

Addressing today's ADME challenges in the translation of in vitro absorption, distribution, metabolism and excretion characteristics to human: A case study of the *SMN2* mRNA splicing modifier risdiplam

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Running title

Challenging ADME properties: a case study with risdiplam

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List of abbreviations

ADME, absorption, distribution, metabolism, and excretion

AUC, area under the concentration-time curve

AUC₀₋₄₈, area under the plasma concentration-time curve from time 0 to 48 hours

AUC_{0-inf}, area under the plasma concentration-time curve extrapolated to infinity

BCRP, breast cancer resistance protein

CYP, cytochrome P450

CL_R, renal clearance

C_{max}, maximum plasma concentration

DDI, drug-drug interaction

DME, drug-metabolizing enzyme

ER, efflux ratio

FDA, U.S. Food and Drug Administration

F, bioavailability

f_m, fraction metabolized

FMO, flavin-containing monooxygenase

hADME, human ADME

HH, human hepatocytes

HLM, human liver microsomes

HV, healthy volunteer

K_i, inhibitory constant

k_{inact}, rate of enzyme inactivation

MATE1, multidrug and toxin extrusion protein 1

MDR1, multidrug resistance mutation

MIST, metabolites in safety testing

OCT2, organic cation transporter 2

PBPK, physiologically based pharmacokinetic

PK, pharmacokinetic

SAD, single ascending dose

SMA, spinal muscular atrophy

SMN, survival of motor neuron

TDI, time-dependent inhibition

T_{\max} , time to reach the maximum concentration

Abstract

Small molecules that present complex absorption, distribution, metabolism, and elimination (ADME) properties can be challenging to investigate as potential therapeutics. Acquiring data through standard methods can yield results that are insufficient to describe the *in vivo* situation, which can affect downstream development decisions. Implementing *in vitro* - *in vivo* - *in silico* strategies throughout the drug development process is effective in identifying and mitigating risks while speeding up their development. Risdiplam (EVRYSDI[®]) - an orally bioavailable, small molecule approved by the U.S. Food and Drug Administration and more recently by the European Medicines Agency for the treatment of patients ≥ 2 months of age with spinal muscular atrophy (SMA), is presented here as a case study. Risdiplam is a low turnover compound whose metabolism is mediated through a non-cytochrome P450 enzymatic pathway. Four main challenges of risdiplam are discussed: predicting *in vivo* hepatic clearance, determining *in vitro* metabolites with regard to metabolites in safety testing guidelines, elucidating enzymes responsible for clearance, and estimating potential drug-drug interactions. A combination of *in vitro* and *in vivo* results was successfully extrapolated and used to develop a robust physiologically based pharmacokinetic model of risdiplam. These results were verified through early clinical studies, further strengthening the understanding of the ADME properties of risdiplam in humans. These approaches can be applied to other compounds with similar ADME profiles, which may be difficult to investigate using standard methods.

Significance statement

Risdiplam is the first approved, small molecule, *survival of motor neuron 2* mRNA splicing modifier for the treatment of spinal muscular atrophy. The approach taken to characterize the absorption, distribution, metabolism and excretion (ADME) properties of risdiplam during clinical development incorporated *in vitro-in vivo-in silico* techniques, which may be applicable to other small molecules with challenging ADME. These strategies may be useful in improving the speed at which future drug molecules can be developed.

Introduction

The preclinical investigation of drug candidates is an important step in the drug development process that enables the selection of the most promising molecule and predicts their likely pharmacokinetics in human. This stage provides valuable information on the likely safety and tolerability of a drug candidate before entering clinical trials. Assessing the PK properties, polymorphism risks and drug-drug interaction (DDI) potential of a new drug requires a considerable number of *in vitro* and *in vivo* preclinical studies over an extended time. However, for compounds with challenging absorption, distribution, metabolism and excretion (ADME) properties (e.g. low turnover, metabolism by enzymes which are not well understood, and/or generation of a complex profile of metabolites), the extrapolation of *in vitro* data to humans cannot be performed with high confidence. This is especially the case when traditional *in vitro* systems (e.g. human liver microsomes [HLM], human hepatocyte suspensions [HH]) may not be sufficiently sensitive for prediction of PK parameters such as clearance. In such situations, a combination of *in vitro* experimentation, model-based prediction and *in vivo* confirmation is needed to build up a combined description of drug metabolism and PK (Cleary et al., 2018).

Modeling and simulation-based approaches are now included in all areas of preclinical drug development programs, particularly physiologically-based pharmacokinetic (PBPK) modeling (Jones and Rowland-Yeo, 2013). Through a ‘bottom up’ approach, PBPK models input data from *in vitro* assays with human cells and preclinical animal studies to understand the mechanism of a drug’s absorption and disposition. When *in vitro* data coupled with *in silico* data are sufficiently reliable, PBPK-derived simulations can speed up molecule development by assisting in designing the most informative clinical studies and providing model-based predictions in lieu of others (Fowler et al., 2017). Moreover, PBPK models can extrapolate PK across different populations and disease states which are used to inform clinical trials, dose escalation studies, and possible DDIs (Jones and Rowland-Yeo, 2013). More notably, PBPK models have been used to develop prescribing labels (e.g. DDIs) and are now accepted as part of the clinical

pharmacology regulatory applications submitted to the U.S. Food and Drug Administration (FDA) (Zhang et al., 2020).

Risdiplam (EVRYSDI[®]), a recently approved drug with a challenging ADME profile (Food and Drug Administration, 2020c; European Medicines Agency, 2021), serves as a case study for an ADME strategy used to inform clinical development. Risdiplam is an orally available small molecule for the treatment of spinal muscular atrophy (SMA). SMA is an autosomal recessive neuromuscular disease caused by deletions and/or mutations of the *survival of motor neuron 1 (SMN1)* gene, resulting in reduced levels of functional SMN protein. A second *SMN* gene, *SMN2*, produces only low levels of functional SMN protein that are insufficient to fully compensate for the lack of the *SMN1* gene (Lorson et al., 1999). This results in a progressive loss of spinal motor neurons leading to muscle atrophy and disease-related complications affecting the whole body (Yeo and Darras, 2020) and may be diagnosed in infancy, childhood (Types 1–3) or adulthood (Type 4) (Mercuri et al., 2020). Risdiplam acts by modifying *SMN2* mRNA splicing, ensuring that more full-length transcripts are generated, thus increasing SMN protein levels (Ratni et al., 2018). It is to be expected that molecules with new chemical characteristics may be required to address such novel drug-targeting mechanisms, and that ADME scientists will need to learn about how best to translate the drug properties of these new compounds into *in vivo* situations.

Herein we describe some of the challenges met, and advances made whilst translating the ADME characteristics of an mRNA splicing modifying drug to the *in vivo* environment. By doing so we further report new data on the safety and PK profile of risdiplam. Comprehensive methodological information and additional supporting data are available in the **Supplemental materials**. Four main areas will be examined as shown in **Fig. 1**: (i) predicting *in vivo* hepatic clearance from low *in vitro* turnover, (ii) determining *in vitro* metabolites that are relevant in humans (with regard to metabolites in safety testing [MIST] guidelines), (iii) elucidating enzymes responsible for hepatic and extrahepatic clearance, and (iv) estimating potential DDIs. The work describes challenges encountered in the modern drug discovery setting and highlights strategies that could be adopted to ensure better predictions for future drug compounds.

1. Metabolic clearance prediction for risdiplam, a low clearance compound

Predicting clearance of drug candidates as accurately as possible is important in drug optimization as this shapes several aspects of PK including the oral bioavailability, half-life and effective dose. Risdiplam was optimized towards low intrinsic clearance and avoidance of issues observed in previous drug candidates (e.g. high levels of active metabolite (Ratni et al., 2018)) by making use of HH suspension cultures as an assay system. HH are the system of choice for optimizing metabolism-based clearance due to their full complement of drug metabolizing enzymes (DMEs) (Hutzler et al., 2015) and are available as high-quality cryopreserved preparations from multiple pooled donors which represent a ‘population average’. However, these primary cells lose function over a time span of ~4–6 h (Hutzler et al., 2015), and the lower limit of intrinsic clearance measurement is only ~3 $\mu\text{L}/\text{min}/\text{million cells}$ in suspension culture (Docci et al., 2019).

Recently, considerable effort has been invested by many laboratories into validating novel *in vitro* systems for low clearance measurements. In the case of risdiplam, the parent drug remained largely unmetabolized after incubation in HLM (89% remaining after 1 h) and hepatocytes (98% remaining after 3 h) with an intrinsic clearance <3 $\mu\text{L}/\text{min}/\text{million cells}$. A more advanced *in vitro* system was therefore needed for accurate intrinsic clearance determination. HepatoPac® was chosen as it offered pooled donor hepatocyte cultures, high reproducibility of intrinsic clearance determinations between experiments and thorough in-house validation (Kratochwil et al., 2017; Docci et al., 2020; Umehara et al., 2020) where it improved the accuracy and precision of hepatic intrinsic metabolic clearance predictions compared with hepatocytes in monoculture. HepatoPac® is a well-established long-term HH co-culture system, which can maintain viability and functional expression of DMEs for up to seven days (Khetani and Bhatia, 2008; Underhill and Khetani, 2018). In addition, reduced error in the *in vivo* intrinsic clearance prediction was observed using the HepatoPac® system when compared with hepatocytes in suspension culture (Umehara et al., 2020). The HepatoPac® long-term incubation system substantially lowers the intrinsic clearance quantification limit to 0.1–0.3 $\mu\text{L}/\text{min}/\text{million cells}$ (Da-Silva et al., 2018; Docci et al., 2019;

Umehara et al., 2020), and this improved sensitivity enabled risdiplam CL_{int} of 0.7 $\mu\text{L}/\text{min}/\text{million cells}$ to be determined. These data were then scaled to predict the *in vivo* clearance, the result of which was in excellent agreement (34% higher) with that observed *in vivo* (**Section 4**). To our knowledge, risdiplam is the first drug to come to the market for which advanced long-term hepatocyte co-cultures were used prospectively for intrinsic clearance measurement and scaling to *in vivo* conditions.

2. Low *in vitro* turnover of risdiplam warranted early confirmation of MIST-relevant metabolites in human plasma samples

According to MIST guidance, drug metabolites present at greater than 10% of total drug-related exposure at steady state in humans are a potential safety concern (Food and Drug Administration, 2016). Such metabolites need to be characterized in greater depth (e.g. ADME parameters or target activity) and need to be included into the pharmacology and safety assessment, e.g. via demonstration of exposure coverage in animal toxicity studies. Hepatocytes (and other subcellular fractions) provide a view of the principal metabolites and metabolic pathways for most compounds. However, due to multiple (and difficult to assess) parameters *in vivo*, determining which metabolite(s) would circulate - and can also reach or exceed 10% of the total drug-related material in plasma - is not easily predictable solely with *in vitro* data (Food and Drug Administration, 2016; Schadt et al., 2018).

Risdiplam showed only low turnover in incubations with HLM (11% in 1 h) and HH (2% after 3 h). Consequently, risdiplam produced metabolites at low mass spectrometry signal intensities in liver microsomes, and even lower in hepatocytes. Although the *N*-hydroxyl M1 was the most abundant metabolite, the mass spectrum peak intensities were measured in low percentages of total drug-related MS signal intensities (1.7% in hepatocytes and 3.8% in microsomes after 3 h; **Fig 2a**). Other oxidative metabolites (M2-M8) were present at even smaller peak intensities or seen at trace levels (M2-M8).

From a single-ascending-dose (SAD) study (18 mg of risdiplam (Sturm et al., 2018)) in healthy volunteers (HVs, NCT02633709), the plasma metabolite profile was assessed using pooled plasma samples (cross-subject pools of individual time points) which revealed that the main components were

parent drug and the *N*-hydroxyl metabolite M1 (**Fig 2.** and **Fig. S1**). It also found that M1 exceeded 10% of the total drug-related MS signal intensities, triggering additional bioanalytical method development for M1. Further exploration of M1 showed that it was devoid of any significant primary (*SMN2* splicing) or secondary (*FOXMI* splicing) pharmacological activity at therapeutic doses of risdiplam (Ratni et al., 2018).

Following the SAD study, a human radiolabeled ADME (hADME) study (oral administration of ¹⁴C-risdiplam to HVs, NCT03036501) was conducted to identify circulating metabolites in plasma samples collected up to 48 h post-dose (**Fig 3**). **Figures 2b and 3** show the plasma time profile for total radioactivity, risdiplam and its metabolites confirming that risdiplam was the major drug-related component found in circulation, and M1 being a major circulating metabolite exceeding 10% of total drug-related material. Four additional low-level metabolites (M2, M7, M9 and M26) from biotransformation of the piperazine moiety were observed in plasma. No individual metabolite accounted for more than 2.2% relative to the area under the concentration-time curve (AUC) of total drug-related material in plasma. All other circulating metabolites were below the limit of detection of the metabolite identification radioprofiling method used in the [¹⁴C]-hADME study.

The metabolism of risdiplam illustrates the challenges drug metabolism scientists face with predicting the relevance of human *in vivo* metabolites from *in vitro* incubations of low turnover compounds. The *in vitro* metabolic profile was comprised of numerous low-level metabolites and the quantitative translation from *in vitro* to *in vivo* was poor. The profile that was finally observed in plasma circulation was mainly risdiplam and M1 (with M1 exceeding 10% of total drug-related material). For low turnover compounds such as risdiplam, *in vitro* systems are limited in accurately anticipating the extent to which metabolites will circulate *in vivo* as distribution and excretion of metabolites is not reflected *in vitro*. However, qualitatively the principal metabolic biotransformation pathways were well reflected *in vitro* and the data could be used to inform potential frontloading activities (e.g. structure elucidation by nuclear magnetic

resonance for lower level metabolites). Therefore, *in vitro* profiles were still of key importance to mitigate the risk of discovering disproportionate metabolites (according to MIST guidance) late in development, despite not being quantitatively predictive.

Major metabolite (M1) coverage in plasma of nonclinical species

The major metabolite M1 was detected in mouse, rat, rabbit, and monkey plasma. The nonclinical coverage of M1 in pivotal nonclinical studies at the respective no observed adverse effect level (NOAEL) for these species is summarized in **Table 1**. Coverage was assessed based on the median M1/parent ratio (0.334) in SMA patients at steady state at pivotal doses.

Metabolite identification at later time points and implications for regulatory approval of risdiplam

In plasma samples from the [¹⁴C]-hADME study, metabolites were identified using a radioprofiling method up to 48 h post-dose because radioactivity levels dropped below the sensitivity of the radioprofiling method used. However, some metabolites may have longer plasma half-lives compared with the parent compound, which would indicate slower elimination rates limiting their clearance (Holmberg et al., 2014). Uncertainties about the levels of metabolites at later time points can be a concern due to potential accumulation after repeated doses, and this was raised during a Health Authority assessment of risdiplam. The Health Authority assessment questioned whether the truncated area under the plasma concentration–time curve from time 0 to 48 hours (AUC_{0-48h}) was representative of area under the plasma concentration–time curve extrapolated to infinity (AUC_{0-inf}) and therefore an investigation was conducted to identify any persistent major metabolites.

An exploratory metabolite identification was performed on selected plasma samples from HVs administered with a single dose of 18 mg risdiplam (SAD study) to identify the major circulating drug-related material. The high-resolution mass spectrometry data acquired from SAD plasma samples of a representative individual up to 216 h post-dose was re-interrogated to address this concern. Of the 21 selected metabolites which met defined relevance criteria, high-resolution mass spectrometry peak areas of 16 metabolites could be extracted in the revisited SAD study raw data and were plotted up to 216 hours (see **Fig. 4**).

Supplemental Table 3 shows a comparison of the metabolite-to-parent AUC ratios for the most abundant metabolites in plasma (M1 and M26) observed in the hADME study, based on ^{14}C and ^{12}C , respectively. Both the risdiplam metabolite-to-parent AUC ratios over 48 h for the hADME study and over 216 h for the SAD study were considered similar. The exploratory metabolite identification data derived from the representative individual over 216 h post-dose in the SAD study increased the confidence that the metabolites identified at the truncated AUC_{0-48} in the hADME study were representative of AUC_{inf} . Additionally, a number of unexplained peaks in the radiochromatogram, due to the low analytical sensitivity of the radioprofiling method, were assigned to several minor metabolites after reanalyzing plasma samples from the SAD study. The analysis of the raw mass spectral data at up to 216 h did not indicate any persistent metabolite with a significantly longer half-life than unchanged risdiplam, indicating that metabolites did not accumulate after repeated doses of risdiplam and resolving the question raised by the Health Authority. Reassessing previously-acquired data therefore revealed extended longitudinal results. The opportunity to reanalyze existing post-acquisition data was pivotal in potentially preventing a costly follow-up clinical trial which could have delayed the drug approval process.

3. Investigating the combination of CYP P450 and flavin-containing monooxygenase contributions to risdiplam metabolism and implications for victim DDIs

In vitro metabolite identification showed that risdiplam was metabolized via an *N*-oxidation and oxidation-mediated piperazine ring degradation. As there was no evidence of hydrolysis and conjugation biotransformation reactions, attention was focused on the metabolism of risdiplam by CYP and flavin-containing monooxygenase (FMO) enzymes. Incubation of 10 μM radiolabeled risdiplam by recombinantly expressed CYP and FMO enzymes showed risdiplam can be metabolized by FMO1 and FMO3 as well as CYPs 1A1, 3A4, and 3A7. Multiple metabolites (including M1) were generated by each of these enzymes. When sensitive liquid chromatography with tandem mass spectrometry methods became available for risdiplam metabolite detection a further assessment of risdiplam and M1 metabolism was made.

Fig 5 shows the relative amount of metabolites M1, M2 and M5, which were detected in high abundance, formed in incubations using a panel of CYP and FMO enzymes. FMO1 and FMO3 and CYPs 1A1, 2J2, 3A4 and 3A5 were the most active enzymes. The circulating metabolite M1 was further metabolized by many CYP and FMO enzymes (CYPs 1A1, 2C8, 2C19, 2J2, 3A4 and FMO1 and FMO3). Among these enzymes, CYP2J2, CYP3A4, FMO1 and FMO3 were most active in M1 metabolism (data not shown). Individual enzyme turnover data can be extrapolated to estimate the fraction metabolized (f_m) when suitable scaling factors are known. However, this was not the case for a number of enzymes active in risdiplam metabolism at the time of performing the studies. To calculate intersystem extrapolation factors or relative activity factors, drug turnover rates and turnover rates for enzyme-selective substrates for the individual enzyme preparations and pooled HLM are required. These approaches have been validated for several CYPs, but others may lack established enzyme-selective probe substrates or may not be well represented by the pooled HLM test system. For instance, CYP1A1 is known to be expressed in extrahepatic tissues and present in liver microsomes from some donors, but is essentially inactive in pooled HLM (Lang et al., 2019). CYP2J2 is expressed in several extrahepatic tissues including the heart and has an important role to play in the regulation of arachidonic acid derivatives (Solanki et al., 2018). Recent studies have reported the discovery of selective CYP2J2 substrates (Zhao et al., 2021) and inhibitors (Phuc et al., 2017). The emerging characterization tools, tissue concentration measurements and drug examples may enable a more quantitative estimation of the contributions of less well-studied enzymes to drug metabolism in future.

Risdiplam was metabolized by human liver, kidney and intestinal microsomes with 24%, 9% and 2% turnover observed, respectively, after 1 hour of incubation (at a higher microsomal protein concentration of 1 mg/mL) (**Table S5**). Under these experimental conditions, midazolam was turned over by >97%, <20% and >97% by liver, kidney and intestinal microsomes, respectively. These results indicated a differential enzymology of risdiplam with this CYP3A marker drug. The high relative rates of the hydroxylamine metabolite M1 formation and that of other metabolites formed by the kidney microsomes

highlighted the possible involvement of FMO1 in the metabolism of risdiplam. Methimazole, an inhibitor of FMO enzymes (Störmer et al., 2000), reduced kidney microsomal metabolism by >90%. The data suggested that risdiplam was mainly metabolized by FMO1 in the kidney with only minor contributions from other enzymes. However, after accounting for the organ weight and blood flow, the scaled whole organ clearance of risdiplam in the kidney was $\leq 3\%$ of hepatic clearance. This indicated that the actual contribution of FMO1 enzyme to risdiplam clearance was low even though the *in vitro* turnover by FMO1 was high, which showed the importance of translating the raw enzyme activities into likely human relevance.

In vitro studies were also performed to investigate the impact of inhibiting CYP3A and FMO enzymes on liver microsomal metabolism. Methimazole reduced human liver microsomal turnover by ~75%, consistent with an important role for FMO3 in liver metabolism. As additional analytical methodologies became available, it was possible to examine the effect of inhibitors on the formation of the most abundant *in vitro* risdiplam primary metabolites M1, M2 and M5 at a more physiologically relevant concentration (1 μM ; **Table S6**). CYP3A inhibition by ketoconazole reduced the formation of these metabolites by 23-62% while methimazole inhibited their formation by 17-46%, dependent upon the metabolite. This demonstrated that FMO and CYP3A enzymes are important contributors to risdiplam metabolism and provided potential f_m ranges for planning of a definitive *in vivo* DDI study. Since the time of performing these studies the first demonstrations of chemical inhibitor effectiveness and application to f_m estimation using long-term hepatocyte cultures have started to emerge (Chan et al., 2020). These approaches will give additional opportunities for f_m estimation, based upon total drug depletion, when such validation is available for a panel of selective inhibitors.

FMOs are important for the metabolic clearance of benzydamine, itopride, pargyline, ranitidine, olopatadine, xanomeline, albendazole, cimetidine and ethionamide, and are minor contributors to the metabolism of many other compounds (Krueger and Williams, 2005; Phillips and Shephard, 2020). However, there are few examples of clinical DDIs and currently no probe inhibitors for use in clinical

DDI studies have been recommended (Food and Drug Administration, 2020b). In a survey of enzymes involved in the metabolism of the FDA new drug applications between 2013 and 2016, FMO enzymes were listed as contributing to the metabolism of only 3 out of 98 cases (where data were available) and investigation with clinical DDI studies was not reported for any of these (Yu et al., 2018). FMOs are generally viewed favorably as contributing enzymes to the metabolism of drugs, recently reiterated in the case study for ABT-126 which is mainly metabolized by FMOs (Liu et al., 2018). FMO induction is not well-known and few drugs which inhibit FMOs have been identified, reducing the likelihood of DDIs (Cashman and Zhang, 2006). These positive aspects are also reflected in the smaller body of *in vitro* and clinical data available for FMOs compared with CYPs. At the same time, the paucity of literature examples of FMO metabolism impacted preclinical confidence in the accurate extrapolation of risdiplam *in vitro* data.

Although FMO3 activity can be assessed using benzydamine *N*-oxidation as a probe reaction (Lang and Rettie, 2000; Störmer et al., 2000) the scaling of FMO contributions to overall metabolism has not been well established to date. Obstacles here include a lack of clinical comparator data and uncertainties about the percentage of FMO3 enzyme activity remaining in HLM preparations due to thermal lability. With additional case reports of *in vitro* f_m prediction and *in vivo* f_m confirmation, confidence will be built in the estimation of FMO contributions to metabolism. A step in this direction was provided by Jones et al. (Jones et al., 2017) who reported on the scaling of intrinsic clearance data for FMO-cleared drugs using HLM and HH intrinsic clearance data, which showed human clearance could be reasonably predicted, consistent with our risdiplam experience. Risdiplam is therefore an important addition to the list of drugs with clinically important contributions from FMO enzymes and demonstrates that clearance scaling for an FMO/CYP substrate could be made effectively. This should help in the build-up of validation datasets needed for intrinsic clearance and f_m predictions for FMO substrate drugs to become better accepted.

4. Translating *in vitro*-*in vivo*-*in silico* data to first-in-human trials

A PBPK model was used to calculate appropriate doses of risdiplam for the entry-into-human study. The safety and tolerability of these predicted doses were assessed in the SAD study (Sturm et al., 2018). PK data and DDI potential with risdiplam were also obtained to verify *in vitro* results and *in silico* predictions. Details regarding the PBPK model can be found in **Table 2**.

The PBPK model predicted almost complete absorption after oral administration of 0.6 to 18 mg risdiplam as oral solution (fraction absorbed; $F_A > 0.9$), and a volume of distribution at steady state of 4.1 L/kg in adult humans (**Table 2**). The predicted metabolic clearances in the kidney and intestine based on the turnover study were $\leq 3\%$ of the predicted hepatic CL and therefore not included in the PBPK model. Consequently, the risdiplam CL was modeled with the predicted plasma CL of 7.3 L/h, which was based upon hepatic metabolism scaled from the CL_{int} determined by the turnover in HepatoPac[®]. This indicated low hepatic extraction (hepatic availability, $F_H > 0.9$) and negligible intestinal metabolism (intestinal availability, $F_G > 0.9$ based on CYP3A metabolism, and unbound fraction in enterocyte, $f_{u, gut} = 1$). Overall, high bioavailability (approximately 0.9) was predicted for 0.6 to 18 mg of risdiplam administered as an oral solution. In the hADME study, approximately 14% and 17% of 18 mg orally administered [¹⁴C]-risdiplam were recovered as unchanged risdiplam in the pooled feces collected over 168 h and 840 h, respectively (**Fig 2C**), which is consistent with the predicted bioavailability.

In the SAD study in HVs, risdiplam was well tolerated up to the highest dose tested (18 mg) and the PK properties were linear over the entire dose range (Sturm et al., 2018). The predicted plasma CL of 7.3 L/h was found to be in good agreement with the apparent CL (CL/F) of 5.68 L/h estimated by a population PK model developed on the SAD study data (**Table S7 and Fig S5**), given the predicted high oral bioavailability of risdiplam. The total hepatic clearance was adjusted to the population PK model estimates and included a renal clearance (CL_R) of 0.33 L/h, which corresponded to approximately 5% of the total clearance.

Potential DDI by CYP3A inhibition were investigated by co-administration of risdiplam with itraconazole in HVs (Sturm et al., 2018). Based on this data, the PBPK model estimated a fraction of risdiplam

metabolized by CYP3A ($f_{m_{CYP3A}}$) of 20% (Sturm et al., 2018). The remainder of the metabolic clearance was assigned to hepatic metabolism through FMO3 enzyme according to the *in vitro* enzymology study for risdiplam ($f_{m_{FMO3}} = 75\%$). The plasma concentration-time profiles and PK parameters simulated by the PBPK model of risdiplam showed good agreement with the observations in all doses investigated in the SAD study (Sturm et al., 2018) as shown in **Fig 6** and **Table 3**, respectively. Subsequently, the PBPK model was extrapolated to adult and pediatric SMA patients (Cleary et al., 2021c) to support dose selection for therapeutic studies and DDI risk assessments (Cleary et al., 2021a).

5. DDIs risk profile – clinical relevance of CYP3A time-dependent inhibition and emerging transporters

DDIs occur when drugs taken in combination with other drugs cause unexpected side effects or efficacy due to modified drug exposure; their detection is therefore essential for patients on concomitant medications. These effects happen when DMEs such as CYP enzymes or drug transporters are inhibited or induced, which affects the PK of the administered drug. This can be conceptualized as ‘victim’ drugs (whose PK is directly affected) or ‘perpetrator’ drugs (inhibits or induces enzymes/transporters which affects the PK of the victim). The case of terfenadine is one example of the victim-perpetrator DDI effect. Terfenadine was withdrawn from the market due its elevated concentrations in plasma that resulted in life threatening and/or fatal cardiac arrhythmia in some patients who were co-medicated with ketoconazole (Honig et al., 1993). However, in some cases perpetrator drugs have been co-administered as an intentional strategy to prolong victim drug plasma concentrations. Such is the case for HIV-protease inhibitors, which are primarily metabolized by CYP3A. For example, when co-administrated with ritonavir (strong CYP3A inhibitor), plasma exposures of protease inhibitors are boosted. It is now recommended, as part of first-line treatment, that low-dose ritonavir is co-dosed with HIV-protease inhibitors (Hull and Montaner, 2011).

Regulatory bodies require a thorough evaluation of any possible drug interactions against both DME and membrane transporters prior to market approval (European Medicines Agency, 2012; Food and Drug Administration, 2018; Ishiguro et al., 2020). It has been recommended to use *in vitro* systems to investigate any potential DDIs. *In silico* studies (e.g. PBPK models) can be used and are accepted in lieu of some prospective DDI studies, to predict moderate or weak perpetrator drugs on the exposure of the parent drug (Food and Drug Administration, 2020a). As the major metabolite of risdiplam, M1, was found to be MIST-relevant, risdiplam and M1 were both examined for CYP induction in HH as well as for reversible (direct) and irreversible (time-dependent inhibition [TDI]) inhibition in HLM. There was no significant induction of CYP1A2, 2B6, 2C8, 2C9, 2C19 and 3A4 at the mRNA level after incubation of risdiplam and M1 in primary HHs. Risdiplam and M1 also did not show direct inhibition of major CYP enzymes (1A2, 2B6, 2C8, 2C9, 2C19, and 2D6) except for CYP3A at concentrations of up to 12.5 μM and 10 μM , respectively. These concentrations are much higher than the C_{max} values (184 ng/mL [0.46 μM]) after oral administration at the therapeutic dose of risdiplam, indicating no clinically relevant direct inhibition on CYP substrates.

In vitro TDI parameters of risdiplam and M1 on CYP3A were measured and the inhibitory constant (K_I) = 13 μM and *in vitro* inactivation rate constant (k_{inact}) = 0.065 min^{-1} ; and K_I = 13.7 μM , k_{inact} = 0.063 min^{-1} determined, respectively; **Table S8**. However, there is some uncertainty over these values because the TDI effect did not reach saturation at the concentrations of risdiplam tested due to solubility limitations. Indeed, the estimated K_I was higher than the maximal test substance concentration (12.5 μM) in the study, which indicated a level of uncertainty with the fitting performance. This could result in the over-prediction of the TDI potency in the initial perpetrator DDI risk assessment. Moreover, *in vitro* TDI data measured from standard HLM assays frequently overpredict the magnitude of DDI (Eng et al., 2021) and fail to correctly categorize a drug as a weak, moderate, or strong inhibitor *in vivo* (Mao et al., 2012). A model system of human hepatocytes suspended in human plasma has been proposed to be an alternative *in vitro* system to more accurately assess CYP3A mediated TDI-DDI risk. The human hepatocytes

suspended in human plasma demonstrated a more reliable clinical DDI prediction for CYP3A TDI (Mao et al., 2012; Mao et al., 2016; Food and Drug Administration, 2020a).

With a positive TDI result of risdiplam in HLM, the PBPK model can be used to predict the magnitude of the DDI and to investigate any DDI risk in future clinical studies. The model predicted an approximately 2-fold increase in AUC for midazolam (prototypical CYP3A substrate) in healthy adults, which was considered clinically relevant (FDA threshold; AUC ratio ≥ 1.25 (Food and Drug Administration, 2020b)) and warranted further clinical investigations. However, results from the clinical DDI study in healthy adults showed that, in the presence of risdiplam, C_{\max} and AUC of midazolam were increased by 16 and 11%, respectively (Cleary et al., 2021b). The finding was not clinically relevant and therefore dose adjustment for concomitantly used CYP3A substrates is not necessary.

In vitro-in vivo extrapolation methods to investigate membrane transporter interactions are mostly unavailable - with the exception of MDR1 (multidrug resistance mutation 1) - and therefore can present a challenge when studying and/or validating potential DDIs (Giacomini et al., 2010; Yoshida et al., 2017). Risdiplam and M1 were screened in the relevant drug transporter assay as substrates. Both compounds were determined to be highly permeable molecules (>300 nm/s in LLC-PK1 or MCKII cells). Risdiplam was not a substrate of human MDR1 and a weak substrate of human breast cancer resistance protein (BCRP; efflux ratio [ER] = 3.1) (Ratni et al., 2018). M1 was a weak-to-good substrate of human MDR1 (ER = 5.5) and a weak substrate of human BCRP (ER = 4.1). The *in vitro* hepatic uptake of risdiplam in HH was not sensitive to an organic anion transporting polypeptide inhibitor (RifSV, 100 μ M) and, therefore, risdiplam was not considered an organic anion transporting polypeptide substrate. The hepatic uptake is likely to be driven predominantly by passive diffusion, which is consistent with its high passive permeability. Overall, risdiplam and M1 did not present a clinically significant DDI risk related to drug transport proteins as victims.

When risdiplam and M1 were screened against a panel of relevant transporter proteins as perpetrators, the results showed that risdiplam inhibited organic cation transporter 2 (OCT2), MATE1 and MATE2-K

while M1 inhibited BCRP and MATE1 (**Table 4**). In order to assess the clinical relevance of these *in vitro* inhibition risks, regulatory bodies recommend: (1) for intestinal efflux (BCRP): calculating the drug level in the gastrointestinal tract as the oral dose divided by 250 mL; (2) for hepatic efflux (BCRP) and renal uptake (OCT2, MATE1 and MATE2-K): calculating the ratio of unbound plasma C_{\max} to the *in vitro* IC_{50} ($[I]/IC_{50}$) (European Medicines Agency, 2020; Food and Drug Administration, 2020a). The transporter-related clinical DDI risk can be excluded if the ratio $[I]/IC_{50}$ is lower than 0.02.

The approach to assess clinical relevance of BCRP inhibition was not applicable to risdiplam as M1 is a circulating metabolite and not detected in the feces of humans. For renal uptake, the static model recommended by the regulatory bodies revealed a potential clinically relevant risk only for risdiplam inhibition of both MATE1 and MATE2, with a calculated ratio exceeding the recommended 0.02 threshold (**Table 4**). However, static models are known to overpredict clinical DDIs (Filppula et al., 2019). Rather, a PBPK model could be an alternative strategy to further evaluate the DDI risk. MATE proteins are often referred to as “emerging” transporters and clinical case examples are rare. To date, as MATE-related DDIs are rare, there is no validated PBPK model available to assess MATE-related DDIs, which further prevented any quantitative extrapolation (Yoshida et al., 2017). Currently, the most validated victim is metformin, and the methodology to extrapolate a possible DDI to other substrates remains to be validated due to a lack of clinical comparator data (Food and Drug Administration, 2020b). Most of the existing clinical evidence concerning MATE-related DDIs pertain to the inhibition of metformin renal clearance by cimetidine, pyrimethamine or dolutegravir (Chu et al., 2018). The highest metformin AUC ratio (2.68) has been observed with pyrimethamine. With metformin elimination dependent mostly on a renal secretion by an active transporter and pyrimethamine acting as a strong and selective MATEs inhibitor, this concrete clinical case can be considered as the worst-case example known thus far. Fexofenadine, dofetilide, cephalexin and procainamide are among other rare victim drugs affected by MATE-mediated DDIs with clinical data (Hillgren et al., 2013). As these drugs are not a part of the standard of care for patients with SMA, the risk of DDI due to MATE was deemed to be minimal.

Quantitative extrapolation and clinical significance of *in vitro* findings for emerging transporters remain a challenge. New biomarker-informed strategies, for example assessing early clinical samples for serum creatinine levels for MATEs, can serve as an additional filter to minimize false negative and false positive predictions, and can enable quantitative DDI predictions (Mathialagan et al., 2020).

6. Conclusion

Assessing small molecules with complex ADME properties is not always straightforward. However, new technology and advancements to existing methods are allowing scientists to overcome longstanding challenges. Moreover, when *in vitro* studies, PBPK modeling and clinical investigations are successively combined, a cohesive picture of the overall PK profile of a drug can be determined. This was exemplified here in the development of risdiplam. Despite low turnover, *in vitro* clearance of risdiplam was determined using a long hepatocyte co-culture system. The PBPK model, with a robust description of ADME process, helped to define the doses to be assessed in the SAD study. The SAD trial was instrumental in assessing the safety and tolerability of risdiplam, but also in verifying metabolic clearance, renal secretion and potential DDI risks. The *in vivo* metabolic profiling of plasma samples from the SAD study, which were supplemented by *in vitro* data, appropriately informed the MIST-relevance of metabolites. This prevented potential delays in the program (e.g. bioanalytical method development or synthesis of standards etc.), as these could be identified early on in development. The SAD trial data were also crucial, several years after its first analysis, for investigating metabolites in plasma for up to 216 h to ensure there were no persistent metabolites. Through this *in vitro-in vivo-in silico* strategy, the clinical development of risdiplam was accelerated; multiple PK questions were swiftly answered and regulatory requirements for drug filing were fulfilled, all of which led to the FDA approval (Food and Drug Administration, 2020c) and recent EC approval (European Medicines Agency, 2021) of risdiplam. We hope insights and perspectives can be gained from this case study to help investigators developing small-molecule drugs with challenging ADME profiles for the clinic.

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Authorship Contributions

Participated in research design: AP, AB, HK, SF, LM, DT, NK

Conducted experiments: AB, CH, MS, SF, KH, KU, MU, NK

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Wrote or contributed to the writing of the manuscript: SF and AB have contributed equally to this work.

YC and AP have contributed to writing sections of the text. AG, KH, CH, HK, NK, LM, MS, CS, DT,

MU, KU have critically reviewed the manuscript. All authors approved the final draft.

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Footnotes

a) This work was supported by F. Hoffmann-La Roche.

b) SF, AG, KU, KH, NK are employees and AB, YC, CH, HK, LM, AP and CS are employees and shareholders of F. Hoffmann-La Roche. DT is an employee and shareholder of F. Hoffmann-La Roche AG. MU was an employee of F. Hoffmann-La Roche at the time of the study. MS is an employee of Unilabs York Bioanalytical Solutions and was commissioned and paid by F. Hoffmann-La Roche to perform metabolism and DMPK studies.

Figure legends

Figure 1. Four main challenges of risdiplam

Figure 2. Overview of risdiplam disposition metabolism and excretion in man after a single oral dose of 18 mg. A) *in vitro* metabolites identified in incubations of HLM and HH after 60 and 180 minutes, respectively. B) high performance liquid chromatography and microbeta-scintillation count analysis of the metabolite profile of risdiplam in pooled human plasma of six healthy individuals at 24 h after oral administration (18 mg) of [¹⁴C]-risdiplam. C) Overview of risdiplam disposition, metabolism and excretion after a single oral dose of 18 mg in six healthy volunteers

An asterisk on the structure of risdiplam denotes the carbon 14 labeling position in the molecules. Unit (plasma, feces and urine): % of total drug-related material (radioactivity) in the given matrix (of AUC₀₋₄₈ in plasma and over 0–168 h in excreta). Approximately 14% and 17% of orally administered 18 mg of [¹⁴C]-risdiplam was recovered as unchanged in the pooled feces collected over 168 h and 840 h, respectively.

* Percent radioactivity recovered after 0–168 h

† Individual contributions: 0.2–3.5% of drug-related material in feces (cumulated = 9.6% of the dose), 0.4–4.5% of drug-related material in urine (cumulated = 5.3% of the dose)

‡ Trace level components each accounting for <0.9% of the dose (feces) or ≤1.0% of the dose (urine)

§ Recovered dose after 0-840 h

Figure 3. Plasma time profile for total radioactivity, risdiplam and its metabolites M1, M2 and M7, M9 and M26 in human plasma after oral administration (18 mg) of [¹⁴C]-risdiplam.

LSC, liquid scintillation counting; MSC, microplate scintillation counting.

Figure 4. Post-acquisition, exploratory metabolite identification analysis in plasma levels of risdiplam and metabolites following single oral dose of 18 mg risdiplam in 1 healthy volunteer.

Figure 5. Generation of specific metabolites on incubation of 1 μ M risdiplam with individual CYP and FMO enzymes and human liver microsomes

Figure 6. Simulated and observed plasma concentrations of risdiplam after 0.6, 2, 6 or 18 mg as a single dose. The observations (open circles), 5th to 95th percentiles (grey shades) and geometric means (grey dotted lines) of the simulated concentrations are shown.

Table 1. M1 Coverage in nonclinical pivotal toxicity studies at the respective NOAEL

| Species | Dose at NOAEL [mg/kg/day] | | M1 Exposure at NOAEL [AUC ₀₋₂₄ in ng·h/mL] | | Animal vs Human M1 Exposure Ratio ^a | |
|--------------|------------------------------|-----|----------------------------------------------------------|-------------------|---------------------------------------------------|------|
| | M | F | M | F | M | F |
| Monkeys | 1.5 | 1.5 | 285 ^b | 314 ^b | 0.43 | 0.47 |
| Adult rats | 1 | 3 | 796 ^b | 3195 ^b | 1.19 | 4.78 |
| Rats (PND31) | 1.5 | 1.5 | 439 | 406 | 0.66 | 0.61 |
| RasH2 mice | 9 | 9 | 1690 | 1580 | 2.53 | 2.37 |
| Rabbits | NA | 4 | NA | 303 | NA | 0.45 |

AUC, area under the curve; F, females; M, males; NA, not applicable; NOAEL, no observed adverse effects level; PK, pharmacokinetics; PND, postnatal day

^a The M1 AUC_{0-24,ss} at the mean exposure guidance in SMA patients (2000 ng·h/mL; parent AUC_{0-24,ss}) was extrapolated by multiplying the parent AUC_{0-24,ss} with the median M1/parent ratio (0.334) in SMA patients at pivotal doses (i.e. M1 AUC_{0-24h,ss} = 2000 ng·h/mL × 0.334 = 668 ng·h/mL). The animal versus human M1 exposure ratio is the ratio of the animal M1 AUC_{0-24,ss} on the last day of dosing at the NOAEL dose divided by the extrapolated M1 AUC_{0-24,ss} at the mean exposure guidance in SMA patients (668 ng·h/mL).

^b Plasma samples taken before incorporation of M1-preservation measures into bioanalytical methods. The M1 exposure was estimated from a corresponding PK bridging study as follows: M1 AUC₀₋₂₄ = (parent AUC₀₋₂₄ on the last day of dosing at the NOAEL dose) × (M1/parent ratio from PK bridging study).

Table 2. Input parameters of risdiplam PBPK model.

| Parameter | Value | Sources |
|---------------------------------------|----------------------------|---------------------------------------------------------------------------|
| Molecular weight | 401.5 g/mol | |
| Compound type | diprotic base | Measured |
| | pKa1 = 4.52 | |
| | pKa2 = 6.82 | |
| LogD* | 2.51 (pH=7.4) | Measured |
| Permeability (P_{eff}) | 20.4×10^{-6} cm/s | Measured |
| B:P ratio | 1.3 | Measured |
| Protein binding | 89% | Measured |
| Absorption | | |
| F_A | >0.9 | Predicted by ADAM model |
| $f_{u,gut}$ | 1 | (Yang et al., 2007) |
| Distribution | 4.1 L/kg | Full PBPK model with predicted Kp values (Rodgers and Rowland, 2006) |
| Metabolism | | |
| $CL_{int,CYP3A4}$ (μ L/min/pmol) | 0.018 | According to itraconazole DDI study results (Sturm et al., 2018) |
| $CL_{int,FMO3}$ (μ L/min/pmol) | 0.364 | Calculated based on <i>in vitro</i> and clinical study results. |
| Elimination | | |
| CL_R (L/h) | 0.33 | According to the observations in healthy individuals (Sturm et al., 2018) |

*The corresponding LogP is 2.61.

Table 3. Comparison of predicted and observed PK parameters of risdiplam

| Dose | C_{max} (ng/mL) | t_{max} (h) | AUC_{inf} (ng • h/mL) |
|--------------------|--------------------------------|----------------------------|--------------------------------------|
| 0.6 mg | | | |
| Observation | 2.82 | 3.0 | 86.7 |
| Prediction | 2.86 | 2.45 | 112 |
| 2 mg | | | |
| Observation | 8.33 | 3.0 | 294 |
| Prediction | 9.53 | 2.45 | 373 |
| 6 mg fasted | | | |
| Observation | 24.5 | 2.0 | 1080 |
| Prediction | 28.6 | 2.45 | 1120 |
| 18 mg | | | |
| Observation | 93.2 | 2.0 | 3290 |
| Prediction | 85.8 | 2.45 | 3360 |

Geometric means are presented except for t_{max} (median).

Table 4. Human DDI risk assessment for transport proteins inhibition by risdiplam or M1

| Compound / Protein | IC ₅₀ [μM] ^a | Total C _{max} in SMA patients [ng/mL] ^a | [I] [μM] ^b | [I]/IC ₅₀ | Threshold ^c |
|---------------------|------------------------------------|-------------------------------------------------------------|-----------------------|----------------------|------------------------|
| Risdiplam / OCT2 | 8.7 | Median = 184 (0.2 mg/kg, <2 years) | 0.049 | 0.006 | 0.02 |
| | | Max observed = 364 | 0.097 | 0.011 | |
| Risdiplam / MATE1 | 0.15 | Median = 184 (0.2 mg/kg, <2 years) | 0.049 | 0.33 | |
| | | Max observed = 364 | 0.097 | 0.65 | |
| Risdiplam / MATE2-K | 0.09 | Median = 184 (0.2 mg/kg, <2 years) | 0.049 | 0.54 | |
| | | Max observed = 364 | 0.097 | 1.08 | |
| M1 / MATE1 | 14.8 | Median = 61 (0.2 mg/kg, <2 years) | 0.011 | 0.0007 | |
| | | Maximum = 122 | 0.022 | 0.0015 | |
| M1 / BCRP | 2.3 | Median = 61 (0.2 mg/kg, <2 years) | 0.011 | 0.0047 | |
| | | Maximum = 122 | 0.022 | 0.0094 | |

Grey shaded areas indicate values above the [I]/IC₅₀ threshold.

^a Risdiplam and M1 plasma concentrations at steady state at pivotal doses (SUNFISH study [NCT02908685]): 0.25 mg/kg (body weight <20 kg) or 5 mg, FIREFISH study (NCT02913482): 0.2 mg/kg).

The assessment was based on the median and maximum observed C_{max} values in the FIREFISH study; C_{max} values were lower in older patients in the SUNFISH study. Median M1 percentage vs parent of 33.4 = ~30% was assumed for calculating M1 C_{max} values.

^b [I] is the unbound plasma concentration (assuming free fraction in human plasma 10.7% for risdiplam and 7.4% for M1).

^c Most conservative [I]/IC₅₀ threshold above which the Sponsor should further investigate the DDI potential by conducting a clinical DDI study (European Medicines Agency, 2020; Food and Drug Administration, 2020a)

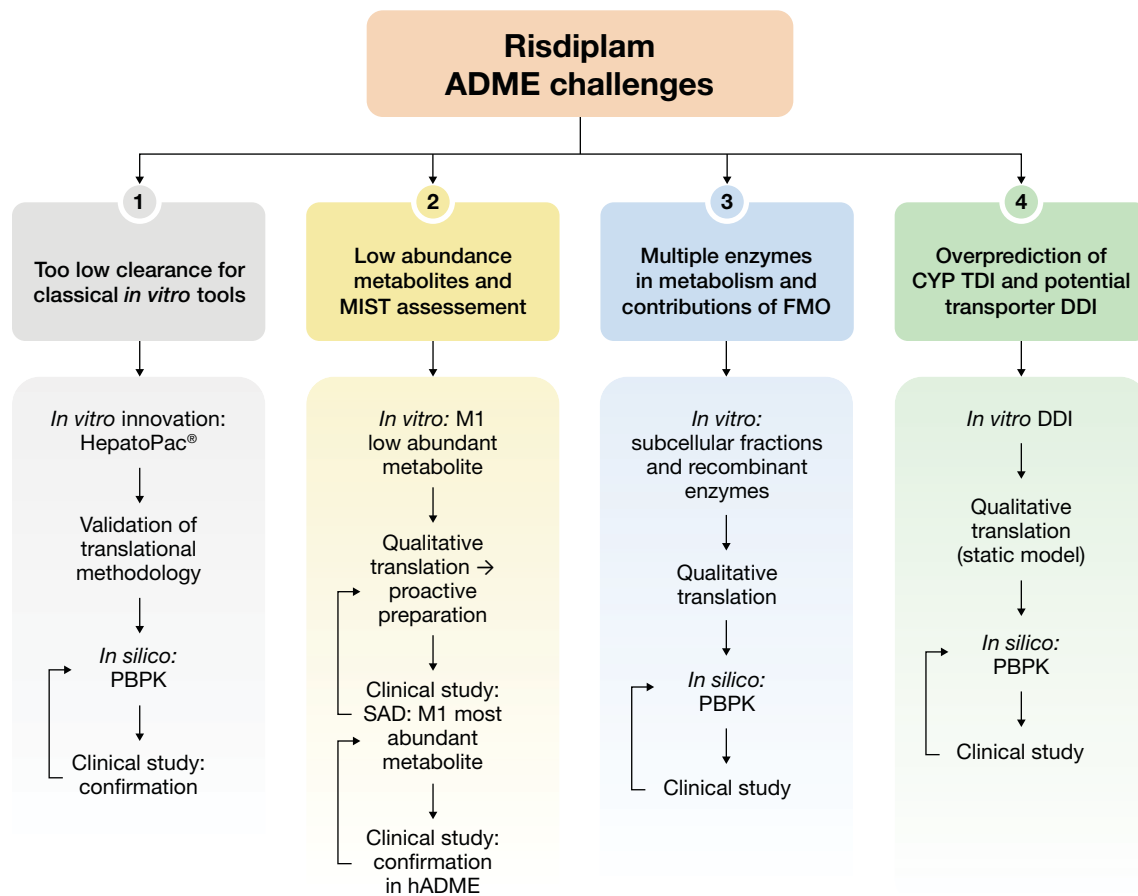


Figure 1

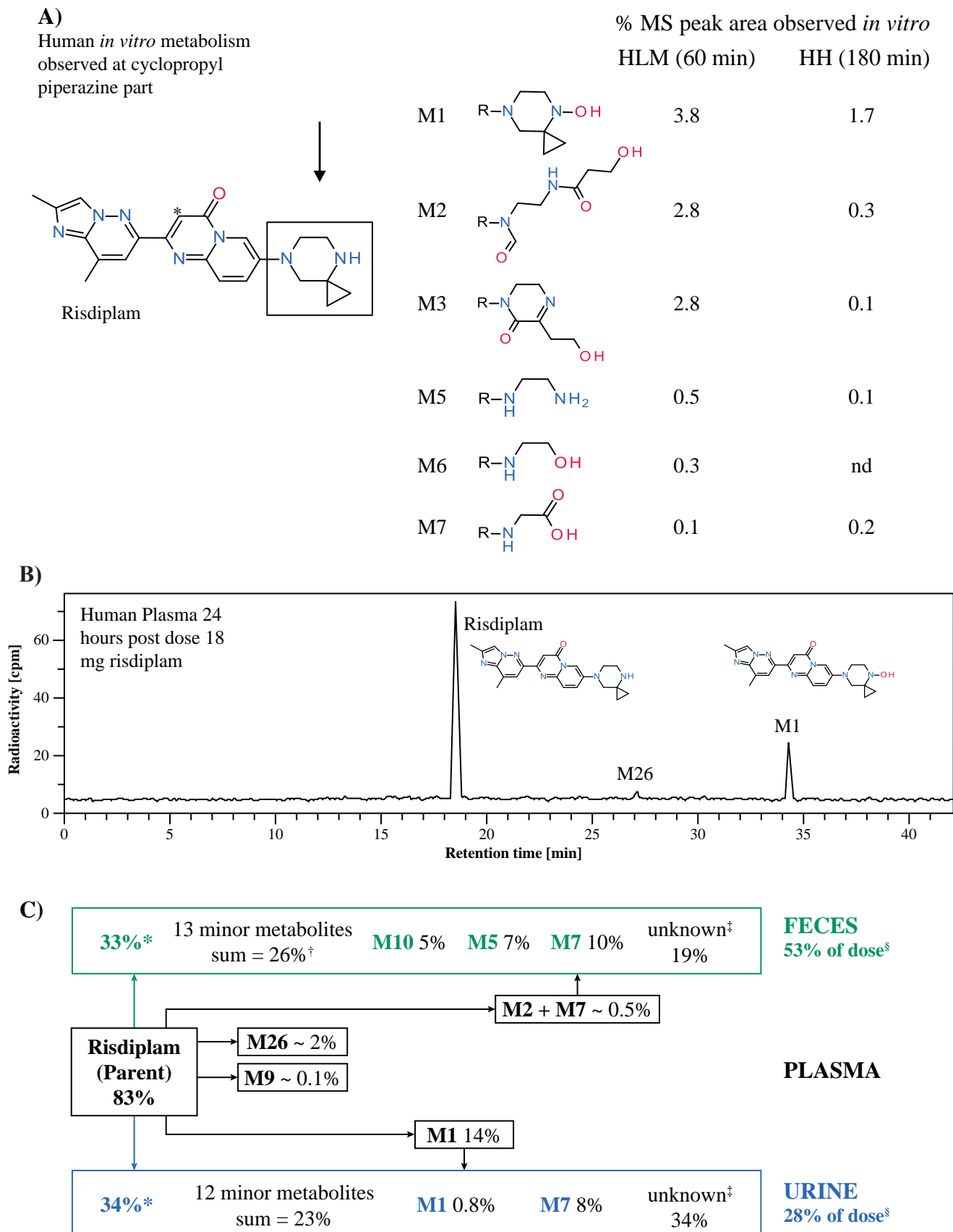
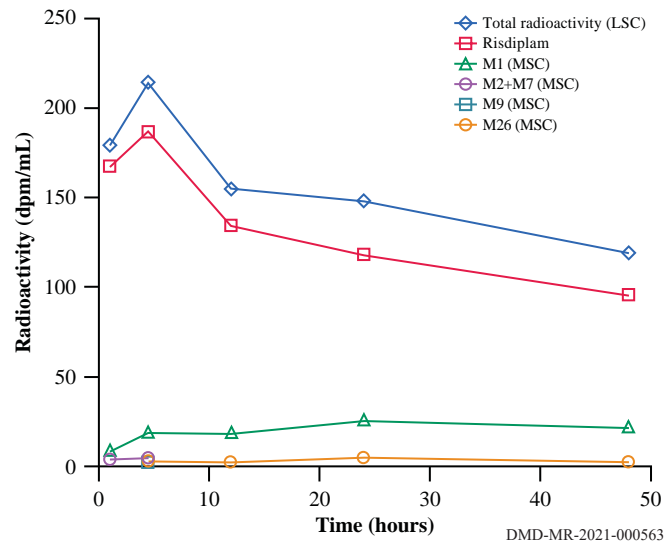
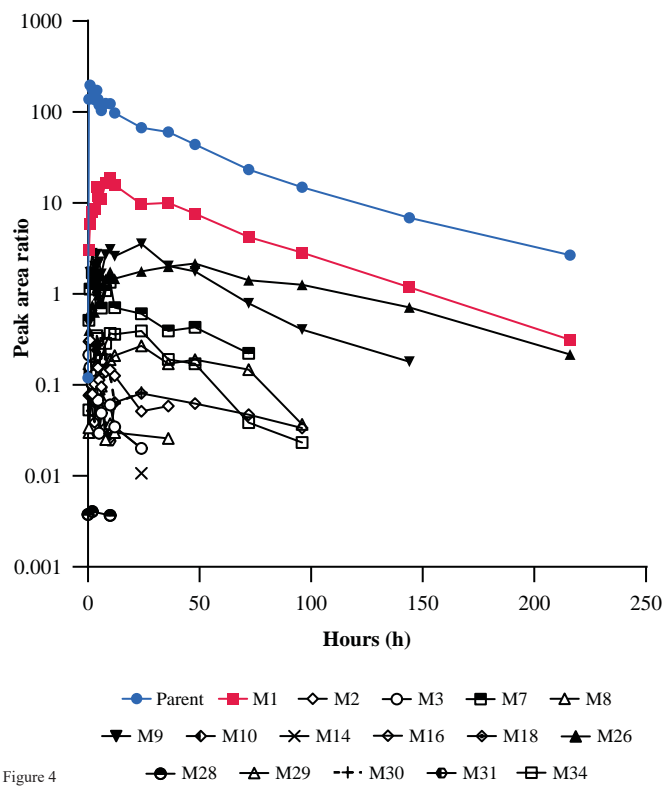


Figure 2





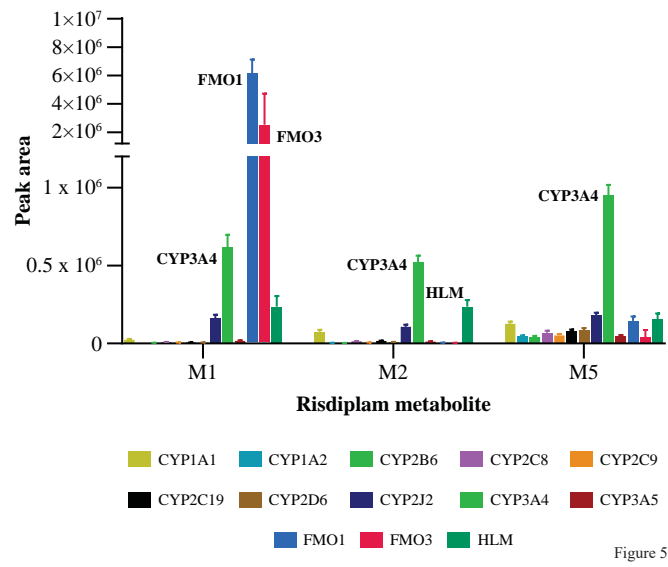


Figure 5

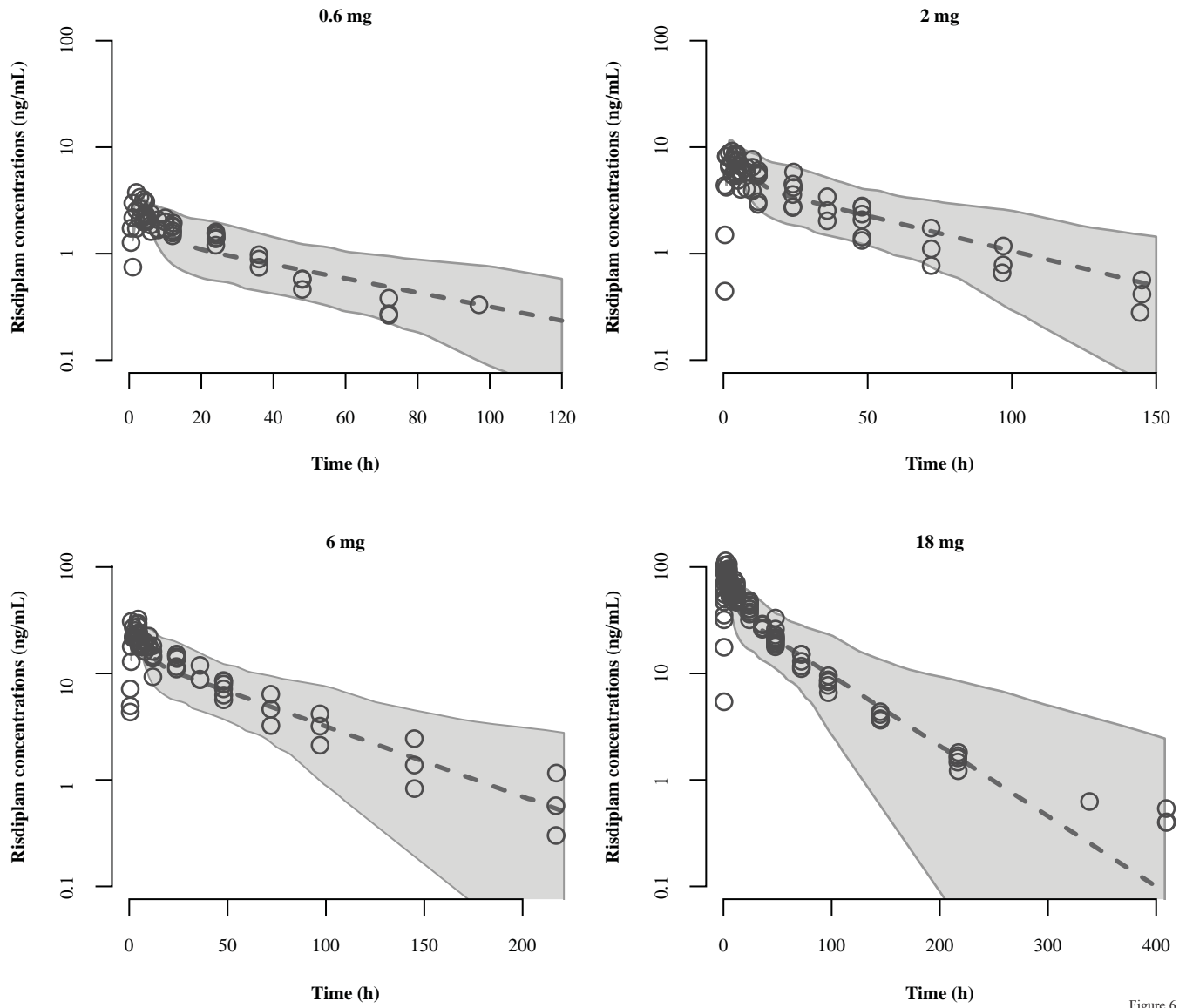


Figure 6