	43.9 ± 18.1**
	100 µM	76.4 ± 11.3***	76.1 ± 18.1***
Ketoconazole	10 µM	23.5 ± 12.3	23.6 ± 5.8
	100 µM	76.4 ± 11.6***	57.0 ± 12.2***
Probenecid	2 mM	61.3 ± 18.2***	29.2 ± 15.5
Rifampicin	10 µM	24.3 ± 8.6	50.0 ± 6.6***
	100 µM	26.4 ± 4.4	52.5 ± 14.0***
Salicylic acid	10 µM	0.6 ± 2.7	<0
	100 µM	9.9 ± 5.1	<0
Troglitazone	10 µM	76.2 ± 22.3***	3.8 ± 11.4
	100 µM	81.0 ± 9.5***	49.3 ± 6.5***

[a] data are expressed as means ± SD of three independent experiments and have been analysed though ANOVA and Dunnett's post-hoc test (**, $p<0.01$ and ***, $p<0.001$ when compared to control cells not exposed to drugs).

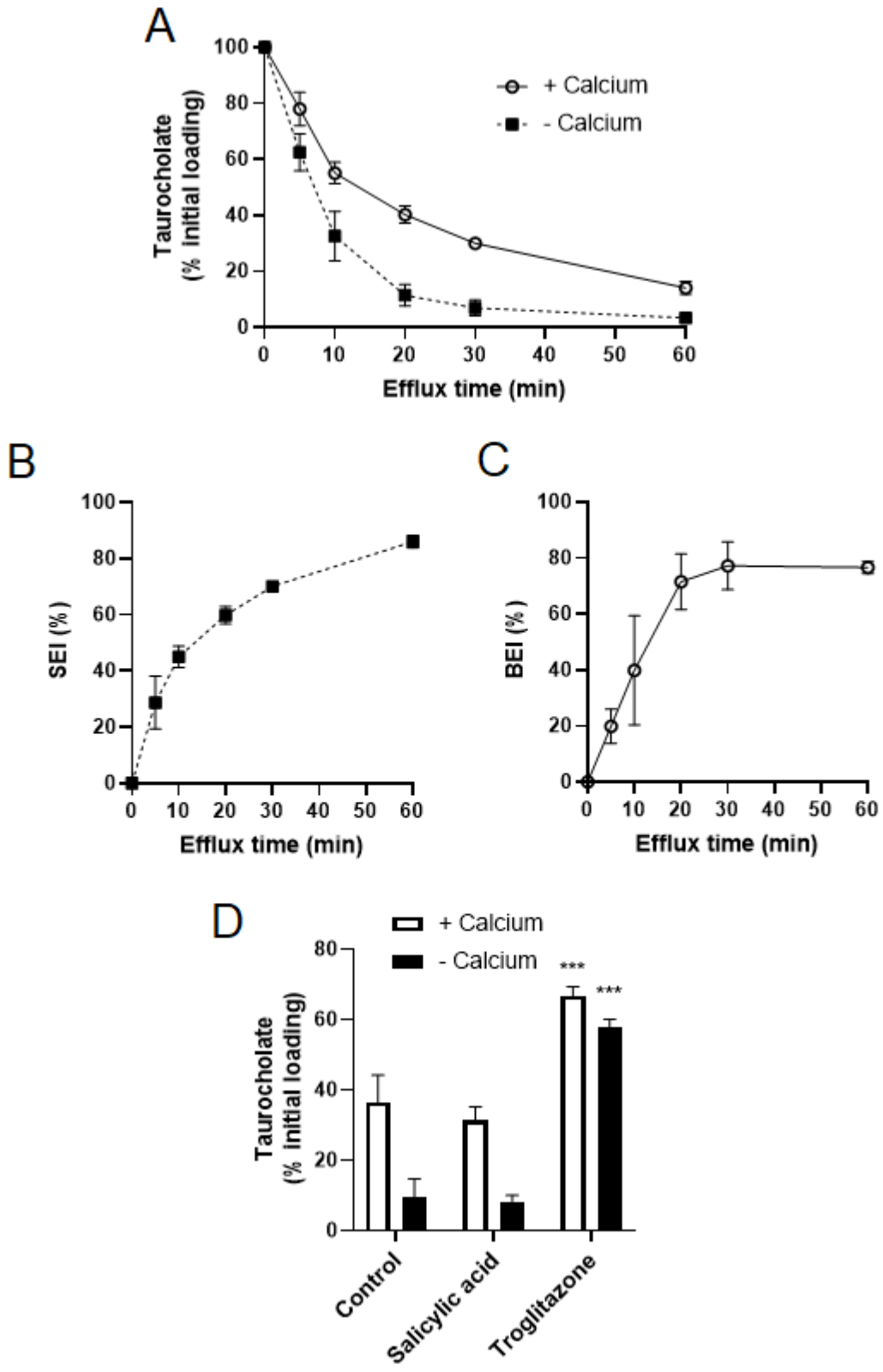
A
Taurocholate (% initial loading)
+ Calcium
- Calcium
Efflux time (min)
B
SEI (%)
Efflux time (min)
C
BEI (%)
Efflux time (min)
D
+ Calcium
- Calcium
***
***
Taurocholate (% initial loading)
Control
Salicylic acid
Troglitazone

Figure 1