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Structure–metabolism–relationships in the microsomal clearance of piperazin-1-ylpyridazines†

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In this study, we provide insight into the metabolic profile of a series of piperazin-1-ylpyridazines suffering from rapid *in vitro* intrinsic clearance in a metabolic stability assay using liver microsomes (e.g. compound 1 MLM/HLM $t_{1/2}$ = 2/3 min). Aided by empirical metabolite identification and computational predictive models, we designed the structural modifications required to improve *in vitro* intrinsic clearance by more than 50-fold (e.g. compound 29 MLM/HLM $t_{1/2}$ = 113/105 min).

Introduction

The pyridazine core is considered a privileged scaffold in medicinal chemistry,¹ and in particular, piperazin/piperidin-1-ylpyridazine-3-carboxamides have proven worthy of investigation in diverse drug discovery projects (Fig. 1).^{2–7} We have previously disclosed a series of piperazin-1-ylpyridazines as part of our effort to discover potent inhibitors of the human dCTP pyrophosphatase 1 (dCTPase).^{2,8,9} This chemical series can be depicted as a left-hand-side (LHS) region, composed of sections A (aromatic ring) and B (piperazine), and right-hand-side (RHS) region, composed of sections C (pyridazine) and D (aromatic ring) (see prototype compound 1, Fig. 1).

With a view to *in vivo* pharmacological experiments, we characterised the *in vitro* microsomal half-life ($t_{1/2}$) of compound 1, as a predictive parameter of *in vivo* intrinsic clearance.¹⁰ 1 was rapidly metabolised in a metabolic stability assay using mouse and human liver microsomes (MLM/HLM), resulting in extremely short *in vitro* microsomal half-lives ($t_{1/2}$ ~ 3 min, Table 1). This finding was in contrast to Zhang's *et al.* characterisation of the related carboxamide 2 (Fig. 1) as a rather stable compound in rat liver microsomes (RLM), with 97% compound remaining after a 30 min incubation.³ This divergence in microsomal stability could be attributed

to inter-species variability or inherent to the subtle structural differences between 1 and 2.

To better understand the metabolic fate of 1, we performed LC-MS/MS metabolite identification (MetID) (Fig. 2). Identification of metabolites is one of the most time-consuming steps during the drug discovery and development (DDD) process. Recent progresses in biochemistry, molecular biology and especially in analytical methods *i.e.* liquid chromatography (LC) and mass spectrometry (MS) enable such studies to be performed at an early, preclinical stage of the DDD process. The metabolic profile of 1 was similar in MLM and HLM, with a lower metabolic turnover in HLM. The major metabolites of 1 were two mono-hydroxylation products at benzene ring(s) (M1a/b). Oxidation at one of the nitrogen atoms in section B or C was the second most abundant metabolite (M2), followed by the double-oxidation metabolite M3. Traces of hydrolysis were observed too. The difference in microsomal half-lives observed between the screening assay (Table 1) and the Met ID assay (Fig. 2) can be explained by the 10-fold higher compound concentration used in the MetID experiment. This was necessary to improve metabolite detection, which probably saturated the microsomal enzymatic system and resulted in a lower metabolic turn-over.

In order to compare our empirical MetID data with predictive Phase I metabolism models, we performed calculations using Meteor Nexus,¹¹ MetaPrint2D,^{12–14} SMARTCyp¹⁵ and XenoSite P450.¹⁶ Given the similar metabolic profile of compound 1 in MLM and HLM, we also considered predicted human Phase 1 metabolism sites. The model predictions were generally aligned with the MetID findings (Fig. S1 to S4†), with metabolic “hotspots” in ring A (benzylic methyl and/or benzene ring), ring B (carbons adjacent to piperazine N1-position and/or N4-position), RHS benzylic position and/or

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Fig. 1 Examples of pharmacologically active piperazin/piperidin-1-ylpyridazine-3-carboxamides, pharmacological target and originating group/company.

benzene ring D. Meteor Nexus and MetaPrint2D were the only models that provided predictions in rodent microsomes, and in both cases, the benzylic methyl was described as a probable position for oxidation. MetaPrint2D also highlighted benzene ring D and piperazine N4-position as possible sites of metabolism.

With this information in hand, we set out to develop molecules with an improved microsomal stability profile, aiming for MLM/HLM $t_{1/2} > 60$ minutes and low *in vitro* extraction ratios (E) ~ 0.3 , setting the goal for the structure–metabolism-relationships presented in this manuscript (Tables 1 and 2).

Synthesis

Compounds in this chemical series (7–29) can be prepared following different synthetic routes tailored to deliver LHS or RHS structural diversity, as described previously,² and illustrated in Schemes 1–4 (see Experimental procedures for information on the synthetic route used for each final compound). Briefly, when LHS diversity was required, section C (carboxylic acids) and section D (amines) were coupled with propylphosphonic anhydride (T3P). The resulting RHS (31) were coupled with section B (BOC-protected cyclic amines) *via* an aromatic nucleophilic substitution (32) or Suzuki coupling (46), and subsequently de-protected using acidic conditions to deliver synthetic intermediates composed of sections B + C + D. The resulting amine hydrochlorides (33 and 47) were coupled with the required section A (sulfonyl chlorides) to afford the final products. When further functionalisation was required on the LHS pyridine ring, the parent chloro-aryl precursor (28) was subjected to palladium catalysed cross-

couplings (22, Scheme 4), with concomitant formation of the hydrolysis product (27), or to an aromatic nucleophilic substitution (25). For RHS diversity (Scheme 2), section A (sulfonyl chlorides) and section B (amines) were combined *via* sulfonamide coupling, and the resulting LHS (Scheme 2) were BOC de-protected using acidic conditions. The LHS amine hydrochloride (36) was then reacted with section C *via* an aromatic nucleophilic substitution. The resulting carboxylates composed of sections A + B + C were either hydrolysed (38) and coupled with amines using T3P conditions, or directly reacted with amines (41), to provide the final products. Compound 12 was prepared from commercial mesylate 42 *via* nucleophilic substitution with 2,3-dichlorobenzenethiol, to yield thioether 43 (Scheme 3). A subsequent *m*-CPBA-mediated oxidation delivered the required sulfone LHS 44. After BOC de-protection using acidic conditions, the LHS was coupled with the appropriate RHS *via* an aromatic nucleophilic substitution to give 12. The *N*-oxide 24 was prepared from 23 using standard *m*-CPBA-mediated oxidation conditions (Scheme 5).

Results

We started the structure–metabolism-relationships exercise by challenging the metabolic oxidation of ring D “fluorine-blocking” the *para*-position of the benzene ring (7), which led to a 13-fold increase in MLM *in vitro* $t_{1/2}$. Benzene replacement with a mono-substituted thiophene ring (8) yielded an equally unstable compound to 1, but this could be ameliorated by introducing an endocyclic nitrogen atom, as seen in thiazole 9. Next, it was found that removal of the benzylic methyl in section A did not increase microsomal stability (10

Table 1 Structure metabolism relationships. Colour code: green = low (<0.3), yellow = intermediate (0.3–0.7), red = high (0.7–1) extraction ratio¹⁰

	clog P	PSA	Species	In vitro Cl _{int}	Degradation t _{1/2}	E
1	2.1	103.9	M	754	2	0.97
			H	429	3	0.93
7	2.2	103.9	M	53	26	0.70
			H	41	34	0.56
8	2.0	132.1	M	813	2	0.97
			H	362	4	0.92
9	0.9	145.0	M	75	18	0.77
			H	46	30	0.59
10	2.3	103.9	M	845	2	0.97
			H	268	5	0.89
11	3.0	103.9	M	96	14	0.81
			H	87	16	0.73
12	3.4	100.6	M	69	20	0.75
			H	51	27	0.61
13	2.3	103.9	M	91	15	0.80
			H	113	12	0.78
14	2.3	103.9	M	21	68	0.47
			H	113	12	0.78
15	2.7	103.9	M	45	31	0.66
			H	118	12	0.84
16	2.8	103.9	M	118	12	0.84
			H	118	12	0.84
17	2.0	103.9	M	458	3	0.95
			H	622	2	0.95
18	2.4	100.6	M	282	5	0.94
			H	570	2	0.96
19	2.1	103.9	M	840	2	0.98
			H	987	1	0.97

vs. 1), indicating that this position is not the key metabolic liability. As expected, decreasing the electron density of ben-

zene ring A (11) resulted in increased in MLM *in vitro* t_{1/2}, possibly due to reduced aromatic hydroxylation rate.

In accordance with MetaPrint2D, the carbons adjacent to the sulfonamide bond are a likely site of metabolism, and replacement with a sulfone group led to a moderate improvement in microsomal t_{1/2} (12 vs. 11). Next, we simultaneously altered sections A and D, adding additional fluorine atoms at the 4- or 5-positions of benzene ring A while “fluorine-blocking” benzene ring D (13, 14). In the case of 13, microsomal clearance increased (vs. 7), indicating that the metabolic emphasis had shifted to another part of the molecule. On the other hand, 14 displayed an encouraging MLM *in vitro* t_{1/2} > 60 minutes (vs. 7). Unfortunately, this increase in stability did not translate to HLM, and the fluorination approach led to low aqueous solubility (data not shown, visual inspection of compound solutions in assay buffer). Compounds 15 and 16 contained an electron-poor benzene ring A and a “fluorine-blocking” benzene ring D. Despite the combined structural modification, MetID experiments (Fig. S5 and S6†) showed that “fluorine-blocking” decreased% of metabolic oxidation at benzene ring D, but did not for benzene ring A (vs. 1). Interestingly, the metabolic profile shifted towards oxidised metabolites at sections B and C (M1 and M2), possibly suggesting N-oxidation followed by conversion to lactam.¹⁷

It is worth noting that replacement of the pyridazine ring in section C with a pyrazine led to an 8-fold decrease in metabolic stability (17 vs. 1), highlighting the pyridazine's stability. Furthermore, modifications on the piperazine ring, such as removal of the N1-nitrogen (18) or introducing angle strain in the region adjacent to the nitrogen atoms (19), did not ameliorate the short microsomal t_{1/2} observed so far.

In order to lower the log P and restore some of the aqueous solubility lost upon molecular fluorination (13 to 16), we replaced the LHS benzene ring with a pyridine ring. Compound 20 displayed a shorter half-life than its benzene analogue 7, indicating that the pyridine ring could be a new metabolic “hotspot”. Methyl replacement with a cyclopropyl (22) did not improve microsomal clearance, and neither did modulating the pyridine ring electronics with electron withdrawing (21, 23, 28) or donating (25, 26) groups *ortho*- to the pyridine nitrogen

Fig. 2 Observed metabolites of 1 in mouse and human liver microsomes after 60 minutes.

Table 2 Structure metabolism relationships. Colour code: green = low (<0.3), yellow = intermediate (0.3–0.7), red = high (0.7–1) extraction ratio¹⁰

		clog P	PSA	Species	In vitro Cl _{int}	Degradation t _{1/2}	E
20		1.2	116.8	M	183	8	0.89
21		2.1	116.8	M	61	23	0.73
22		2.0	116.8	M	215	6	0.90
23		0.7	140.6	M	267	5	0.92
24		0.8	153.1	M	86	16	0.79
25		0.5	142.8	M	74	19	0.77
26		0.9	129.2	M	698	2	0.97
27		0.8	137.0	M	19	72	0.46
28		1.5	116.8	M	103	13	0.82
29		1.5	116.8	M	12	113	0.35
				H	13	105	0.29

atom, with the exception of the –OH group in 27 (MLM $t_{1/2}$ > 60 min) possibly due to the more favourable 2-pyridone tautomeric form in aqueous environment. Despite the well-known connection between lipophilicity and cytochrome P450 (CYP) metabolism,¹⁸ we found no correlation between clog *P* or polar surface area (PSA) and MLM *in vitro* extraction ratio in this chemical series (Fig. S7†).

To elucidate which nitrogen atom was metabolised, compound 23 was “pre-oxidized” *via* a mild chemical oxidation with *m*-CPBA. Two products with MW = 497 (23 + 16 Da), assumed to be *N*-oxides 24 and 24b (Fig. 3, 24b tentative structure), were isolated in a ratio 15 : 1. ¹H-NMR analysis revealed that the pyridine hydrogen atoms (green boxes) in 24 did not experience a downfield shift in comparison to 23, indicating that the pyridine ring remained unaltered. The pyridazine hydrogen atoms (red boxes) in 24 shifted following a pattern consistent with pyridazine *N*-oxidation. Additionally, a remarkable downfield shift of the amide proton (orange boxes) was observed, consistent with hydrogen bonding. Based on these facts, it was assumed that the pyridazine N1 nitrogen was oxidised, and engaged with the neighbouring amide proton in an intramolecular hydrogen bond to generate a 6-membered ring (Fig. 3). The aliphatic protons of the piperazine ring (purple and turquoise boxes) of compound 24 remained essentially unaffected in comparison to 23, whereas

the aliphatic protons in compound 24b shifted, suggesting an alteration of the piperazine ring. Unfortunately, 24b was not isolated in sufficient amount and purity for unequivocal characterization and testing. MetID of 24 (Fig. S8†) revealed that the piperazine ring was still the major metabolic site in this chemical scaffold (23 vs. 24).

To our satisfaction, when three key structural corrections were applied, a desirable microsomal profile was achieved. In compound 29, we “fluorine-blocked” the *para*-position of ring D, introduced an electron-poor pyridine in region A, and replaced the piperazine ring C with the strained diazasp[3.3]heptane system, to obtain MLM/HLM half-lives >100 minutes. Compound 29 showed fewer and different metabolic sites, compared with 1, in Meteor Nexus and MetaPrint2D. In the case of SMARTCyp, a similar metabolic profile to 1 was observed, and for XenoSite P450, more sites of metabolism with lower predictive score were postulated. It is worth noting that the computational models used aim to predict which atoms are likely to be metabolised, but do not rank compounds according to metabolic stability.

Based on the improvements achieved during this structure–metabolism–relationships exercise, Meteor Nexus, MetaPrint2D and XenoSite P450 provided the most relevant predictions to address Phase I metabolism. Despite the fact that three out of four models indicated RHS benzylic oxidation as a major metabolic reaction, we did not observe such process under our assay conditions, and dramatic improvements could be achieved without altering the benzylic position. For this series of compounds, it was found that amending only one or two metabolic sites was generally not enough to have a positive impact on the *in vitro* clearance half-life.

Conclusion

Here, we have demonstrated that through systematic exploration of structure–metabolism–relationships guided by MetID experiments, it was possible to improve *in vitro* microsomal half-lives of a series of piperazin-1-ylpyridazines by >50 fold, after only a few rounds of iterative design. Among the predictive Phase I metabolism models employed here, Meteor Nexus, MetaPrint2D and XenoSite P450 provided the most relevant calculations. Microsomal clearance in the two species was generally aligned for the compounds tested in both species, with the exception of compound 14. The structural guidelines provided in this study may be applicable to related chemotypes, and help in the design of metabolically stable drug candidates.

Experimental

In vitro microsomal stability assay

Performed according to literature procedure.¹⁹

Metabolite identification procedure

10 μM of test compound (from 10 mM DMSO stock) was incubated with 1 mg ml^{−1} human or mouse liver

Scheme 1 Synthetic route A. Synthesis of compounds 22, 25, 27, and 28. Reagents and conditions: (a) 4-fluorobenzylamine (1.1 eq.), T3P (2.2 eq.), Et₃N (2.5 eq.), CH₂Cl₂, 0 °C to r.t., 18 h, 55%. (b) 1-Boc-piperazine (1 eq.), Et₃N (2 eq.), 1,4-dioxane, 100 °C, 18 h, quant. (c) 4 M HCl in 1,4-dioxane, r.t., 18 h, quant. (d) 2-Chloropyridine-3-sulfonyl chloride (1.1 eq.), Et₃N (2.2 eq.), r.t., 18 h, 29%. (e) Cyclopropylboronic acid (3 eq.), Pd(OAc)₂ (0.2 eq.), P(Cy)₃ (0.4 eq.), K₃PO₄ (3.5 eq.), toluene/water, 100 °C, 18 h, (22) 18% (27) 16%. (f) 35% NH₄OH in MeOH, 120 °C, 18 h, 73%.

Scheme 2 Synthetic route B. Synthesis of compounds 8 and 17. Reagents and conditions: (a) 2-methylbenzene-1-sulfonyl chloride (1.1 eq.), Et₃N (2.2 eq.), r.t., 18 h, 91%. (b) 4 M HCl in 1,4-dioxane, r.t., 18 h, 59%. (c) 36 (1 eq.), Et₃N (2 eq.), 1,4-dioxane, 100 °C, 18 h, 40–51%. (d) 0.5 M aq. NaOH/THF (1:3), 60 °C, 1 h, 67%. (e) 2-Thiophenemethylamine (1.1 eq.), T3P (2.2 eq.), Et₃N (2.5 eq.), CH₂Cl₂, 0 °C to r.t., 18 h, 50%. (f) 4-Fluorobenzylamine (4 eq.), EtOH, 120 °C, 72 h, 46%.

microsomes (XenoTech LLC, KS, USA) in 0.1 M phosphate buffer pH 7.4, 1 mM NADPH for different time point, in most cases 30 and 60 min, at 37 °C. The reaction was stopped with 1 volume (0.5 ml) of ice-cold acetonitrile. Control sample was treated with acetonitrile prior to the addition of NADPH. The sample was centrifuged and

supernatant transferred to a new vial, and evaporated using a SpeedVac centrifuge. The sample was then reconstituted in mobile phase A (0.1% formic acid, 5% acetonitrile) and analysed by fullscan MS and MS/MS. Analysis was performed using a Waters XEVO TQ coupled to an Acquity UPLC system (Waters Corp, MA, USA).

Scheme 3 Synthetic route C. Synthesis of compound **12**. Reagents and conditions: (a) 2,3-dichlorobenzenethiol (1.1 eq.), K_2CO_3 (1.1 eq.), DMF, 70 °C, 18 h, 90%. (b) *m*-CPBA (3 eq.), CH_2Cl_2 , r.t., 2 h, 88% (c) TFA in DCM, r.t., 18 h, quant. (d) *N*-Benzyl-6-chloropyridazine-3-carboxamide (1 eq.), Et_3N (2 eq.), *n*-BuOH, 130 °C, 18 h, 63%.

Scheme 4 Synthetic route D. Synthesis of compound **18**. Reagents and conditions: (a) *tert*-butyl 4-(tetramethyl-1,3,2-dioxaborolan-2-yl)-1,2,3,6-tetrahydropyridine-1-carboxylate (1.1 eq.), $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ (0.02 eq.), K_2CO_3 (3 eq.), 1,4-dioxane/water (3:1), 60 °C, 18 h, 78%. (b) 4 M HCl in 1,4-dioxane, r.t., 18 h, 90%. (c) 2-Methylbenzene-1-sulfonyl chloride (1.1 eq.), Et_3N (2.2 eq.), r.t., 18 h, 86%.

Scheme 5 Synthesis of compound **24**. Reagents and conditions: (a) *m*-CPBA (1.2 eq.), CH_2Cl_2 , r.t., 18 h, 75%.

Column BEH C18 1.7 μm 2 $\times$ 50 mm and mobile phases A: 0.1% formic acid, 5% acetonitrile/B: 0.1% formic acid, 100% acetonitrile were used.

General information for synthetic procedures

All commercial reagents and solvents were used without further purification. Analytical thin-layer chromatography was performed on silica gel 60 F-254 plates (Merck) and visualized under a UV lamp. Flash column chromatography (FCC) was performed in a Biotage® SP4 MPLC system using Merck silica gel 60 Å (40–63 mm mesh). 1H NMR spectra were recorded on a Bruker DRX-400. Chemical shifts are

expressed in parts per million (ppm) and referenced to the residual solvent peak. Analytical HPLC-MS was performed on an Agilent MSD mass spectrometer connected to an Agilent 1100 system with: method acidic pH: column ACE 3 C8 (50 $\times$ 3.0 mm); H_2O (+0.1% TFA) and MeCN were used as mobile phases at a flow rate of 1 mL min $^{-1}$, with a gradient time of 3.0 min; or method basic pH: column X-Terra MSC18 (50 $\times$ 3.0 mm); H_2O (containing 10 mM NH_4HCO_3 ; pH = 10) and MeCN were used as mobile phases at a flow rate of 1 mL min $^{-1}$, with a gradient time of 3.0 min. Preparative HPLC was performed on a Gilson HPLC system; basic pH: column Xbridge Prep C18, 5 μm CBD (30 $\times$ 75 mm); H_2O (containing 50 mM NH_4HCO_3 ; pH = 10) and MeCN

Fig. 3 Chemical structures and ^1H -NMR spectra for compounds 23, 24 and 24b.

were used as mobile phases at a flow rate of 45 mL min^{-1} , with a gradient time of 9 min. Acidic pH: column ACE 5 C8 ($150 \times 30\text{ mm}$); H_2O (containing 0.1% TFA) and MeCN were used as mobile phases at a flow rate of 45 mL min^{-1} , with a gradient time of 9 min. For HPLC-MS detection was made by UV using the 180–305 nm range and MS (ESI+). For preparative HPLC, detection was made by UV at 254 or 214 nm. All final compounds were assessed to be $>95\%$ pure by HPLC-MS analysis. All final compounds were prepared following literature procedures.² Details of the compound characterisation can be found in the ESI.†

Conflict of interest

The authors declare no competing interests.

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