Non-alcoholic Steatohepatitis (NASH) Drug Discovery – Building a Consensus on ADME Screening Tools and Clinical Pharmacology Strategies to Aid Candidate Development

Ranjeet Prasad Dash[1,3,4], R. Jayachandra Babu[1], Nuggehally R. Srinivas[2,5]

[1]Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, AL 36849, USA. [2]Drug Metabolism and Pharmacokinetics, Zydus Research Centre, Ahmedabad 382210, Gujarat, India. [3]Johns Hopkins Drug Discovery, Johns Hopkins University, 855 North Wolfe Street, Baltimore, MD 21205, USA. [4]Department of Neurology, Johns Hopkins University, 855 North Wolfe Street, Baltimore, MD 21205, USA. [5]Innovation and Technology, Jubilant Life Sciences, D-12 Sector 59a, Noida 201301, Uttar Pradesh, India.

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ABSTRACT - Number of drugs with different mechanisms of actions is undergoing clinical trials for non-alcoholic steatohepatitis (NASH). Given the complexity of the disease with respect to pathophysiology in the liver and associated changes in the renal function, it becomes apparent that a clear ADME (absorption, distribution, metabolism and excretion) strategy needs to be put in place for a successful nomination of a drug candidate for NASH. This review discusses using *in vitro* and *in vivo* ADME screens to understand the properties of drugs and to establish whether or not the chosen drug(s) can overcome the challenges related hepatic and renal transporters covering both uptake and efflux mechanisms imposed by NASH. A complete panel of *in vivo* preclinical experiments including a ^{14}C-labeled study are proposed in NASH animal models to delineate the problematic areas for early drug development. Furthermore, a framework is provided with respect to the clinical pharmacology studies early in clinical development to characterise in an unbiased manner, the altered pharmacokinetics of drug in NASH patients for optimizing the dose selection for late phase clinical development. Because NASH patients have other co-morbid conditions and are prescribed co-medications for treating blood pressure, type 2 diabetes mellitus, obesity, dyslipidemia and many more disorders, it is also suggested to examine the drug-drug interaction potential by performing a cocktail probe study to cover a broad range of cytochrome P450 (CYP) enzymes and transporters.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an escalating health problem with a projected global predominance of 25% (1). NAFLD is associated with various metabolic disorders namely obesity, insulin resistance or type 2 diabetes, dyslipidemia and hypertension (2-3). The pathological abnormalities of NAFLD span from simple steatosis to advanced fibrosis or cirrhosis. Approximately, 7%–30% of NAFLD patients are identified with non-alcoholic steatohepatitis (NASH) symptoms. The predisposition to develop hepatic steatosis differs among the various races. The Hispanic population (45%) present the highest incidence, followed by Americans of European descent (33%) and African-Americans (24%), although the causes for these race/ethnic differences in incidence of hepatic steatosis are poorly elucidated (4).

NASH is a subset of NAFLD characterized by fatty liver followed with hepatocellular damage, inflammation and fibrosis (5-6). NASH is most commonly seen in obese individuals with adipose tissue dysfunction and inflammation, where the lipid storage ability of adipocytes is compromised. Adipose tissue dysfunction results in exposure of liver to the free fatty acids generated from adipocyte lipolysis as well as trigger the release of cytokines (Tumour Necrosis Factor-TNF) from the adipose tissues, which in conjunction promote hepatic steatosis (7). The TNF released during the process impedes adipocyte insulin signalling, resulting in insulin resistance that subsequently increases the flux of free fatty acid from the adipose tissue to liver. Furthermore, the upregulation of hepatic fatty acid transporters CD36 and FATP5 results in increased hepatic uptake of free fatty acid from the circulation. Also in NASH, the insulin-mediated hepatic lipogenesis is observed which is stimulated by lipogenic transcription factor SREBP1 (7).

Corresponding Author: Nuggehally R. Srinivas, Jubilant Life Sciences, Innovation and Technology, D12 Sector 59, Noida, Uttar Pradesh, India. Email: srinivas.nuggehally@jubl.com

Furthermore, high stress on endoplasmic reticulum (ER) functioning also contributes to the progression of NASH which can be categorised from a pathophysiology perspective as ER stress-mediated stimulation of hepatic lipogenesis and ER stress-mediated inhibition of hepatic lipid export. All these above factors trigger in totality and contribute to the development and progression of NASH (7).

The pathogenesis of NASH is very complex involving multiple pathways which requires targeting of several cellular and molecular events to control the disease symptoms. Furthermore, based on the disease stage, either single or multiple therapeutic modalities may be opted to obtain desired therapeutic benefits. Unfortunately, there are no US FDA-approved NASH therapeutics. Currently, the anti-oxidant, vitamin E and pioglitazone are considered as the first line therapy for NASH (8-10). Various clinical studies have been conducted with different mechanisms and as well as part of repositioning strategy for the existing therapeutics to obtain symptomatic relief by targeting various axis of the disease pathology as shown in Table 1.

Scope

We conducted a review of published preclinical and clinical studies underlining the impact of NASH/NAFLD on various physiological processes that modulate the disposition of therapeutics in NASH/NAFLD. Since it is a developing field in terms of understanding the impact of pharmacokinetics of drugs in NASH/NAFLD patients, we attempted to put together the current state of knowledge in the field by gathering published data (*in vitro versus in vivo*; preclinical *versus* clinical) Additionally, we have not segregated the data with respect to preclinical *versus* clinical and/or *in vitro versus in vivo* because of lack of availability of large data sets to apportion accordingly to each of the sub-headings.

The literature review was done using Pubmed® search (NCBI 2016), SCIFINDER® and Google Scholar databases with specific key words such as NASH, NAFLD, preclinical, clinical, pharmacokinetics, absorption, distribution, metabolism, excretion, bioavailability, disposition, transporter, biliary, renal, drug-drug interaction, and human to collect the related full-length articles and abstracts. The literature search covers the period until November 2018.

Case studies highlighting impact of NASH on ADME properties

Altered activity of the hepatic drug metabolising enzymes in NASH

Several case studies are presented that represent heterogeneity in the species and *in vitro versus in vivo* comparisons to provide some conjecture(s) on the possible impact of NASH on hepatic drug metabolising enzymes.

Table 1. Investigational therapies for management of NASH

Therapeutic targets	Mode of action	Drugs
PPAR agonists	• Increasing the insulin sensitivity • Increasing fatty acid oxidation	Fibrates, Thiazolidinediones, Elafibranor
Farnesoid X receptor (FXR) agonist	• Inverse regulation of bile acid synthesis • Decreasing hepatic gluconeogenesis, lipogenesis and steatosis	Obeticholic acid
Stearoyl-CoA desaturase-1 inhibitor	• Inhibiting the synthesis of monounsaturated fatty acids	Aramchol
GLP-1 receptor agonists	• Decreasing insulin resistance by enhancing insulin biosynthesis • Increasing peripheral insulin sensitivity	Exenatide, Liraglutide
Pan-caspase inhibitor	• Inhibiting TNFα mediated hepatocyte cell injury and caspase-regulated apoptosis	Emricasan
Phosphodiesterase inhibitor	• Reducing the release of inflammatory cytokines	Pentoxyfylline
Anti-fibrotic agent	• Targeting lysyl oxidase-like • 2 (LOXL2), responsible for collagen formation and resulting in fibrosis	Simtuzumab
Miscellaneous agents	Orlistat (lipase inhibitor), Solithromycin (macrolide antibiotic), Bicyclol (anti-oxidant), Vitamin D (anti-inflammatory and anti-fibrotic)	

Both expression and activity of various hepatic drug metabolising enzymes are likely to be altered due to the pathophysiological condition observed in severe hepatic damage. *Ex vivo* studies conducted in human fatty and non-fatty NASH liver tissues showed decrease in the protein expression and functional activity of CYP3A4 (11). An *ex vivo* study by Woolsey et al. (2015) demonstrated a 69% decrease in the CYP3A4 mRNA levels in NASH (12). This was confirmed from an *in vivo* study in NASH subjects where a 2.5-fold decrease in CYP3A4 activity was observed (12). *In vitro* studies using Huh7 hepatoma cells showed 80 and 38% decrease in CYP3A4 mRNA levels and CYP3A4 activity, respectively (12). A study in methionine and choline deficiency (MCD) rat model showed down-regulation of rat Cyp2B1 (rat ortholog of human CYP2B6) activity, mRNA and protein expressions (13). Since CYP2B6 is less copious, assessing the influence of heterogeneous NAFLD on its expression and activity poses a challenge.

The murine ortholog of CYP2A6, Cyp2A5, was found to be elevated in the presence of steatosis (14-15) which was similar to the observations made in human hepatic tissues (16). A reduced activity and mRNA expression of CYP2A6, CYP2B6, CYP2C9 and CYP2D6 have been reported in human hepatocytes isolated from fatty liver grafts (17). The level of Cyp2D6 decreased in NASH as confirmed from preclinical *in vivo* (18) and *ex vivo* studies using human liver tissues (11). Li et al. (2011) demonstrated a 2.5- and 1.5-fold reduction in the expression of Cyp3A2 and 2D2, respectively, in high-fat emulsion NASH models (19). This altered enzyme expression affected the metabolism of selected cationic drugs as Cyp3A2 substrates metoprolol and antipyrine and Cyp2D2 substrates propranolol, metoprolol and atenolol (19). A 2-fold reduction in hepatic extraction ratio using perfused rat livers for antipyrine and atenolol was observed in NASH rodents as compared to control animals. The hepatic extraction ratios for metoprolol in NASH and control animals were 0.72 and 0.84, respectively (19). No significant difference in the hepatic extraction ratio for propranolol was observed in between NASH and control animals. The mean transit time for propranolol, metoprolol, antipyrine and atenolol increased by 22, 12, 38 and 42%, respectively in NASH rodents perfused liver as compared to control animals (19).

The expression of Cyp2B1 reduced significantly (5-fold) in liver microsomes of MCD rats (NASH model) as compared to control animals which altered the enzyme-kinetic and pharmacokinetic parameters for Cyp2B1-mediated bupropion metabolism (20). *In vitro* metabolic conversion of bupropion to hydroxybupropion in hepatic microsomes showed a 3.5-fold reduction in the intrinsic clearance in NASH hepatic microsomes as compared to control animal hepatic microsomes (20). A significant reduction in the V_{max} (NASH: 48.0 and control: 60.7 pmol/min/mg protein) and increase in the K_m (NASH: 294 and control: 102 μM) was observed in NASH hepatic microsomes as compared to control animal hepatic microsomes (20). Pharmacokinetic studies showed reduced Cyp2B1-mediated metabolic conversion of bupropion to hydroxybupropion which could be inferred from 2-fold higher AUC levels of bupropion and 1.7-fold lower AUC levels of hydroxybupropion as compared to control animals. Also, the T_{max} of hydroxybupropion increased from 30 min in control animals to 60 min in NASH rats, thus suggesting altered enzyme kinetics mediated delayed absorption (20). The expression and activity of CYP2E1 was found to increase in NASH in both humans and rodents (21-23). The down-regulation of CYP1A2 in NAFLD appeared to be one of the most consistent despite some incongruities (24).

Microsomal protein expression of CYP2C19 also decreased with the successive progression of the liver disease from NAFLD to NASH (11). Na et al. demonstrated from *in vitro* and *in vivo* Cyp cocktail assays, Cyp1A, Cyp2B, Cyp2C and Cyp3A were significantly decreased, whereas Cyp2D remained unchanged in MCD diet mice as compared to normal mice (25). Jamwal et al. (2018) studied the association of NAFLD, NASH and diabetes mellitus on CYP3A4 activity in human liver tissue from brain dead donors (26). The results showed a 1.9 and 3.1-fold decrease in CYP3A4 activity in NAFLD and NASH, respectively. Also, the intrinsic clearance was 2.7 and 4.1-fold lower in NAFLD and NASH liver donors, respectively with an overall decrease in CYP3A4 expression by 2-fold in both NAFLD and NASH (26). The CYP3A4 activity in livers from diabetic donors with NAFLD and NASH was 4.5 and 5.7-fold lower as compared to the normal diabetic liver. The intrinsic clearance in diabetic livers with NAFLD and NASH was 1.4-fold lower as compared to diabetic liver without fatty disease (26). The CYP3A4 expression in diabetes+NAFLD and diabetes+NASH was 4.2 and 2.9-fold lower as compared to normal diabetic liver (26).

With respect to the phase II metabolizing enzymes, Zhang et al. (2013) showed that valproic acid upregulated the mRNA levels of uridine 5'-

diphospho-glucuronosyltransferase (UGT)1A1, UGT1A1, 1A6, 1A7, and UGT2B1 in rat liver microsomes (27). The protein expression and enzymatic activity of UGT1A6 were significantly increased in rats treated with valproic acid alone (27). Upregulation of UGT1A1 and UGT1A6 increased the urine recovery of valproic acid glucuronide by 42% following 8-weeks dosing of 500 mg/kg/day of valproic acid in rats (27). The observed microvesicular fatty liver and hepatic dysfunction was modulated by valproic acid mediated modulation of mitochondrial β-oxidation of fatty acids (27). However, the sulfotransferase1A2 (SULT1A2) was found to be downregulated in NASH (28). Furthermore, Yalcin et al. (2013) also observed that SULT activity decreased significantly with the progression of liver disease from steatosis to cirrhosis (29). With respect to glutathione S-transferase (GST), a decrease in the enzyme activity has been observed in ob/ob mice (30-31) and human liver samples (32). Kyriakides et al. (2014) showed that methotrexate aggravated the NASH pathological condition in NASH rodents (33). Methotrexate showed dose-dependent metabolic consequences affecting gut microbial, energy, nucleobase, nucleoside, and folate metabolism (33). Furthermore, it affected the metabolic phenotyping by elevating the hepatic phenylalanine, urocanate, acetate, and both urinary and hepatic form iminoglutamic acid (33). This systems level metabonomic analysis of the hepatotoxicity of methotrexate in NASH perspective unravelled a novel mechanistic view of potential drug-drug interaction (33).

Impact of NASH in modulating the efflux and uptake transporters

NASH pathological conditions have significant impact on the expression and activity of uptake and efflux transporters which can be involved in the modulation of the drug disposition. This has been proved from multiple nonclinical and clinical studies (34). A clinical study in 32 and 41 patients with early and advanced NASH complications, respectively, showed a significant elevation of jejunal mRNA and protein expression of microsomal triglyceride transfer protein as compared to the control subjects which subsequently increased the intestinal absorption of palmitic acid (35). Furthermore, the NASH patients exhibited higher serum levels of ApoB-48 levels relative to normal subjects, thus suggesting increased palmitic acid transport *via* chylomicrons in these patients (35). Ali et al. (2017) demonstrated that the expression and activity of hepatic transporters namely OATPs and MRPs were significantly altered in NASH subjects (36). ^{99m}Tc-mebrofenin (MEB) was used as a probe to evaluate OATP1B1/1B3 and MRP2 function in NASH patients (36). NASH patients showed 1.4- and 1.6-fold higher systemic and hepatic exposure of MEB as compared to healthy subjects whereas the biliary clearance and volume of central compartment decreased by 2-fold, thus suggesting that the MEB hepatic uptake was reduced in NASH subjects which could be because of either reduced expression and activity of the uptake transporters namely OATP1B1 and OATP1B3; also, altered MRP2 expression that governs the efflux mechanisms modulating the biliary transport (36). The impaired function of the OATP1B1 and OATP1B3 as well as MRP2 was attributed to the impaired N-linked glycosylation of these drug transporters (37). N-linked glycosylation of proteins is crucial for appropriate protein folding and trafficking to the plasma membrane. A mechanistic study by Clarke et al. (2017) showed that genes involved in protein processing in the ER and biosynthesis of N-glycans were significantly downregulated with the progression of NAFLD causing retardation of the functioning ability of these transporters in NASH (37).

The NASH pathology apart from affecting the liver transporters is also likely to affect the kidney transporters primarily OCT1, OCT2, and MATE1 (38). In a study carried out in three mice cohorts: WT/MCD (NASH only), ob/control (diabetes only) and ob/MCD (NASH+diabetes); significant reduction in the urinary excretion of metformin was observed in the ob/MCD group as compared to WT/control mice (38). The urinary excretion in WT/control, WT/MCD, ob/control and ob/MCD animals was 82, 60, 70 and 28%, respectively. Furthermore, the plasma concentration of metformin (AUC levels) was 4.8-fold higher in ob/MCD mice compared with WT/control, thus indicating potential loss of function or reduced expression of the renal transporters in NASH (38). Previous work has confirmed that renal excretion of metformin is *via* OCT1, OCT2, and MATE1 (39-41).

Because the pathophysiology of NASH in adult patients cannot be directly translated to the paediatric NASH patients, there is dilemma in the therapeutic paediatric dose. Canet et al. (2012) conducted a pharmacokinetic study to understand the disposition of acetaminophen (APAP) and its glucuronide and sulfate metabolites in NASH paediatric patients (42). Patients with NASH showed increased serum and urinary levels of APAP-glucuronide along with decreased serum

levels of APAP-sulfate (42). The probable reason for the elevated APAP-glucuronide in urine samples was attributed to the induction of hepatic MRP3. The altered canalicular localization of the biliary efflux transporter, MRP2 contributed to the increased plasma APAP-glucuronide levels (42). However, the serum APAP-sulfate levels did not increase following administration of APAP in NASH paediatric patients which might be due to decreased expression of the sulfate uptake transporter, SLC26A1, as well as decreased expression of cysteine dioxygenase type 1 (CDO1) and sulfite oxidase (SUOX) in the liver, indicating that there is a reduced probability for sulfate activation from intracellular sources (42). These findings were in agreement to the preclinical results as demonstrated by Lickteig et al. (2007) in NASH rodent model (43).

Methotrexate, a substrate for Mrp2, Mrp3, Oat1 and Oat3, manifested increased toxicity in NASH rodents primarily affecting the liver and kidney but lesser damage was caused to the intestine (44). The probable reason might be attributed to the reduced functionality of Mrp2 due to impaired glycosylation in NASH, which subsequently reduced the biliary excretion (44). However, due to the compensatory mechanism, Mrp3 induction was observed in NASH resulting in the elevated systemic exposure of methotrexate (44). The increased renal uptake of methotrexate that resulted in renal toxicity was attributed to Oat1 and Oat3 transporters. The lower intestinal damage might be due to reduced biliary clearance into the small intestine because of impaired Mrp2 function (44).

Another study was conducted by Clarke et al. (2014) to understand the disposition of simvastatin and its bioactive metabolite simvastatin acid in rodent NASH model (45). NASH animals showed increased plasma retention and decreased biliary excretion of simvastatin acid which was attributed to the decreased protein expression of multiple hepatic OATPs (OatP1B2, 1A1 and 1A4 expression decreased 1.3-, 1.5- and 6-fold, respectively as compared to control animals) (45). Simvastatin being a lipophilic compound is less likely to be transported by active transport mechanism, however, transporters play a key role in the disposition of simvastatin acid of being more hydrophilic. The lipophilic nature of simvastatin contributed to its larger volume of distribution and subsequent partitioning to the muscles of NASH animals, thus decreasing the plasma concentrations without affecting the amount of simvastatin excreted into the bile (45). From the metabolism perspective, both enzymatic (CYP2C11 and CYP3A1/23) and non-enzymatic (esterases and paraoxonases) reactions are important for simvastatin (46-48). Although, the protein expression for Cyp2C11 and Cyp3A1/23 decreased by 5- and 6-fold in NASH rodents as compared to control animals, no significant difference in the sums of the AUCs for simvastatin and simvastatin acid in plasma or bile was observed, thus suggesting metabolism may not be the key determinant for the altered disposition of simvastatin and simvastatin acid in NASH rodents. Hence, the altered disposition can be considered as a transporter-mediated process affecting primarily the disposition of simvastatin acid and to some extent for simvastatin (45).

Fisher et al. (2009b) demonstrated that the expression of Oatp1A1 and Oatp1B2 was significantly lower in NASH rodents as compared to control animals (16). Furthermore, the liver samples of NASH rodents showed increased levels of interleukin-1β (IL-1β), a pro-inflammatory cytokine known to reduce expression of Ntcp, Oatp and Oat transporters, suggesting a probable mechanism for the observed transporter alterations in NASH (16).

Renal elimination and biliary excretion in NASH

NASH pathological condition also altered the renal elimination process by modulating the renal transporters. A study by Laho et al. (2016) showed the effect of NASH on the renal filtration and secretion of adefovir in NASH rodents (49). Protein expressions studies showed 40 and 25% increase in Mrp4 and Oat3, respectively, with no influence on Oat1. Over expression of Oat3 is likely to result in the enhanced renal uptake of adefovir (49). Following, intravenous dosing of adeforvir (7 mg/kg; 35 lCi/kg) in both control and NASH rodents, no significant difference in the plasma concentration of adefovir was observed (49). Although, the total clearance of adefovir remained unaffected in NASH as compared to control animals, the impact of renal excretory pathways to its elimination was significantly altered. A 50% reduction in the glomerular filtration rate was observed in the NASH rodents as compared to the control animals; however, this was counterbalanced by the elevated net tubular secretion, mediated *via* induction of renal Mrp4, the major kidney efflux transporter for adefovir (49). As a result, total clearance of adefovir was not altered, but its concentration profiles in urine were dissimilar. In contrast, a decrease in the expression of hepatic Mrp4 also resulted in the lower liver levels of adefovir (49).

NASH has a significant impact on the biliary excretion of drugs. The down regulation of canalicular Mrp2 in NASH is likely to affect the biliary excretion of drugs which was confirmed from a study by Dzierlenga et al. (2016) (50). The findings of this study showed no cumulative biliary elimination of pemetrexed, an antifolate chemotherapeutic in Mrp2-/- rats, suggesting that disposition of pemetrexed is completely dependent on Mrp2-mediated biliary clearance (50). Furthermore, the biliary excretion of pemetrexed was reduced by 60% in NASH rats as compared to normal animals. This study unravelled Mrp2 as the exclusive biliary elimination mechanism for pemetrexed making it a suitable *in vivo* probe substrate for Mrp2 function, and thereby, effectively accounting for the loss of function in NASH (50). Toth et al. attempted to elucidate the altered functionality of the Bcrp and Mrp2 in preclinical model of NASH using SN-38 (active metabolite of irinotecan and Bcrp substrate) and SN-38 glucuronide (Mrp2 substrate) as probe substrates (51). The biliary efflux of SN-38 decreased to 31.9%, and efflux of SN-38G decreased to 38.7% of control, but no change was observed in WT-MCD and knockout control animals suggesting that Bcrp is not solely responsible for SN-38 biliary efflux, but rather implicate a combined role for Bcrp and Mrp2 (51). The impact of NASH on ADME properties based on preclinical and clinical reports are summarized in Table 2.

Factoring acquired knowledge in drug discovery and clinical pharmacology strategy

In vitro ADME screens

Many ADME related activities are typically set up in drug discovery programs to ensure various stage gates of candidate nomination with objectivity and rigour. We believe that certain additional screens need to be added that specifically covers the properties that may benefit in NASH. Table 3 provides a comprehensive list of all the activities that may enable selection of the right candidate for NASH treatment.

In vivo preclinical ADME experiments

Selection of appropriate animal model for predicting drug disposition in NASH

The complex pathophysiology of NASH is characterised by hepatocellular damage resulting in dysregulation of hepatic biotransformation and transport mechanisms. This is likely to alter the ADME profile of the drug candidates leading to altered pharmacokinetics. Hence, it is critical that the early candidate optimization has to be carried out in animal models that closely resemble the human NASH pathophysiology both from pharmacodynamics and pharmacokinetics perspective. Canet et al. (2014) demonstrated that MCD and atherogenic rats, as well as ob/ob and db/db mice, developed NASH (52). The mRNA and protein expression studies showed that the efflux transporters (Mrp1, Mrp2, Mrp3, Mrp4, Mdr1A, Mdr1B, Bcrp) were induced and uptake transporters (Oatp1A1, Oatp1A4, Oatp1B2, Oatp2B1) were intimidated in the rat MCD and the mouse ob/ob and db/db models (52). Furthermore, the transporter mRNA and protein expression pattern in mouse and rat MCD models as well as mouse ob/ob and db/db NASH models were similar to that of humans, thus indicating that these animal models may be a good fit to conduct ADME studies for compounds intended to be developed as NASH therapeutics (52).

Characterization of pharmacokinetics and DDI potential

Table 3 provides certain preclinical pharmacokinetic studies that may aid in the elucidation of key problematic area(s) for the early clinical development of the drug. We believe that pharmacokinetics derived in NASH animals may aid in gauging the drug-drug interaction potential at hepatic transporter and/or renal transporter areas. The bile duct cannulated (BDC) study in NASH rats *versus* control rats need to be planned to understand the degree of alteration of the pharmacokinetics of the drug/metabolite(s). It is also recommended that a radiolabelled mass balance study and tissue distribution study (preferably whole-body autoradiography) conducted in NASH animals *versus* healthy controls. This study, in addition to understanding the mass balance of the radioactive drug, would also provide critical data in the distribution of radioactivity in key organs/tissues (liver, kidney, intestine etc.) and impaired Cyp metabolism, and/or altered glucuronide biliary clearance, if any. This may aid in correlation of the exposure(s) of the drug and/or metabolite to the transporters (uptake/efflux) expressed in the organs/tissues of concern as compared to healthy controls. Furthermore, it may be possible to de-risk the propensity of drug-drug interaction and/or drug-transporter interaction by doing a cocktail probe study in NASH animals (53-54).

Table 3. A suggestive comparative ADME screening tools for conventional and NASH therapeutics development

Screening stages	Conventional ADME screening tools	NASH ADME screening tools*
1st ADME screen	• Metabolic stability in liver microsomes (mouse, rat, dog, monkey, human) • Plasma stability • Cyp liability (regular panel) • Covalent binding • Permeability (Caco-2, PAMPA, MDCK)# • Efflux (P-gp, Bcrp) • Plasma protein binding	• Metabolic stability in NASH liver microsomes (mouse, rat, human) • NASH serum/ plasma stability • Specific Cyp liability (upregulated in NASH) • Intestinal inverted sac in NASH animal model • Plasma protein binding in NASH serum/ plasma
2nd ADME screen	• Metabolic stability in hepatocytes • Metabolism – finger print – liver microsomes/ hepatocytes/ liver slices • Cyp inhibition (IC_{50})$ • Cyp induction • Polymorphic metabolism	• Metabolic stability in NASH hepatocytes/ liver slices • Metabolism – finger print – NASH liver microsomes/ hepatocytes/ liver slices • Cyp inhibition (IC_{50})** in NASH liver microsomes • Polymorphic metabolism in NASH liver microsomes
3rd ADME screen	• Cyp phenotyping • GSH adduct • Transporters • *In vivo* PK/brain penetration • Excretion	• NASH specific Cyp phenotyping • GST, UGT and SULT metabolites monitoring • Hepatic transporters assay primarily uptake transporters (Oatp1A1, Oatp1A4, Oatp1B1, Oatp1B3, Oat2B1, Ntcp, Oct1) and efflux transporters (Mrp2, Mrp3, Mrp4, Mrp6, Mdr1a, Mdr1b, Bcrp, Bsep, Mate1) • Renal transporters assay primarily uptake transporters (Oat1, Oat3, Oct1, Oct2) and efflux transporters (Mate1/2, Mrp4, Mrp2) • *In vivo* PK/brain penetration in NASH rodents • Excretion in NASH rodents
4th ADME screen	• RBC uptake • Time dependent inhibition • PK in two species • Metabolite ID • Limited TD in rodents	• RBC uptake in blood obtained from NASH rodents • PK in NASH mouse and rat models • Metabolite ID in NASH rodents

Cut-off point to enable decision
$ Only relevant ones based on 1st screen
* Experiments in addition to regular/ conventional studies
** Relevant to NASH

Clinical pharmacology strategy

After gathering of the standard clinical pharmacology data during the first-in-human single dose and rising dose tolerance studies, it may be critical to examine the human pharmacokinetics and plan an exploratory single dose safety/pharmacokinetic study in NASH patients possibly at two doses, deemed to be therapeutic. Such early clinical pharmacology data would provide valuable information on any altered pharmacokinetics of the drug that need to be considered during the clinical development in NASH patients. We also strongly recommend conducting ^{14}C-labeled study of the drug in NASH patients rather than healthy subjects as a 3rd or 4th study during phase 1 development. Radiolabeled study, amongst other things, would provide a succinct picture of the mass balance of the administered radioactivity (renal *versus* faecal route of elimination) in addition to the characterisation of the pharmacokinetics of parent drug/metabolite(s) along with radioactivity

profiles. Using the data in NASH patients, it is possible to determine the doses for phase 2 study. Furthermore, we believe that any clinical drug-drug interaction study, if deemed necessary (especially in a polypharmacy situation in phase 2 because of the patient pool) may be performed prior to the initiation of phase 2 study in NASH patients rather than healthy subjects simply because the altered disposition due to transporters and/or CYP enzymes cannot be assessed using healthy subject data. In this context, we also suggest evaluation whether or not the drug is a perpetrator in NASH patients, using the cocktail approach for CYP enzymes/transporters. Recent literature has provided the impetus for planning and execution of such cocktail probe studies in NASH patients (55-57).

DISCUSSION

Given the high interest level in therapeutic approvals for NAFLD/NASH areas, there is unprecedented frenzy occurring in the conduct of clinical trials especially in the USA. Since most of the late phase clinical trials are examining the applicability of different mechanisms including re-purposing options for certain drugs in the management of NASH, it is still largely unknown as to what mechanism(s) has a major role to play as the etiology and pathogenesis of NASH is complex. If one critically examines the drugs in late phase development in NASH, it may be readily apparent that the initial screening and candidate nomination of such drugs may not have focussed on the utility of discovery/early development ADME screens which would be more appropriate for NASH. However, it is our understanding that standard industry ADME (*in vitro* and *in vivo*) screens would have been applied to ensure that the proposed drugs met the criteria for drug developability without any major red flags.

The key question to introspect: would lack of applying specific ADME screens for NASH in adjudicating a drug for clinical development lead to either low/moderate response or therapeutic failure during clinical trials in relevant NASH patients? This question has been asked after rosuvastatin showed poor response in NASH (55). It was reasoned that due to altered transporter related disposition in NASH, the uptake of rosuvastatin into the liver may have been reduced with the concomitant faster basolateral efflux facilitating excretory mechanisms including biliary clearance as qualitatively suggested in the schematic represented in Figure 1 (59-61). In addition, faster urinary elimination of the drug may have resulted in inadequate liver drug levels to achieve therapeutic benefits in NASH (60-61). The credence to support decreased liver uptake and increased basolateral efflux in NASH comes from recently published data of simvastatin and morphine (45, 62). In experimental NASH animals, it was found that there was an increase in systemic simvastatin hydroxyl acid exposure correlating directly with the reduced hepatic uptake of the drug (45). In human NASH patients, Ferslew et al. (2015) showed there was an enhanced systemic circulation of morphine-3-glucuronide relative to healthy subjects due to higher basolateral efflux and/or reduced MRP2 efflux associated with NASH (62). Likewise reduced hepatic uptake and impaired biliary excretion should be expected for mycophenolic acid glucuronide, whose transport is governed by MRP2 efflux (63). Another notable example is that of raloxifene-6-glucuronide whose disposition was potentially altered in reduced Mrp2 efflux environment (64).

The recent report has substantiated importance of metabolism (CYP related) screens from a NASH perspective (24, 34). Fisher et al. (2009a, 2009b) evaluated mRNA levels, protein expression and enzymatic activity of a variety of CYP enzymes using liver microsomes obtained from NASH patients representing the spectrum of the disease progression *versus* healthy human liver microsomes (11, 16). While the functional activity of CYP1A2, CYP2D6, CYP3A4 and CYP2C19 were decreased, the activity of CYP1A6, CYP2C9 was increased. The CYP2C8 activity appeared to be similar across NASH patient spectrum *versus* healthy human microsomes; whereas, no data was reported for the enzymatic activity of CYP2B6 in NASH patients. This work showed the importance of inclusion of the CYP phenotyping index as part of the ADME using both NASH patient and NASH animal liver microsomes to judge the liability of the drug(s) in question (11, 16).

The critical interplay of various hepatic transporters – uptake and efflux need to be considered in the discovery and development of drugs in this class. While the expression of key liver uptake transporters are reduced in NASH patients (36); the disease can synergistically combine with genetic polymorphism observed in uptake transporters to further worsen the hepatic uptake of drugs leading to increased systemic exposure as evident in the disposition of pravastatin in NASH animals (65). The role of several efflux transporters such as MRP2, BCRP, MDR, MATE1 needs to be factored in totality for alteration of the drug disposition in NASH

patients. As indicated for mebrofenin, the decrease in hepatic exposure was directly correlated to the decreased biliary excretion due to reduced efflux capacity of Mrp2 (36). Therefore, several drugs such as methotrexate, enalapril, fexofenadine, valsartan, olmesartan etc., that depend on efflux role of MRP2 for efficient biliary clearance should be expected to show altered pharmacokinetics in NASH patients (66). A recent example of decreased biliary efflux of both SN-38, active metabolite of irinotecan, and SN-38 glucuronide suggested the need of dose consideration in cancer patients when they are on irinotecan based chemotherapies (51).

Likewise, the roles of several key renal transporters have to be considered in the disposition of drugs in NASH. Because of diminishing glomerular filtration rate accompanied by altered functioning of uptake (Oct1/2) or efflux transporters (Mate1/2, Mrp1/2 etc.), higher systemic exposure of drugs that are primarily renally eliminated should be expected as is the case with metformin (38-39).

Utmost caution in the extrapolatability of pharmacokinetic data in NASH animals to NASH patients need to be exercised. However, similarity in dispositional characteristics of drugs in NASH provides an opportunity to evaluate the likely altered pharmacokinetics in animal model and possibly to an extent de-risk the clinical development program with appropriate strategies. For example, in NASH mice, the biliary clearance of acetaminophen glucuronide and acetaminophen were reduced leading to systemic accumulation of the drugs (43). A cohort of NAFLD paediatric patients showed higher acetaminophen glucuronide showing similar impairment of hepatic basolateral efflux and other transporters (67). Another example was that of ezetimibe, a useful lipid lowering drug (68).

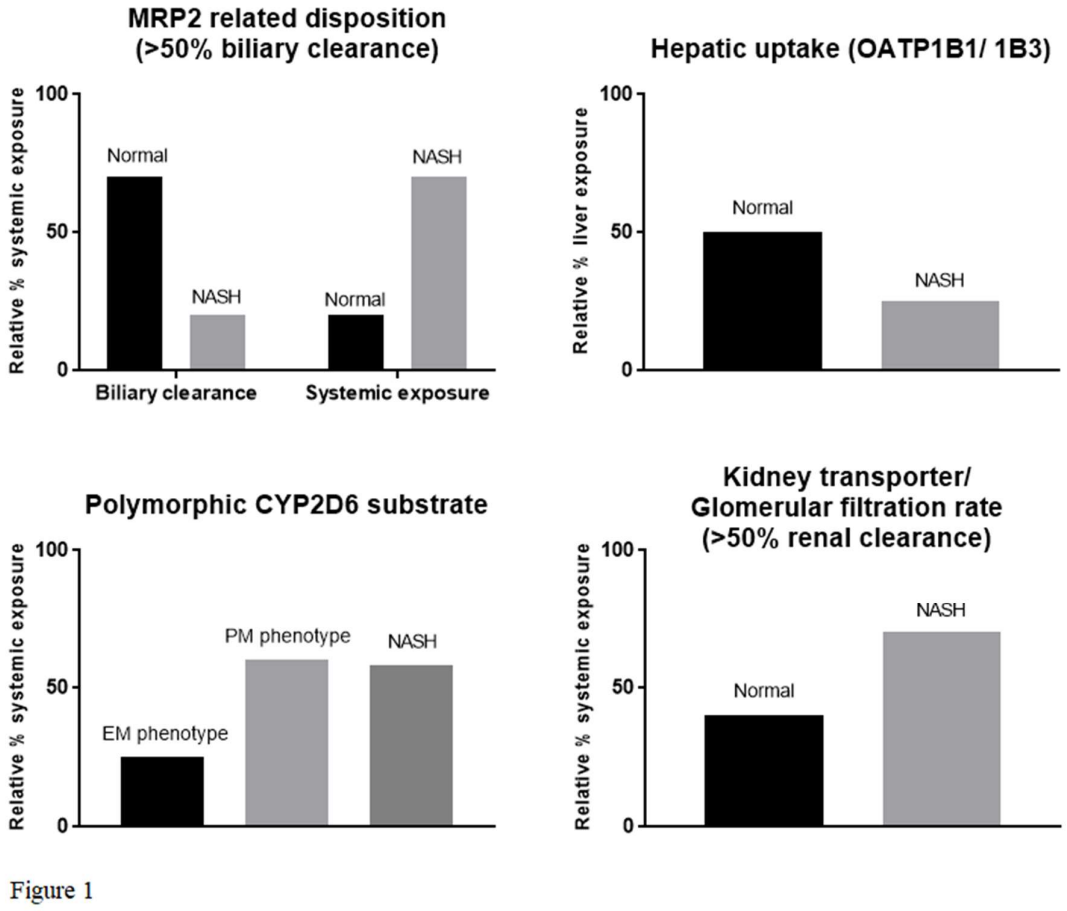

Figure 1

Figure 1. Hypothetical qualitative representation of NASH related physiological factors that likely modulate the disposition of therapeutics in NASH subjects (relative to normal subjects). While the scale is not quantitative in nature, the hypothetical data are represented in terms of relative % systemic and/or liver exposure to visualise the likely impact NASH may have on the appropriate pharmacokinetic parameter. Translatability of the data may vary with specific protein activity of the transporters and/or enzymes and the nature of the investigational drugs (victim drugs and/or perpetrator

drugs) in the diseased condition and therefore, it should not be generalized. EM: Extensive metabolizers, PM: Poor metabolizers.

In rodent NASH model systemic levels of the glucuronide of ezetimibe was increased due to decreased biliary efflux (68). Given the importance of biliary efflux in humans to ensure delivery of the ezetimibe glucuronide metabolite to small intestine which contributes for a continuous suppression of the absorption of dietary cholesterol, the blockade of this efflux pathway in NASH patients would reduce the efficacy of ezetimibe (68).

Because of the polypharmacy situation in the clinical therapy of NASH patients who are prescribed drugs to manage other co-morbid conditions, it becomes relevant to understand the pros and cons of the various co-medications with respect to disposition from both CYP enzymes and hepatic/renal transporters. Few examples are presented: a) Drugs that are similar to fasiglam (TAK-875) that inhibit MRP2/MRP3, OATP and NTCP (i.e., perpetrator drug) would need dose adjustment(s) during the clinical therapy of NASH patients (69); b) Drugs like bosentan which is metabolized by CYPs 3A4 and 2C9; and owing to its inhibition of uptake transporters and bile acid transporters, should be dosed with caution in NASH patients (70); c) The newly approved LCZ696 (a novel, crystalline complex comprising sacubitril and valsartan) may present as both a perpetrator/victim drug in NASH patients owing to the involvement of inhibition of uptake transporters and dependence on MRP2 for its excretion (64, 71).

As suggested in Figure 1, from a qualitative perspective various pharmacokinetic nuances come into play in the selection of right candidates for NASH. Figure 1 was not drawn from a quantitative view but is a mere reflection of various hypothetical conjectures that may likely occur in NASH and therefore, in totality may influence the disposition of the drug. Since the drug is targeted to liver, the uptake of the drug through OATP1B1 and 1B3 is critical. Because of the lower expression of hepatic uptake transporters in NASH, it should be expected that there will be reduced liver localization of the drug. Hence, the chosen drug should be potent such that despite reduction in hepatic uptake in NASH, it should not compromise its pharmacological activity. Also, during candidate selection stage, both MRP2 and renal contribution for the excretion of the drug/metabolite(s) need to be considered. One screening strategy with respect to excretion in the drug selection process is proposed with an arbitrarily chosen cut-off to illustrate the example. If the drug exhibits hypothetically >50% biliary clearance (in regular biliary cannulated rats) or >50% renal excretion in regular rats, it is not a viable option to purse with the drug candidate for NASH. In addition, although no reported pharmacokinetic data are available for drugs that are subjected to polymorphic CYP metabolism, it may be critical to keep this factor in the drug nomination process. Because of the reduced expression of two polymorphic CYP enzymes, namely CYP2D6 and CYP2C9, the likely impact on the pharmacokinetics of drugs that are substrates to polymorphic enzymes need to be considered in NASH. As NASH can increase the risk of chronic kidney disease, the propensity of drug-drug interaction and dosage adjustment needs to be factored in the therapeutic management (72). Recent data suggesting the likelihood of lower expression of Cyp3A/CYP3A4 in the livers of NASH preclinical disease models and human NASH/NAFLD patients may be pertinent from therapy perspective of other concomitant drugs that are metabolized by CYP3A4 in humans (51). Finally, since NAFLD has been considered to present diagnosis of total metabolic syndrome from the liver manifestation; the co-existence of obesity, lipid disorders, blood pressure, and diabetes mellitus may further complicate the drug disposition of the chosen drug and present opportunities for victim and/or perpetrator kind of drug interactions (73).

In summary, the potential of the influence of either NAFLD or NASH on pharmacokinetics is not fully understood, given the disease complexity in terms of its progression. However, we have attempted to present a well thought-out and balanced *in vitro* and *in vivo* ADME screening strategy along with clinical pharmacology considerations with exclusive focus on NASH therapeutics. We believe the adoption of such experimental framework may enable in the decision process and reduce attrition rates of drugs due to lack of right pharmacokinetic properties in late stage clinical development for NASH.

CONCLUSIONS

There is an unprecedented frenzy in the number of clinical trials for NASH indication in the USA. Despite the best research efforts, there is still void in small molecule therapeutics for treating NASH. In this context, several drugs with varied mechanisms are undergoing clinical development for NASH. Since NASH needs effective liver targeting, it is utmost important to equip the drug

candidates with right ADME attributes for treating this complex disease. A number of factors need to be considered in setting up effective *in vitro* ADME screens to gauge the properties of drugs and whether or not it can overcome the challenges imposed by NASH. The screens of particular importance should encompass hepatic and renal transporters covering both uptake and efflux mechanisms. Additional *in vitro* ADME screens should consider the propensity of drugs to be metabolized by various CYP enzymes including polymorphic enzymes CYP2C9 and CYP2D6. Several *in vivo* ADME experiments including biliary/renal excretion studies may be undertaken using NASH animals to obtain data for risk assessment. A ^{14}C-radiolabeled whole body autoradiography study is also proposed in NASH animals *versus* control animals.

In terms of clinical pharmacology strategy, it is proposed to conduct single dose safety/ pharmacokinetic study in NASH patients possibly at two doses, to gauge altered pharmacokinetics and aid dose selection in NASH patients. It is also proposed to consider the possibility of ^{14}C-labeled study of the drug in NASH patients rather than healthy subjects to obtain clarity on the mass balance in addition to the characterisation of the pharmacokinetics of parent drug/metabolite(s) and metabolic profiling. Using the data in NASH patients, it is possible to determine the doses for phase 2 study. Finally, the evaluation of whether or not the drug is a perpetrator in NASH patients may be undertaken using the cocktail approach for CYP enzymes/transporters.

CONFLICT OF INTEREST

The authors declare no conflicts of interest that might be relevant to the contents of this manuscript.

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Table 2: Impact of NASH on ADME properties*

Absorption	• Upregulation of jejunal microsomal triglyceride transfer protein ⟶ Increased fatty acid absorption • Upregulation of P-gp and BCRP ⟶ Reduced absorption of P-gp and BCRP substrates • Upregulation of intestinal, liver and kidney MRP3 ⟶ Increased basolateral efflux and higher plasma levels
Distribution	• Downregulation of OATP1B1 and OATP1B3 ⟶ Decreased hepatic uptake, increased free plasma levels and tissue distribution • Impaired N-glycosylation of proteins ⟶ Altered plasma protein levels and plasma protein binding
Metabolism	• Down regulation of CYP enzymes (CYP3A4, 1A2, 2B6, 2C9, 2C19, 2D6) ⟶ Impaired CYP mediated metabolism and DDI potential • Upregulation of CYP enzymes (CYP2A5, 2E1) ⟶ Enhanced metabolism of CYP2A5, 2E1 substrates and DDI potential • Impaired N-glycosylation ⟶ Decreased functionality of CYP and non-CYP enzymes • Down regulation of GST and SULT enzyme ⟶ Impaired phase-II metabolism
Elimination	• Down regulation of renal transporters OCT1, OCT2 ⟶ Less luminal uptake and lower tubular secretion • Upregulation of efflux transporters MRP3, MRP4, MATE1/2 ⟶ Increased urinary excretion • Decreased glomerular filtration rate ⟶ Decreased renal elimination • Upregulation of BCRP and down regulation of BSEP and MRP2 ⟶ Decreased cannalicular biliary excretion

* composite of published NASH patients/ NASH animals data