Evaluating Reactive Acyl Glucuronides Formation from Diclofenac using a Resazurin/Resorufin Assay with Primary Rat Hepatocytes

N. Hari Singh[1], M. McMillian[1], Y. Qu[1], C. W. Ng[2], Y. Zhou[3], H. Yu[2] and A. Ananthanarayanan[1]

1 InvitroCue Pte Ltd, Singapore; 2 Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; 3 Centre for Mechanobiology, National University of Singapore, Singapore

Abstract #1998

INTRODUCTION

Previously we reported that increased resazurin reduction to resorufin and/or decreased removal of resorufin in response to carboxylated drug candidates is consistent with reactive acyl glucuronides (RAGs) generated from parent drugs in HepG2 cells (Hari Singh et al, 2015). NQO1 appears to be the critical enzyme inhibited by RAGs in HepG2 cells, resulting in inhibition of resorufin fluorescence removal, with the increased fluorescence providing an easy, convenient screen for evaluating compounds for RAG activity (Hari Singh et al, 2015). However, HepG2 cells are metabolism deficient and although enough glucuronidation is present to detect RAGs, the sensitivity of the resazurin assay is low. In the present study, we have taken advantage of the much higher metabolic capacity (glucuronidation and Cyp activities) in freshly isolated primary rat hepatocyte cultures, to examine the sensitivities of three NSAIDs in the RAG resazurin assay. Diclofenac, diflunisal and flufenamic acid all produced RAGs at concentrations similar or far below their therapeutic concentrations. At low concentrations, diclofenac (unlike the other two NSAIDs examined) is well known to be metabolized by Cyps rather than glucuronidated. Cyp inhibitors reduce diclofenac cytotoxicity by diverting diclofenac away from its cytotoxic metabolites to the formation of RAGs (Kretz-Rommel and Boelsterli, 1993). We observed potentiation of the resorufin fluorescence intensity with Cyp inhibitor ketoconazole (15 µM) at two concentrations of diclofenac, which are close to its therapeutic concentration (consistent with Kretz-Rommel and Boelsterli, 1993). The sensitivity of the assay to diflunisal and flufenamic acid were not similarly increased by ketoconazole. We used SKF 525A (40 µM, pre-incubated for an hour), a non-selective inhibitor of Cyp 450 enzymes and observed a huge potentiation of the resorufin fluorescence intensity at various concentrations of diclofenac and attributed it to better Cyp inhibition.

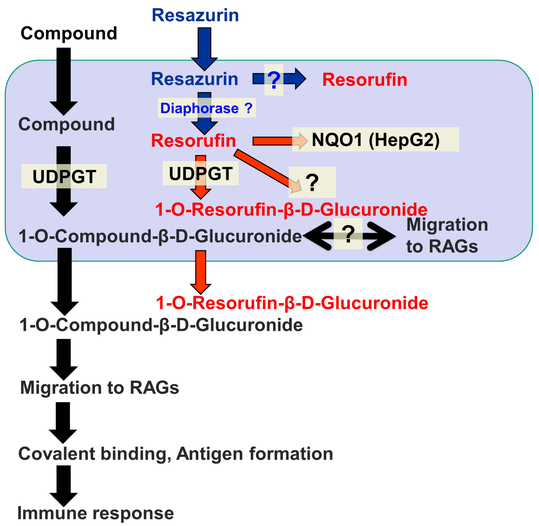


Figure 1. *Scheme depicting possible pathways involved in the disposition of carboxylated drugs and the transformation of resazurin and resorufin in vitro.*

Carboxylated drugs (including NSAIDs) are some of the most commonly prescribed and over-the-counter drugs. Around 25% of drugs withdrawn from marketplaces around the world due to severe toxicity have been carboxylic acids (Bailey et al, 2003). In hepatocytes and HepG2 cells, a carboxylated compound enters the cell at a certain rate (1), is glucuronidated in the ER at a compound-specific rate (2), and the 1-O-compound–β-D-glucuronide is pumped out from the cell in a different compound-specific rate. Additionally the 1-O-compound–β-D-glucuronide may be handled differently within the cell (3), particularly if metabolized to other reactive species. Potential adverse effects of RAGs are currently predicted from the migration rate of the glucuronide. RAGs bind covalently to proteins presumably in relation to reactivity. This may in turn have significant biological impact by possibly initiating immune response and cellular dysfunction. Thus there are a number of cellular processes besides innate reactivity of the formed RAG which contributes to the safety of a carboxylated compound.

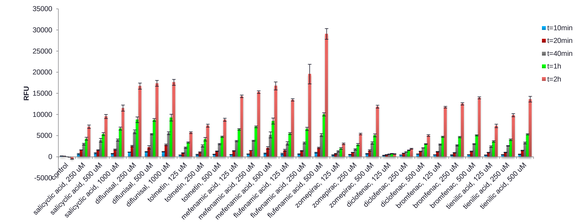


Figure 2. *Time course of fluorescence changes (relative fluorescent units, RFU) in resazurin/resorufin response to NSAIDs in primary rat hepatocytes at different times. Note that the values for the controls are negligible hence we observed massive increase with all tested compounds.*

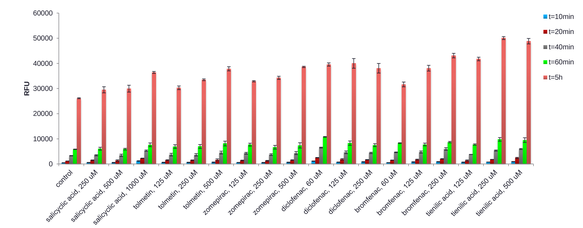


Figure 3. *Time course of fluorescence changes (RFU) in resazurin/resorufin responses to NSAIDs in HepG2 cells at different times. Note that the changes are small relative to the control.*

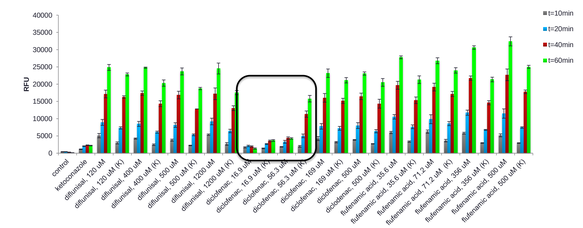


Figure 4. *Time course of fluorescence changes (RFU) in resazurin/resorufin responses to NSAIDs in primary rat hepatocytes at different times with and without ketoconazole (K) 15 µM pre-incubation. Note that the potentiation was observed only at low concentrations of diclofenac.*

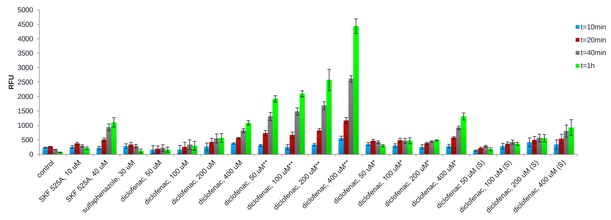
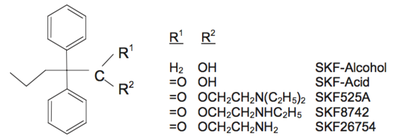


Figure 5. *Time course of fluorescence changes (relative fluorescent units, RFU) in resazurin/resorufin response to NSAIDs in primary rat hepatocytes at different times. Note the huge potentiation observed at the various concentrations of diclofenac used with SKF 525A at 40 µM (**) but not at 10 µM (*) and not with sulfaphenazole (S) at 30 µM.*



R^1	R^2	
H_2	OH	SKF-Alcohol
=O	OH	SKF-Acid
=O	$OCH_2CH_2N(C_2H_5)_2$	SKF525A
=O	$OCH_2CH_2NHC_2H_5$	SKF8742
=O	$OCH_2CH_2NH_2$	SKF26754

Figure 6. *Structures of SKF 525A-related Cyp 450 inhibitors (Franklin and Hathaway, 2008). We get a huge potentiation in resorufin fluorescence with SKF 525A at 40 µM after an hour of pre-incubation. We suspect that the potentiation might be due to the SKF-Acid and it could be independent of Cyp inhibition. Before we expand to other compounds, we want to determine the mechanism for the SKF 525A potentiation. The SKF-Acid could form RAGs which may interfere with the removal of resorufin. Obviously other mechanisms are possible and are currently being studied.*

CONCLUSION

Potentiation of resorufin fluorescence intensity by diclofenac is observed by several Cyp inhibitors and this suggests that inhibition of the Cyp metabolism diverts more of the diclofenac away from its cytotoxic metabolites to more RAGs (complimentary to Kretz-Rommel and Boelsterli's observation with diclofenac cytotoxicity). It has been pointed out that SKF 525A is metabolized to a potent diclofenac antagonist but more important from the perspective of the resazurin/resorufin assay to a SKF-Acid (Franklin and Hathaway, 2008). Pre-incubation of SKF 525A for an hour at relatively high concentration (40 µM vs 10 µM) implies RAG formation from SKF 525A, however further studies will be done to confirm the basis of the observed potentiation. The RAG resazurin assay in rat hepatocytes shows extreme sensitivity to these NSAIDs (diclofenac, diflunisal and flufenamic acid) and may help identify compounds early in Drug Development where Cyp metabolism is more important (as with diclofenac) than glucuronidation at low concentrations.