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Metabolism Study and Biological Evaluation of Bosentan Derivatives

Susan Lepri,[‡] Laura Goracci,[‡] Aurora Valeri and Gabriele Cruciani.^{*}

Department of Chemistry, Biology and Biotechnology, University of Perugia, Via Elce di Sotto 8, 06123 Perugia, Italy.

*Corresponding author. Gabriele Cruciani: phone, +39 075 585 5629; Fax, +39 075 45646; E-mail, gabriele.cruciani@unipg.it.

[‡] These authors contributed equally to this work.

Abstract

Bosentan, the first-in-class drug used in treatment of pulmonary arterial hypertension, is principally metabolized by the cytochromes P450, and it is responsible for cytochromes induction and drugdrug interaction events with moderate to severe consequences. A strategy to reduce drug-drug interactions consists of increasing the metabolic stability of the perpetrator, and fluorinated analogues are often designed to block the major sites of metabolism. In this paper bosentan analogues were synthesized, and their metabolism and biological activity were evaluated. All synthesized compounds showed an improved metabolic stability towards CYP2C9, with one maintaining a moderate antagonist effect towards the ET_A receptor.

Keywords: bosentan, perfluorinated analogues, metabolic specificity, phase I metabolism, HLM.

List of abbreviations

Cl Clearance, CYP Cytochrome P450, DDI drug-drug interactions, ERA Endothelin Receptor Antagonist, ET-1 Endothelin 1, ET_A Endothelin receptor A, ET_B Endothelin receptor B, PAH pulmonary arterial hypertension.

1. Introduction

The molecular mechanism of pulmonary arterial hypertension (PAH) is still uncertain, but it is likely that the excessive vasoconstriction of the pulmonary arterioles related to endothelial dysfunctions results in an imbalance of various endothelial vasoactive mediators (such as endothelin-1, or ET-1), affecting the growth of the smooth muscle cells, and may facilitate the structural remodeling characteristic of PAH [1]. Since this condition arises from a complex interplay of molecular and genetic abnormalities, in clinical practice PAH can be controlled by coadministration of multiple drugs [2]. Combination of several treatments on the one hand might increase their clinical effectiveness but, on the other hand, it is also likely to increase the risk of adverse drug-drug interactions (DDIs) with a reduced treatment efficacy or increased side effects [3]. As mentioned above, with high levels of ET-1 detectable in lung tissues of PAH patients, the administration of endothelin receptor antagonists (ERAs) helps to reduce symptoms of disease [4]. The first-in-class ERA used in PAH treatment is bosentan (1), a sulfonamide-based drug, antagonist for both endothelin receptor A (ET_A) and B (ET_B) [5, 6]. Bosentan is a well-known strong perpetrator in pharmacokinetic drug-drug interaction, as it is an inducer of drug transporters and metabolizing enzymes, and an inhibitor of P-glycoprotein and organic anionic transporting polypeptides [7-9]. Moreover, a possible perpetrator effect of bosentan metabolites has been recently studied [7].

Thus, to decrease the risk of adverse events associated with DDI during bosentan administration, a thorough knowledge of the pharmacokinetic profile of this drug is mandatory. Unfortunately, despite its large and valuable therapeutic use, the bosentan metabolism profile is not optimal. Bosentan is commonly reported to be metabolized in the liver by the cytochrome P450 enzymes CYP2C9 and CYP3A4 producing three metabolites (Figure 1): the aliphatic hydroxylation at the tert-butyl group produces Ro 48-5033 (2), a pharmacologically active metabolite that may contribute to 10 - 20 % of the total activity of the parent compound; the oxidative demethylation of guaiacol moiety by CYP3A4 forms phenol derivative Ro 47-8634 (3), whereas metabolite Ro 64-1056 (4) is a minor product formed from both primary metabolites [10]. Recently, a new metabolite [11] was identified in human liver microsomes (HLM) as shown in Figure 1. In bosentan metabolism the involvement of CYP2C9 may represent an issue when the metabolic pathway of a drug is investigated. Indeed, CYP2C9 is a polymorphically expressed enzyme showing high human variability in the frequency of variant CYP2C9 allele (about 60 allelic variants have been reported) [12]. Recently, several studies have been conducted on allelic variants of CYP2C9 [13-16], demonstrating a great interethnic and intra-ethnic variability; most recently, due to this CYP2C9 polymorphism, Chen et al. [17] have highlighted the importance of directing more attention when administering bosentan to a subject carrying some infrequent CYP2C9 alleles. The genetic polymorphism of CYP2C9 has been also associated to bosentan-induced liver injury [18] although this point is still under debate [11].

In addition, clinical trials have also demonstrated that bosentan is an inducer of isoenzymes CYP2C9 and CYP3A4, making DDI studies even more complex when bosentan is co-administrated with drugs that are metabolized by these CYP isoforms (e.g. warfarin [19], cyclosporine [20], oral estrogens [21], simvastatin [22], and sildenafil [23]). Although the mechanism of isoenzyme induction by bosentan is still unclear, the optimization of the metabolic stability of bosentan towards CYP3A4 and mainly towards CYP2C9 could represent a valid strategy to reduce DDI. In

this study four bosentan derivatives were designed and synthesized to evaluate the impact of slight changes on the bosentan scaffold in phase I metabolism in human liver microsomes (HLM), and in CYP3A4 and CYP2C9 isoforms. Metabolism by CYP2D6 was also investigated to explore a possible role of this enzyme in the metabolism of the synthesized compounds. For the most interesting compounds the possible agonist and antagonist activities against endothelin receptors was studied.

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Figure 1. Proposed metabolic pathway of bosentan in human. The site of metabolism is highlighted by blue arrows [10]. The major metabolic pathway, indicated by a thicker arrow, leads to the

formation of metabolite **2**. The formation of a recently identified metabolite is indicated by a dashed arrow [11].

2. Results and Discussion

2.1 Design

An enhancement of the metabolic stability can be achieved by modifying the chemical structure of the parent compound. For example, a common strategy requires blocking the site of detrimental metabolism with the introduction of fluorinated groups. The benefits of fluorine addition are numerous: the size of fluorine is similar to that of hydrogen (fluorine radius is just 20% larger and H₃C-F bond is 27% longer than H₃C-H bond [24]) and indeed it has been shown enzymes often do not distinguish between a natural substrate and its fluorinated analogue [25]. Moreover, since H_3C -F bond is stronger than that of H₃C-H by 5.0 Kcal/mol [24], the metabolic oxidation by CYP450 is reduced. This blocking effect has been widely used to prevent deactivation of active molecules due to their metabolic instability. For instance, fluorinated analogues of buspirone [26] and vitamin D₃ [27] have shown improved metabolism respect to their hydrogenated compound; moreover, incorporation of fluorine is reported to enhance the drug potency (e.g. the ezetimibe, fluorinated antitumor benzothiazoles 5F 203 and GW 610, fluroquinolones, etc. [28]). In this study, since the tert-butyl group of bosentan is the main site of metabolism, this group was replaced with fluorinated substituents. Despite their different volumes (see Table S1), a large number of fluorinated isosteres of the *tert*-butyl group have been used in drug discovery, ranging from trifluoromethyl (CF₃) [29], pentafluorosulfanyl (SF₅) [30, 31], bicyclo[1.1.1]pentanyl (BCP) [32, 33], and cyclopropyltrifluoromethyl (cyclopropyl-CF₃) [34]. Thus, rigid rules for the replacement of the *tert*-butyl group in metabolic stability studies cannot be applied. In the present study, we investigated the effect of hexafluoro-2-hydroxyprop-2-yl, hexafluoro-2-methoxyprop-2-yl, and heptafluoro isopropyl, to give

compounds **5a-c**, respectively (Figure 2, block I). In particular, in compound **5a** a further acidic function adjacent to the two trifluoromethyl functionality, with a pK_a value of 8.60, was introduced.



Figure 2. Proposed modification of bosentan moieties to evaluate the effect on metabolism and the biological response.

The fourth analogue (**5d**, Figure 2 block II) was designed to test if the metabolism of bosentan by CYP2C9 could be reduced by a slight change in scaffold decoration, without modifying the structure at the site of metabolism (i.e. the *tert*-butyl group, as shown in Figure 1).

The MetaSiteTM software [35, 36] was used to obtain information about which interactions play a role in the exposition of bosentan towards the heme in the CYP2C9 cavity. Indeed, MetaSiteTM allows selection of the desired site of metabolism (SoM) of bosentan and to orient the molecule in the CYP2C9 cavity, computing the bosentan-CYP hydrophobic complementarity and the complementarity of charges and H-bonds between the ligand and the enzyme. Such complementarities are then used to assign a contribution score (for the selected SoM) to the different atoms in the substrate. This innovative strategy to design derivatives with tailored

metabolism towards a specific enzyme recently proved to be effective to modulate the metabolism of bepridil by FMO3 [37].

In the case of bosentan, the pose of this compound within the CYP2C9 cavity was modeled by fixing the *tert*-butyl group orientation towards the heme. The contribution scores reported in Figure 3 highlight that the molecular groups influencing the bosentan aliphatic hydroxylation at the *tert*-butyl group are the nitrogen of the sulfonamide function (interacting with Arg 108) and the pyrimidinoxyethanol group, which interacts with the carbonyl group of Val 292. Based on these considerations, the 2-hydroxyethoxy moiety at the 6 position of the substituted pyrimidine ring was replaced by an ethoxy group, attempting to disfavor the exposition of the *tert*-butyl group towards the heme, and to consequently increase metabolic stability.



Figure 3. Contribution scores (circles) generated by MetaSiteTM when the *tert*-butyl group of bosentan is exposed to the heme in CYP2C9 cavity. The two CYP2C9 amino acids Val 292 and Arg 108 are predicted to be responsible for the favourable exposition of the *tert*-butyl group towards the heme, interacting with the 2-hydroxyethoxy moiety and the sulfonamide moiety, respectively.

2.2 Synthesis

Schemes 1–3 illustrate the synthesis of compounds described herein. To obtain the common intermediate dichloropyrimidine **9** we followed the known literature protocols [38, 39]. Briefly, condensation of aryloxymalonate **6** with the pyrimidylamidine **7** afforded pyrimidinepyrimidone **8**, which was converted into the chlorinated intermediate **9** upon treatment with PCl_5 in refluxing acetonitrile. In particular, the aryloxymalonate **6** and pyrimidylamidine **7** were prepared by *O*-alkylation of guaiacol and amination of 2-cyanopyrimidine, respectively (Scheme 1).





^a Reagents and conditions: (a) Dimethyl 2-chloromalonate, MeONa, MeOH, 45 °C, 1 h [38]; (b) *i*. MeONa, MeOH, rt, 24 h; *ii*. AcOH, NH₄Cl, rt, 24 h [39]; (c) MeONa, MeOH, rt, 10 h [38]; (d) PCl₅, *N*,*N*-diisopropylethylamine, CH₃CN, reflux, 20 h [38].

As shown in Scheme 2, the nucleophilic attack of *N*-lithiated sulfonamides **10a-b** to dichloropyrimidine **9** afforded the monochlorosulfonamides **11a-d** (respectively), afterwards converted into the target bipyrimidines **5a-d** by nucleophilic substitution of the second chlorine atom by sodium glycolate (for **5a-c**) or sodium ethoxide (for **5d**).



^aReagents and conditions: (a) *i. n*-BuLi, THF, -78 °C, 15 min ; *ii.* DMF, 50 °C, 16 h; (b) Na, ethylene glycol 110 °C (for **5a-c**) or EtOH 75 °C (for **5d**), 5 h [38].

The synthesis of sulfonamides **10a-c** is worth describing briefly (Scheme 3), whether sulfonamide **10d** is commercial available.

The diazotization of commercial 2-(4-aminophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol with sodium nitrite in a hydrogen chloride-acetic acid mixture, followed by reaction with CuCl-SO₂ complex in acetic acid solution, gave the arylsulfonyl chloride intermediate **12a** which was afterwards transformed in the desired sulfonamide **10a** by simple addition of aqueous ammonium hydroxide to the reaction mixture (Scheme 3). The sulfonamide **10c** was prepared analogously from 4- (perfluoroisopropyl)aniline **15** which, in turn, was obtained by alkylation of aniline with 2-iodoperfluoropropane [40]. On the contrary, a different strategy was adopted to obtain the methoxylated sulfonamide **10b**. Butyllithium-promoted bromine/lithium exchange followed by the addition of gaseous hexafluoroacetone, transformed 1,4-dibromobenzene into fluorinated bromoarene **13**. The latter was methylated by methyl iodide upon treatment with sodium hydride in DMF to give the methoxylated bromoarene **14**. Bromoarene **14** was smoothly transformed into the desired sulfonamide **10b** in 4 steps in 20–25% overall yield. In particular, lithiation of bromo compound **14** by butyllithium, followed by addition of gaseous sulfur at -60 °C, gave the lithium

sulfinate intermediate [41]; the latter was converted into the desired sulfonamide **10b** upon treatment with *N*-chlorosuccinimide and, eventually, with aqueous ammonia in acetone at 50 $^{\circ}$ C [41].

Scheme 3^a



^a Reagents and conditions: (a) *i*. NaNO₂, HCl, AcOH, -10 °C, 45 min; *ii*. SO₂, CuCl, 0 °C, 10 min [42]; (b) NH₄OH aq., THF, 0 °C, 15 min; (c) *i*. *n*-BuLi, THF, -95 °C, 15 min; *ii*. Hexafluoroacetone sesquidrate H₂O, -95 °C, 10 min; (d) *i*. NaH, DMF, 0 °C, 40 min; *ii*. CH₃I, 40 min, rt; (e) *i*. *n*-BuLi, THF, -78 °C, 15 min; *ii*. SO₂, -60 °C, 5 min; *iii*. NCS, CH₂Cl₂, 0 °C for 15 min, then 25 min rt; *iv*. NH₄OH aq, acetone, 40 °C, 12 h [41]; f) 2-iodoperfluoropropane, Na₂(S₂O₄), NaHCO₃, N(Bu)₄HSO₄, MTBE, water, rt, 8 h [40].

2.3 Metabolism profile

Metabolism of bosentan (1) and its analogs **5a-d** was evaluated upon incubation with HLM and recombinant isoforms (3A4, 2D6, 2C9) at three incubation times (0, 15, 30 minutes; see the

Experimental Section). Although bosentan is a not substrate for CYP2D6, this isoform was also tested to evaluate if this enzyme could be involved in the metabolism of the synthesized derivatives. Samples were analyzed by LC-MS/MS and the raw data were processed with the Mass-MetaSiteTM software (version 3.0.2, Molecular Discovery Ltd., UK [43-45]) to assist in the metabolism elucidation process. Table 1 shows the percentage of unchanged substrate in all matrices and the nature of metabolites found, while a more detailed characterization of the detected metabolites based on the LC-MS/MS parameters is provided in Table 2. In addition, the main MS/MS fragment assignments used for structure elucidation are reported in Supplementary Data (Table S1). MS/MS spectra of 1 and its metabolites were congruent with literature data [46]. Bosentan (1) and compounds 5a and 5c were minimally metabolized by HLM. For compound 5a, the protection of the tert-butyl group induces the formation of the O-demethylated metabolite only, although this does not result in an improved metabolic stability. Interestingly, for compound 5c, the most stable compound, the formation of the corresponding 5c-OH (aromatic)-O-demethyl(guaiacol) metabolite (Table 1) was observed, with an accurate mass of 666.0888 (M+H⁺). The formation of this metabolite was confirmed by the presence of MS/MS fragments 622.0637, 313.0806, and 189.0651 (Tables 2 and S1).

On the contrary, compounds **5b** and **5d** were less stable than bosentan in HLM (with 73.9 and 68.5% of parent compound remaining after 30 minutes of incubation, respectively). It is noteworthy that for compound **5b**, which differs from the analogue **5a** by the additional methylation of hexafluoro-2-methoxyprop-2-yl moiety, a second *O*-demethylation process was also observed to give **5b**-*O*-demethyl(arylsulfonamide). In particular, **5b**-*O*-demethyl metabolites exhibiting a retention time (RT) of 4.72 min and 5.14 min have been attributed to hexafluoro-2-methoxyprop-2-yl arylsulfomamide and guaiacol *O*-demethylation, respectively (see Table S1 for main fragment assignment). Compound **5d**, it resulted the least stable in HLM, possessing a similar metabolism of

bosentan in this matrix, although the corresponding **5d**-OH(*t*-Bu)-*O*-demethyl(guaiacol) was not observed (Table 1).

Metabolism studies in specific CYP isoforms revealed that none of the investigated compounds were substrates of CYP2D6, with the level of unchanged substrates being greater than 99%.

Concerning bosentan (1), the obtained results for CYP3A4 and CYP2C9 are congruent with literature data [10]. In particular, three metabolites have been detected with CYP3A4: 1-OH(*t*-Bu) (Table 1), referring to the aliphatic oxidation on *tert*-butyl moiety as for incubation in CYP2C9; 1-*O*-demethyl(guaiacol), generated by the *O*-demethylation reaction, which matches the literature metabolite **3**; 1-OH(*t*-Bu)-*O*-demethyl(guaiacol), which can be associated to **4**, being generated by the combination of *O*-demethylation and aliphatic oxidation. Only one metabolite, **1**-OH (*t*-Bu), could be detected with CYP2C9, with a m/z of 568.1861 (M+H⁺), attributable to an aliphatic oxidation on *tert*-butyl moiety, according to the literature metabolite **2**. As previously mentioned bosentan (**1**) was also confirmed as a non-substrate for CYP2D6.

Concerning the fluorinated analogues **5a-c**, all of them were found to be substrates for CYP3A4, with the nature of the formed metabolites being identical to that in HLM. Indeed, for all the fluorinated analogues the *O*-dealkylation reaction occurred at the methoxy guaiacol group to give **5a-c**-*O*-demethyl(guaiacol). In addition, for compound **5b**, which differs from the analogue **5a** by the additional methylation of the hexafluoro-2-hydroxyprop-2-yl moiety, a second *O*-demethylation process was also observed to give **5b**-*O*-demethyl(arylsulfonamide), as for the HLM data. This suggests that, despite the introduction of fluorinated moieties to replace the *tert*-butyl group of bosentan, that region of the molecule is still favorable to be exposed to the heme in CYP3A4, and thus our modifications to give **5a-c** only affect the reactivity factor. Also in CYP3A4, only compound **5c** underwent the aromatic oxidation in combination with *O*-demethylation to produce the corresponding **5c**-OH(aromatic)-*O*-demethyl (guaiacol) metabolite.

In our study, the stabilization of the synthesized compounds towards CYP2C9 metabolism was the primarily goal. A significant impact of the fluorination strategy was observed analyzing the metabolism by CYP2C9. Indeed, since bosentan is metabolized by this isoform only at the *tert*-butyl group through an aliphatic hydroxylation, this reaction was impeded for compounds **5a-c** and these compounds were totally stable after 30 min incubation in the presence of the CYP2C9 isoform. Differently from what was observed in CYP3A4, the *O*-demethylation of the hexafluoro-2-methoxypropan-2-yl moiety was not observed in CYP2C9, suggesting that for this enzyme this reaction is less favorable.

Concerning compound **5d**, which was designed to reduce metabolism by CYP2C9 without altering the *tert*-butyl moiety, it resulted to be practically stable after incubation with CYP2C9 (with 99% of substrate remaining and only traces of the hydroxylated metabolite formed), proving that the replacement of the 2-hydroxyethoxyl moiety with an ethoxy group disfavors the exposition of the *tert*-butyl group towards the CYP2C9 heme, as suggested by MetaSiteTM (Figure 3).

For completeness, the binding modes for the synthesized analogues **5a-d** in CYP3A4 and CYP2C9 are available as Supplementary Material (see Figure S1). In particular, the *in silico* predictions in CYP3A4 are in agreement with the experimental results showing that the guaiacol group is the most favourable SoM. The similar analysis for CYP2C9 cannot be applied because MetaSiteTM assumes that the docked compounds are substrates, while compounds **5a-c** are metabolically stable.

Table 1. Bosentan	and its analogue	metabolites analysis i	in HLM, CYP3A4	, and CYP2C9.
	0		,	/

Comp.	Structure	HLM			CYP3A4		CYP2C9	
		% sub. after 30'	Metabolites analysis	% sub. after 30'	Metabolites analysis	% sub. after 30'	Metabolites analysis	
1		91.6	1-OH (t-Bu) 1-O-demethyl (guaiacol) 1-OH (t-Bu)-O- demethyl(guaiacol)	20.9	1-OH (t-Bu) 1-O-demethyl (guaiacol) 1-OH (t-Bu)-O- demethyl(guaiacol)	75.8	1-OH (<i>t</i> - Bu)	

5a		90.1	5a-O-demethyl (guaiacol)	72.8	5a-O-demethyl (guaiacol)	100	ND
5b	Factor NH Coche Hace Grand NH Coche	73.9	5b - <i>O</i> -demethyl (arylsulfonamide) 5b - <i>O</i> -demethyl (guaiacol)	19.2	5b - <i>O</i> -demethyl (arylsulfonamide) 5b - <i>O</i> -demethyl (guaiacol)	100	ND
5c		99.0	5c - <i>O</i> -demethyl (guaiacol) 5c -OH (aromatic)- <i>O</i> - demethyl (guaiacol)	16.3	5c-O-demethyl (guaiacol) 5c-OH (aromatic)- O-demethyl (guaiacol)	100	ND
5d		68.5	5d -OH (<i>t</i> -Bu) 5d -O-demethyl (guaiacol)	15.0	5d-OH (t-Bu) 5d-O-demethyl (guaiacol)	99.0	5d -OH (<i>t</i> -Bu)

Table 2. Compound 1, and **5a-d** and their main metabolites identified by LC-MS/MS and their revealed characteristic fragments.

Comp.	Structure assigned	Exact $[M+H]^+$	Elemental composition	Accurate $[M+H]^+$	Δppm	Characteristic fragments	RT (min)
1 (bosentan)	-	552.1911	C ₂₇ H ₂₉ N ₅ O ₆ S	552.1912	0.06	508.1649, 311.1013 280.0829, 202.0723	5.10
1-OH (<i>t</i> - Bu)	HO N N N N N N N N N N N N N N N N N N N	568.1860	$C_{27}H_{29}N_5O_7S$	568.1861	-0.03	524.1591, 311.1022, 280.0818, 202.0722	3.79
1 - <i>O</i> - demethyl (guaiacol)		538.1755	C ₂₆ H ₂₇ N ₅ O ₆ S	538.1753	0.37	494.1493, 189.0643	4.73
1-OH (<i>t</i> -Bu)- <i>O</i> -demethyl	HO N N N N N N N N N N N N N N N N N N N	554.1704	C ₂₆ H ₂₇ N ₅ O ₇ S	554.1703	0.12	510.1442, 189.0643	4.16
5a	- 0	662.1139	$C_{26}H_{21}F_6N_5O_7S$	662.1140	0.16	618.0875, 311.1015, 280.0832, 202.0727	4.72
5a - <i>O</i> - demethyl (guaiacol)	F3C HOCF3NHOH	648.0983	$C_{25}H_{19}F_6N_5O_7S$	648.0971	0.77	604.0728, 297.0847, 280.0588, 189.0645	4.39
5b	-	676.1295	$C_{27}H_{23}F_6N_5O_7S$	676.1296	-0.19	632.1033, 311.1013, 280.0831, 202.0727	5.48
5b - <i>O</i> - demethyl (sulfonamid e)	$\begin{array}{c c} & & & \\ & & & & \\ & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & &$	662.1139	$C_{26}H_{21}F_6N_5O_7S$	662.1141	-0.31	618.0810, 311.1009, 280.0826, 202.0727	4.72
5b - <i>O</i> - demethyl (guaiacol)	F ₃ C H ₃ CO CF ₃ N N N O O O O O O O O O O O O O O O O O	662.1139	$C_{26}H_{21}F_6N_5O_7S$	662.1136	0.35	618.0876, 297.0860, 280.0640, 189. 0643	5.14



2.4 Biological assays

Compounds metabolized by CYP2C9 and CYP3A4 (**5a-c**) were further investigated for their cytotoxicity and for the agonist and antagonist response towards ET_A and ET_B . Compound **5d**, although very interesting for demonstrating that the tailored metabolic stabilization towards a specific isoform is possible, was not further investigated because it exhibited the worst overall metabolic profile in HLM (Table 1).

2.4.1 Cell viability

Cells viability of human recombinant ovarian (CHO-k1) cells incubated with compounds **1**, and **5a-c** was assessed by MTT assay following 24 h exposure to the compounds. The dose–response curves of each compound in CHO-k1 cells are shown in Figure 4. The IC₅₀ values, graphically determined from each concentration–response curves, were higher than 150 μ M. Thus, the tested compounds start to be slightly cytotoxic at micromolar concentration, which exceeds the nanomolar activity range on ET receptors.



Figure 4. Cytotoxicity of compounds **1**, **5a-c** on CHO-k1 cells and IC_{50} determination by MTT test. Results are expressed as percentages of OD versus vehicle control cells. IC_{50} values were calculated on the best fitting of dose–response inhibition type curves using GraphPad Prism 5 software.

2.4.2 Biological activity

Since one of the most important abnormalities of PAH is an overexpression of ET-1 that acts upon the receptors ET_A and ET_B , which mediate its physiological and pathological effects, the possible agonist and antagonist activities of bosentan analogues towards endothelin receptors were determined by Cerep, France [47]. The *in vitro* cellular function assays were performed for compounds **5a-c** by fluorimetric method using two cells lines: human neuroblastoma cell-line (SKNMC) for ET_A receptor and CHO-k1 cells for ET_B receptor. Agonist and antagonist activities were evaluated by comparing the response of different test compound solutions with a reference compounds. In particular, endothelin-1 was the reference compound used for agonist assays in both receptors (IC₅₀ = 0.67 nM and 0.1 nM in ET_A and ET_B , respectively). The reference compound used for antagonist effect in ET_{A} was BQ-123 (IC₅₀ = 0.62 nM, K_B = 0.082 nM) [48], while BQ-788 (IC₅₀ = 0.41 nM, K_B = 0.50 nM) was used in ET_{B} [49]. In the agonist assay the ability of test compound in stimulating the receptor was evaluated in term of % of control agonist response; in turn, antagonist effect was evaluated in terms of % inhibition of control agonist response and IC₅₀ for active compounds was calculated.



Figure 4. Evaluation of % inhibition of ET_A and ET_B for compounds 5a-c.

None of the bosentan analogues resulted in agonist activities towards endothelin receptors (supplementary data, Table S2). As shown in Figure 4 compound **5a** was completely inactive towards ET_A , and really low for ET_B (% of inhibition of control agonist response ~ 13) suggesting that the acidity of hexafluoro-2-hydroxy-2-propyl group heavily influences compound binding affinity towards ET_A . Compound **5b**, which differs from **5a** for the replacement of acidic hydroxyl group with a methoxy moiety, has a low activity, slightly better for ET_A ($IC_{50} = 693 \mu M$) than ET_B . Compound **5c**, where the 4-position of benzensulfonamide is occupied by a heptafluoroisopropyl group, was found to be the most active in this series with an interesting moderate effect selective for ET_A with an IC_{50} of 75.4 nM. Interestingly, although compound **5c** has a lower potency with respect to bosentan, it was found to be more selective towards the ET_A receptor (for comparison, bosentan binds to human ET_A and ET_B receptor with IC_{50} values of 4.7 and 95 nM, respectively [50]).

3. Conclusions

At present the use of bosentan as part of a combination therapy in PAH treatment has resulted in improvements of life quality and survival. However, due to bosentan ability to induce CYP3A4 and CYP2C9, belonging to CYP450 family, the risk associated for DDI is elevated, and hepatic events are known to occur and patients require regular monitoring with liver function tests [51]. In addition, the interaction of bosentan with the poly-morphically expressed enzyme CYP2C9 is cause of concern, due to the possible inter-ethnic and intra-ethnic variability of the drug response. To investigate possible strategies to enhance the metabolic stability of bosentan, especially towards CYP2C9, four synthetic analogues of bosentan have been designed and synthesized. These compounds were tested for their metabolism, agonistic and antagonistic activities.

The replacement of the *tert*-butyl group with perfluorinated bioisosteres (compounds **5a-c**) impeded, as expected, the *tert*-butyl aliphatic hydroxylation reaction, which is one of the major metabolism pathways of bosentan by CYP3A4 and CYP2C9. Interestingly, in HLM or in CYP3A4 this did not lead to a systematic increase of the metabolic stability, and new metabolic reactions occurred for **5b** and **5c**. In particular, **5b** was metabolized to a greater extent than bosentan in HLM, and a novel *O*-demethylation reaction at the sulfonamide moieties was observed. The occurrence of the *O*-demethylation reaction at the sulfonamide group of **5b** was also observed after incubation with CYP3A4, indicating that the methylated hexafluoro-2-hydroxyprop-2-yl can still be oriented towards the heme of CYP3A4. Despite the variable behaviour observed for fluorinated analogues in HLM and 3A4, these compounds resulted to be totally stable after 30 minutes incubation not only in CYP2D6 (like bosentan) but also in CYP2C9. Although the metabolic stability towards CYP2C9 was our primarily goal, the antagonist activity of compounds **5a-c** on ET_A and ET_B was also tested, with compound **5c** exhibiting a moderate antagonist activity on ET_A. To date there is no evidence that the selective inhibition of ET_A has a clinical advantage over the nonselective one but both

sitaxsentan and ambrisentan, two selective ET_A antagonists already marketed, have shown lower development of abnormal liver function [52].

Finally, an innovative *in silico* driven strategy to improve metabolic stability using MetaSiteTM was applied to design and synthesize an additional analogue of bosentan aiming at specifically reducing metabolism by CYP2C9 without modifying the SoM. The resulting compound **5d**, differing from bosentan by a change in the lateral chain only, proved to be much more stable than bosentan when incubated in the presence of CYP2C9 for 30 minutes, with 99% of unchanged substrate remaining. Thus, although compound **5d** was the least stable compound in HLM, this study has demonstrated that the MetaSiteTM predictions can be used as an efficient approach for tailoring the phase I metabolic pathway of a drug.

4. Experimental section

4.1 Chemistry

4.1.1 Generalities

¹H, ¹³C, and ¹⁹F NMR spectra were recorded at 400, 100.6 and 376.3 MHz, respectively. Spectra were recorded at 298 K if not specified otherwise. Chemical shifts (δ) are given in ppm relative to the internal standards tetramethylsilane and trichlorofluoromethane. HRMS spectra were registered on Agilent Technologies 6540 UHD Accurate Mass Q-TOF LC/MS, HPLC 1290 Infinity. Melting points were corrected using a calibration curve established with authentic standards. Purities of the final compounds were \geq 98% pure and were determined by UHPLC: column, Phenomenex AERIS Peptide 1.2 mm × 1000 mm (1.7 µm); flow rate, 0.8 mL/min; acquisition time, 20 min; DAD 190-650 nm; oven temperature, 45 °C; gradient of acetonitrile in water containing 0.1% of formic acid (0-100% in 20 minutes).

Tetrahydrofuran was stored over potassium hydroxide pellets in the presence of cuprous chloride, from which it was distilled, before being redistilled from sodium wire after the characteristic blue

color of *in situ* generated sodium biphenyl ketyl (benzophenone-sodium "radical anion") had been found to persist. Air and moisture sensitive compounds were stored in Schlenk tubes or Schlenk burettes. They were protected by and handled under an atmosphere of 99.995 % pure nitrogen, using appropriate glassware. All the commercial available products were bought and used without any further purification.

4.1.2 Products

Dimethyl 2-(2-methoxyphenoxy)malonate (6) [38]. A sodium methoxide solution in methanol (2.3 g of Na, 0.10 mol, 1.0 eq in 75 ml of MeOH) was slowly added to a stirred mixture of guaiacole (12.5 g, 0.100 mol, 10 eq) and dimethyl 2-chloromalonate (19.0 g, 0.114 mol, 1.14 eq). After having reacted for 1 h at 45 °C, the volatile material were evaporated and the residue was taken up in toluene, washed with water and NaHCO₃ (1% aqueous solution). The concentrated organic phase afforded 20.0 g (yield 78.7 %) of a viscous oil that was characterized as follows: bp 131 °C (lit. [38] bp 128 °C); ¹H NMR (CDCl₃) δ 7.12 – 7.00 (m, 2H), 6.98 – 6.90 (m, 1H), 6.90 – 6.82 (m, 1H), 5.27 (s, 1H), 3.85 (s, 3H), 3.84 (s, 6H); ¹³C NMR (CDCl₃) δ 166.2 (2C), 150.4, 146.0, 124.4, 120.8, 119.0, 112.5, 78.4, 55.8, 53.0 (2C); HRMS calcd for C₁₂H₁₄O₆ 255.0869 (M+H⁺), found 255.0870 (M+H⁺).

2-Amidinopyrimidine hydrochloride (7) [38]. A MeONa solution in methanol (0.35 g of Na, 15 mmol, 0.10 eq in 112 ml of MeOH) was slowly added to 2-cyanopyrimidine (13.8 g, 0.131 mol, 1.0 eq) and the solution was stirred at rt for 24 h. Acetic acid (0.75 ml, 11.9 mmol, 0.09 eq) was added, followed by NH₄Cl (7.80 g, 0.146 mol, 1.1 eq) and the suspension was stirred for further 24 h. The solid was filtered, the solution concentrated and the residue was triturated with diethyl ether. The solid was solved in methanol (25 ml) and filtered. Ethyl acetate (500 ml) was added and the resulting crystalline precipitate filtered off and dried at 80 °C to afford 7.8 g (yield 37.9%) of a white solid. Mp 126-127 °C; ¹H NMR (D₂O) δ 8.95 (s, 1H), 7.72 (s, 1H), 4.79 (s, HDO); ¹³C NMR

(D₂O) δ 159.8, 158.3, 152.3, 124.8 (2C); HRMS calcd for C₅H₆N₄ 123.0671 (M+H⁺), found 123.0666 (M+H⁺).

4,6-Dihydroxy-5-(o-methoxyphenoxy)-2,2'-bipyrimidine (8) [38]. A methanol solution (25 ml) of dimethyl 2-(2-methoxyphenoxy) malonate **6** (11.0 g, 430 mmol, 1.0 eq) was added to a stirred MeONa solution in methanol (4.85 g of Na, 210 mmol, 5 eq in 50 ml of MeOH). After 30 minutes, 2-amidinopyrimidine hydrochloride **7** (7.50 g, 47.0 mmol, 1.1 eq) was added and the reaction stirred for further 20 hours. The solvent was evaporated, the residue was taken up in NaOH 1 N, acidified with HCl 1 N and the precipitate was filtrated off, washed with water and dried under high vacuum at 80 °C to afford 13.4 g (yield 55.1%) of a yellow solid that was characterized as follows. ¹H NMR (DMSO-*d*₆) δ 12.34 (s, 2H), 9.02 (d, *J* = 4.9 Hz, 2H), 7.71 (t, *J* = 4.7 Hz, 1H), 7.10 – 6.97 (m, 1H), 6.92 (t, *J* = 7.6 Hz, 1H), 6.77 (t, *J* = 7.6 Hz, 1H), 6.65 (d, *J* = 7.6 Hz, 1H), 3.81 (s, 3H); ¹³C NMR (DMSO-*d*₆) δ : 158.4 (2C), 157.1 (2C), 149.6, 148.7 (2C), 146.8, 123.2, 122.2, 121.5, 120.8, 113.5, 113.2, 56.0; HRMS calcd for C₁₅H₁₂N₂O₄ 313.0937 (M+H⁺), found 313.0939 (M+H⁺).

4,6-Dichloro-5-(o-methoxyphenoxy)-2,2'-bipyrimidine (9) [38]. A mixture of 4,6-dihydroxy-5-(o-methoxyphenoxy)-2,2'-bipyrimidine **8** (2.08 g, 6.40 mmol, 1.0 eq), *N*,*N*-diisopropylethylamine (2.20 ml, 22.9 mmol, 3.5 eq) and PCl₅ (2.75 g, 13.2 mmol, 2.0 eq) was refluxed in acetonitrile (20 ml) for 20 h. The cooled mixture was dropped in iced water and extracted with ethyl acetate. The organic phase was evaporated and the residue was chromatographed on Silica gel with diethyl ether, followed by ethyl acetate, to afford 1.68 g (yield 74.9 %) of a yellow solid that was characterized as follows: mp 80-81 °C [lit. [38] mp 79-80 °C]; ¹H NMR (CDCl₃) δ 9.05 (d, *J* = 4.8 Hz, 2H), 7.46 (t, *J* = 4.9 Hz, 1H), 7.21 – 7.05 (m, 1H), 7.00 (dd, *J* = 8.1, 1.5 Hz, 1H), 6.95 – 6.84 (m, 1H), 6.80 (dd, *J* = 8.0, 1.6 Hz, 1H), 3.85 (s, 3H); ¹³C NMR (CDCl₃) δ 156.8, 154.8 (2C), 153.1, 152.0 (2C), 145.8, 141.6, 141.2, 121.5, 118.4, 117.5, 113.4, 109.5, 52.8. HMRS: calcd for C₁₅H₁₀Cl₂N₄O₂ 349.0259 (M+H⁺), found 349.0261 (M+H⁺).

N-(6-Chloro-5-(2-methoxyphenoxy)-2,2'-bipyrimidin-4-yl)-4-(1,1,1,3,3,3-hexafluoro-2-

hydroxypropan-2-yl)benzenesulfonamide (11a). Sulfonamide 10a (2.0 g, 6.2 mmol, 1.1 eq) was solved in dry THF (8 ml) and cooled to -78 °C under a nitrogen atmosphere. n-BuLi (1.62 N solution in hexane, 7.70 ml, 2.2 eq) was added drop-wise. After the addition was completed, the solution was stirred for 15 minutes, then the temperature was allowed to raise at -60 °C and the THF was evaporated under very high vacuum. DMF was added (10 ml), followed by dichloropyrimidine intermediate 9 (2.0 g, 5.7 mmol, 1.0 eq) and the mixture was stirred overnight at 50 °C. The reaction mixture was poured onto a 1:1 mixture of water-ice and the aqueous phase was washed with DEE, then acidified with HCl and the resulting precipitate was filtrated off, washed with water and dried at 110 °C to afford a bright yellow solid (2.22 g, yield 61.2%). Mp 226-229 °C: ¹H NMR (DMSO- d_6 , T = 320 K) δ 11.82 (bs, 1H), 9.00 (d, J = 4.9 Hz, 2H), 8.74 (s, 1H), 8.15 (d, J = 7.5 Hz, 2H), 7.68 (d, J = 8.8 Hz, 2H), 7.63 (t, J = 4.9 Hz, 1H), 7.08 (d, J = 8.2 Hz, 1H), 7.05-6.96 (m, 1H), 6.86 - 6.77 (m, 1H), 6.56 (d, J = 8.0 Hz, 1H), 3.79 (s, 3H); ¹³C NMR (DMSO- d_6) δ 161.9, 158.1 (2C), 156.4, 150.0, 149.1(2C), 146.2, 146.0, 134.4, 133.6, 128.5 (2C), 126.7 (2C), 123.2 (q, J = 288.7 Hz, 2C), 123.2, 122.0, 121.1, 115.0, 114.0, 77.4 (hept, J = 28.7, 27.4 Hz), 56.4; ¹⁹F NMR (DMSO-*d*₆) δ -74.22 (s, 6F). HMRS: calcd for C₂₄H₁₆ClF₆N₅O₅S 636.0543 (M+H⁺), found 636.0548 (M+H⁺).

N-(6-Chloro-5-(2-methoxyphenoxy)-2,2'-bipyrimidin-4-yl)-4-(1,1,1,3,3,3-hexafluoro-2-

methoxypropan-2-yl)benzenesulfonamide (11b). Following the same procedure used for **11a**, but starting from sulfonamide **10b** (150 mg, 0.45 mmol), the titled compound was obtained as a white solid (100 mg, 35.1 % yield. Mp > 290 °C; ¹H NMR (DMSO- d_6) δ 8.93 (d, J = 4.8 Hz, 2H), 8.08 – 7.97 (m, 2H), 7.58 (t, J = 4.9 Hz, 1H), 7.47 (d, J = 8.2 Hz, 2H), 7.06 (dd, J = 8.1, 1.4 Hz, 1H), 6.96 (td, J = 7.7, 1.5 Hz, 1H), 6.78 (td, J = 7.7, 1.5 Hz, 1H), 6.42 (dd, J = 8.1, 1.4 Hz, 1H), 3.81 (s, 3H), 3.36 (s, 3H); ¹³C NMR (DMSO- d_6) δ 162.6, 160.0, 157.9 (2C), 157.1, 149.5, 148.8, 147.8, 146.2, 133.8, 128.7 (2C), 128.1, 127.4 (2C), 122.5, 122.5 (q, J = 288.8 Hz, 2C), 121.7, 120.9, 113.8,

113.5, 82.7 (hept, J = 28.7 Hz) 56.1, 54.7; ¹⁹F NMR (DMSO- d_6) δ -70.71 (s, 6 F); HRMS: calcd. for C₂₅H₁₈ClF₆N₅O₅S 650.0700 (M+H⁺), found 650.0700 (M+H⁺).

N-(6-Chloro-5-(2-methoxyphenoxy)-2,2'-bipyrimidin-4-yl)-4-(11,1,1,2,3,3,3-

heptafluoropropan-2-yl)benzenesulfonamide (11c). The titled compound was obtained following the same procedure used for 11a, but starting from sulfonamide 10c. Mp 287-289 °C; Yield 35% ¹H NMR (acetone + 5% MeOD) δ 9.00 (d, J = 4.9 Hz, 2H), 7.78 (d, J = 8.3 Hz, 2H), 7.66 (t, J = 4.9Hz, 1H), 7.61 (d, J = 8.3 Hz, 2H), 7.00 (dd, J = 8.1, 1.6 Hz, 1H), 6.94 (td, J = 8.1, 7.7, 1.5 Hz, 1H), 6.66 (td, J = 7.7, 7.2, 1.7 Hz, 1H), 6.55 (dd, J = 8.0, 1.5 Hz, 1H), 3.79 (s, 3H); ¹³C NMR (CDCl₃) δ 160.9, 159.4, 158.0 (2C), 154.8, 150.3, 149.4, 148.5, 146.5, 136.6, 127.6 (d, J = 20.6 Hz), 126.5 (2C), 125.5 (d, J = 10.9 Hz, 2C), 122.6, 121.9, 121.7 (qd, J = 283.7 and 30.7 Hz, 2C), 120.4, 114.9, 113.5, 79.2 – 77.6 (m), 55.6; ¹⁹F NMR (acetone- d_6 + 5% MeOD) δ -76.83 (m, 6F), -183.30 (m, 1F); HRMS: calcd. for C₂₄H₁₅ClF₇N₅O₄S 638.0500 (M+H⁺), found 638.0509 (M+H⁺).

4-(*tert*-Butyl)-N-(6-chloro-5-(2-methoxyphenoxy)-[2,2'-bipyrimidin]-4-yl)benzenesulfonamide

(11d). Following the same procedure described for 11a, but starting from commercial 4-*t*-butylbenzenesulfonamide and dichloropyrimidine 9, a pale yellow solid was obtained in 75.1% of yield. Mp 150-151 °C [lit. [38] mp 152 °C]; ¹H NMR (DMSO- d_6) δ 11.81 (bs, 1H), 8.96 (d, J = 4.9 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H), 7.62 (t, J = 4.9 Hz, 1H), 7.33 – 7.25 (m, 2H), 7.10 – 7.04 (m, 1H), 7.00 – 6.93 (m, 1H), 6.76 (td, J = 7.7, 1.5 Hz, 1H), 6.41 (d, J = 8.0 Hz, 1H), 3.80 (s, 3H), 1.22 (s, 9H); ¹³C NMR (DMSO- d_6) δ 162.3, 160.0, 158.1, 156.8, 153.2, 149.4, 148.8, 146.2, 142.6, 134.2, 127.2, 124.6, 122.6, 121.8, 120.8, 113.9, 113.6, 56.2, 34.8, 31.3; HRMS: calcd. for C₂₅H₂₄ClN₅O₄S 526.1316 (M+H⁺), found 526.1313 (M+H⁺).

4-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)-N-(6-(2-hydroxyethoxy)-5-(2-

methoxyphenoxy)-2,2'-bipyrimidin-4-yl)benzenesulfonamide (5a). Chloropyrimidine **11a** (0.317 g, 0.50 mmol, 1.0 eq) was added to a stirred solution of sodium glycolate (from 0.11 g of Na in 3 ml of ethylene glycol) and the resulting mixture was stirred at 110 °C for 5 h. The cooled mixture was

dropped in ice, the aqueous phase washed with diethyl ether and then acidified. The resulting precipitate was filtrated off, washed with water and chromatographed (MeOH/EA 3:7, SiO₂) to afford 302.0 mg as a yellow solid (90.7% yield). Mp 99-100 °C; ¹H NMR (DMSO- d_6 , T = 325 K) δ 9.00 (d, *J* = 4.4 Hz, 2H), 8.72 (bs, 1H, OH), 8.14 (bs, 2H), 7.73 – 7.65 (m, 3H, CH₂ arom + NH), 7.65 – 7.59 (m, 1H), 7.08 – 7.01 (m, 1H), 6.95 (bt, *J* = 7.9 Hz, 1H), 6.76 (bt, *J* = 7.6 Hz, 1H), 6.61 – 6.46 (m, 1H), 4.72 (bs, 1H, OH), 4.35 (bs, 2H), 3.82 (s, 3H), 3.56 (bs, 2H); ¹³C NMR (DMSO- d_6 , T = 325 K) δ 162.7, 161.4, 158.1 (2C), 157.5, 155.5, 149.2, 147.8, 147.3, 133.3, 128.9, 128.4 (2C), 126.7 (2C), 123.10 (q, *J* = 291.5, 289.0 Hz, 2C), 122.5, 121.7, 121.0, 115.0, 114.0, 77.4 (h, J = 29.4 Hz), 68.6, 60.1, 56.5; ¹⁹F NMR (DMSO- d_6) δ -74.17 (s, 6F). HRMS: calcd. for C₂₆H₂₁F₆N₅O₇S 662.1144, found 662.114.

4-(1,1,1,3,3,3-Hexafluoro-2-methoxypropan-2-yl)-N-(6-(2-hydroxyethoxy)-5-(2-

methoxyphenoxy)-2,2'-bipyrimidin-4-yl)benzenesulfonamide (5b). Following the same procedure used for 5a, but starting from 11b (150.0 mg, 0.45 mmol), The titled compound was obtained as a white solid (22 mg, 22.9 % yield). Mp 163-168 °C ¹H NMR (DMSO-*d*₆, 325 K) δ 11.51 (bs, 1H, NH), 9.18 – 8.92 (m, 2H), 8.64 – 8.37 (m, 2H), 7.92 – 7.44 (m, 3H), 7.16 – 6.91 (m, 2H), 6.93 – 6.58 (m, 2H), 4.38 (bs, 2H), 3.80 (s, 3H), 3.52 (bs, 2H), 3.44 (s, 3H), 1.54 (bs, 1H, OH); ¹³C NMR (DMSO-*d*₆) δ 161.8, 158.5 (2C), 158.2, 151.2, 149.4, 149.1, 146.1, 143.6, 131.1, 128.6 (2C), 124.2, 123.2, 122.3 (q, *J* = 290.5 Hz), 122.3, 122.1, 116.8, 114.0 (2C), 113.8, 82.8 (hept, *J* = 27.9 Hz), 69.2, 59.6, 56.5, 55.1; ¹⁹F NMR (DMSO-*d*₆) δ -70.64 (s, 6F); HRMS: calcd. for $C_{27}H_{23}F_6N_5O_7Si$ 676.1301 (M+H⁺), found 676.1303 (M+H⁺).

4-(1,1,1,2,3,3,3-Heptafluoropropan-2-yl)-N-(6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2,2'bipyrimidin-4-yl)benzenesulfonamide (5c). Following the same procedure described for 5a, but starting from chloro intermediate 11c, the titled compound was obtained as a white solid (105 mg, yield 65.3 %). Mp 243-245 °C ¹H NMR (DMSO- d_6 , 325 K) δ 11.60 (s, 1H, NH), 9.07 (d, J = 5.0Hz, 2H), 8.73 – 8.28 (m, 2H), 7.90 – 7.81 (m, 2H), 7.77 – 7.52 (m, 1H), 7.17 – 6.94 (m, 2H), 6.90 – 6.63 (m, 2H), 4.52 (bs, 1H, OH), 4.49 – 4.23 (m, 2H), 3.79 (s, 3H), 3.54 (s, 2H); ¹³C NMR (acetone- d_6) δ 161.6 (d, J = 20.6 Hz), 158.4, 157.8 (2C), 155.5, 151.0 (d, J = 137 Hz), 149.8, 146.0, 143.5, 130.7 (2C), 130.1 (d, J = 20.6 Hz), 126.6, 126.0, 125.9, 124.6, 120.9, 120.3 (qd, J = 314.0, 27.8 Hz, 2C), 118.5 , 112.8 (2C), 91.4 (dhept, J = 201.9, 32.5 Hz), 70.1, 60.6, 55.6. ¹⁹F NMR (DMSO- d_6) δ -74.82 (d, J = 8.6 Hz, 6 F), -181.01 (m, 1F); HRMS: calcd. for C₂₆H₂₀F₇N₅O₆S 664.1101 (M+H⁺), found 664.1104 (M+H⁺).

4-(*tert*-**Butyl**)-*N*-(**6**-ethoxy-**5**-(**2**-methoxyphenoxy)-[**2**,**2**'-bipyrimidin]-**4**-yl)benzenesulfonamide (**5d**). Following the same procedure used for **5a**, but starting from chloro intermediate **11d** and ethanol, the titled compound was obtained as a white solid (yield 69.3 %) that was characterized as follows: mp 140-142 °C. ¹H NMR (CDCl₃) δ 8.69 (d, J = 4.9 Hz, 2H), 7.84 (s, 1H), 7.52 (d, J = 8.0Hz, 2H), 7.06 – 6.98 (m, 3H), 6.92 – 6.72 (m, 2H), 6.60 (t, J = 7.7 Hz, 1H), 6.37 (d, J = 8.1 Hz, 1H), 4.38 (q, J = 7.3 Hz, 2H), 3.77 (s, 3H), 1.15 (t, J = 7.0 Hz, 3H), 1.13 (s, 9H); ¹³C NMR (CDCl₃) δ 163.5, 162.1, 161.8, 157.7 (2C), 155.3, 153.6, 148.7, 146.8, 140.9, 126.3 (2C), 125.1, 124.9, 124.7 (2C), 122.1, 120.7, 120.6, 112.5, 62.4, 56.2, 34.6, 31.0 (3C), 14.4; HRMS: calcd. for C₂₇H₂₉N₅O₅S 536.1968 (M+H⁺), found 536.1973 (M+H⁺).

4-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)benzene-1-sulfonyl chloride (12a) [42]. To a mixture of 2- (4-aminophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (10.0 g, 38.6 mmol, 1.0 eq), HCl (37 % in water, 20 ml) and acetic acid (6 ml), NaNO₂ (2.93 g, 42.5 mmol, 1.1 eq) in water (3.3 ml) was added drop-wise at -15 °C. The temperature of the reaction was kept below -5 °C for 45 minutes. Meanwhile, a saturated solution of sulfur dioxide in acetic acid was prepared, by bubbling into acetic acid SO₂ for 15 minutes. Freshly prepared CuCl (0.920 g, 9.2 mmol, 0.25 eq) was added to the solution at rt and while stirring, SO₂ introduction was continued for 20 minutes. At 0 °C, the diazotization reaction mixture was added in small portion to the SO₂-CuCl complex solution. After addition was completed, stirring was continued for 10 minutes while temperature was maintained under 10 °C. The reaction mixture was poured onto a 1:1 mixture of water-ice (330 ml) and stirred

until ice was melted. The mixture was then extracted with DEE (3x70 ml) and the combined organic extracts were washed with water (2x70 ml), saturated NaHCO₃ (caution, vigorous gas evolution) and brine. The organics were then dried over Na₂SO₄ and concentrated under reduced pressure. Flash chromatography of residue (100% DCM, SiO₂) gave 4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)benzene-1-sulfonyl chloride (7.10 g, 55.5 % yield). ¹H NMR (CDCl₃) δ 8.13 (d, *J* = 8.1 Hz, 1H), 8.01 (d, *J* = 9.0 Hz, 2H), 3.97 (s, 1H); ¹³C NMR (CDCl₃) δ 142.4, 133.1, 125.0 (2C), 123.7 (2C), 118.73 (q, *J* = 287.1, 2C), 73.85 (hept, *J* = 28.6 Hz); ¹⁹F NMR (CDCl₃) δ -75.67 (s, 6 F).

4-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)benzenesulfonamide (**10a**). A solution of sulfonyl chloride **12a** (5.00 g, 14.4 mmol, 1.0 eq) in THF (25 ml) was cooled with an ice bath. Aqueous ammonium hydroxide was added drop-wise (25% water solution, 4.5 mL). After the addition was completed, the solution was stirred at rt for 15 min. The solvent was removed in vacuum and the residue was dissolved in NaOH 2.0 M solution, washed with DEE. The aqueous phase was acidified with H₂SO₄ and then extracted with EA, washed with water and the organic phase was dried over Na₂SO₄. The solvent was evaporated to afford pure 4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)benzenesulfonamide (3.50 g, yield 74.2 %) as a white solid. ¹H NMR (DMSO-*d*₆) δ 9.00 (s, 1H), 7.95 (d, *J* = 8.8 Hz, 2H), 7.87 (d, *J* = 8.4 Hz, 2H), 7.47 (s, 2H); ¹³C NMR (DMSO-*d*₆) δ 142.7, 131.2, 124.7 (2C), 123.0 (2C), 119.71 (q, *J* = 288.4 Hz, 2C), 73.80 (hept, *J* = 28.3 Hz); ¹⁹F NMR (DMSO-*d*₆) δ -74.25 (s, 6F); HMRS: calcd for C₉H₇F₆NO₃S 324.0129 (M+H⁺), found 324.013 (M+H⁺).

2-(4-Bromophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (**13**). Gaseous hexafluoroacetone was generated by adding cautiously the sesquihydrate (6.56 g, 34.0 mmol, 2.0 eq) to concentrated (98% aq.) sulfuric acid at 50 °C and it was condensed, through Teflon tubing, into a cold trap cooled to - 75 °C. In a second Schlenk tube, *n*-butyllithium (1.62 N hexane solution, 10.5 ml, 17.0 mmol) was added drop-wise, in 15 min, to a solution of 1,4-dibromobenzene (4.00 g, 17.0 mmol, 1.0 eq) in

tetrahydrofuran (40 ml) at -95 °C. Warming the cold trap slowly to 25 °C made the hexafluoroacetone stream through a Teflon canola, ending at 5 mm above the liquid surface, into the Schlenk tube containing the reaction intermediate. When the addition was complete, the mixture was kept for further 10 min at -95 °C. Upon chromatography on silica gel, using a 1:20 (v/v) mixture of diethyl ether and hexanes as the eluent, a viscous colorless oil was collected; 3.68 g (67.1% yield). ¹H NMR (CDCl₃) δ 7.60 (s, 4H), 3.57 (s, 1H); ¹³C NMR (acetone-*d*₆) δ 131.9 (2C), 128.3 (2C), 126.7, 125.0, 122.4 (q, *J* = 288.4 Hz, 2C), 74.4 (hept, *J* = 30.3 Hz); ¹⁹F NMR (CDCl₃) δ -76.09 (s, 6F).

1-Bromo-4-(1,1,1,3,3,3-hexafluoro-2-methoxypropan-2-yl)benzene (14). To a stirred suspension of hexane washed NaH (60% in mineral oil, 238 mg, 6.20 mmol, 1.15 eq) in dry DMF (5 ml) alcohol **13** (1.72 g, 5.4 mmol, 1.0 eq) solution in dry DMF (5 ml) was added under nitrogen atmosphere at 0 °C. After 40 minutes, methyl iodide (1.2 ml, 16.2 mmol, 3.0 eq) was added and the mixture was further reacted at rt for 40 minutes. Then, the mixture was added to ice and extracted with DEE (3x30 ml). The organic phases were reunited, the solvent removed under vacuum and the residue distilled at 19 mmHg (96 °C) to afford a colorless oil (1.630 g, 89.6% yield) of 1-bromo-4-(1,1,1,3,3,3-hexafluoro-2-methoxypropan-2-yl)benzene. ¹H NMR (CDCl₃) δ 7.70 – 7.52 (m, 2H), 7.57 – 7.35 (m, 2H), 3.48 (hept, J = 1.0 Hz, 3H); ¹³C NMR (Acetone-*d*6) δ 132.2 (2C), 130.1 (2C), 126.7, 124.7, 122.3 (q, J = 289.6 Hz, 2C), 74.5 (hept, J = 30.3 Hz), 54.0; ¹⁹F NMR (CDCl₃) δ - 71.41 (s, 6 F).

4-(1,1,1,3,3,3-Hexafluoro-2-methoxypropan-2-yl)benzenesulfonamide (10b) [41]. *n*-Butyllithium (1.62 N hexane solution, 2.75 ml, 4.45 mmol) was added drop-wise, in 15 min, to a solution of bromo derivate **14** (1.50 g, 4.45 mmol, 1.0 eq) in tetrahydrofuran (15 ml) at -78 °C and the mixture wasreacted, while stirring, for 10 min before the bath temperature was allowed to rise to -60 °C. Sulfur dioxide was bubbled into the mixture until its pH value reached 6-7. The cold bath was removed and the temperature was allowed to rise to 25 °C. Hexane (20 mL) was added and the

white precipitate formed was filtered, washed with hexanes and dried at 50 °C. The dry solid was suspended in dichloromethane (15 mL), the mixture was cooled at 0 °C and *N*-chlorosuccinimide (0.83 g, 6.2 mmol, 1.4 eq) was added in portions. The mixture reacted at 0 °C for 15 min, while stirring, and then further 25 min at room temperature. The resulting suspension was filtered off on Celite, the solvent was evaporated at reduced pressure and the solid residue was solved in acetone (15 mL). Ammonium hydroxide solution (0.39 ml, 10 mmol, 2.4 eq) was added and the mixture was kept at 40 °C for 12 h while stirring. After the solvent evaporation at reduced pressure, flash chromatography of the residue on silica gel (eluent EA/PE 3:7) gave the expected product (334 mg, 22.3% yield) which was characterized as follow. ¹H NMR (CDCl₃) δ 8.02 (d, *J* = 8.3 Hz, 2H), 7.72 (d, *J* = 8.2 Hz, 2H), 5.80 (s, 2H), 3.49 (s, 3H); ¹³C NMR (DMSO-*d*₆) δ 146.6, 130.4, 129.2 (2C), 126.9 (2C), 122.4 (q, *J* = 290.2 Hz, 2C), 82.7 (hept, *J* = 28.1 Hz), 55.0; ¹⁹F NMR (DMSO-*d*₆) δ - 70.83 (s, 6 F); HMRS: calcd for C₁₀H₉F₆NO₃S 338.0286 (M+H⁺), found 338.0303 (M+H⁺).

4-(1,1,1,2,3,3,3-Heptafluoropropan-2-yl)aniline (15) [40]. To a mixed solution comprising MTBE (20 ml) and water (20 ml), aniline (1.0 g, 10.8 mmol, 1.0 eq), Na₂(S₂O₄) (2.2 g, 13.0 mmol, 1.2 eq), NaHCO₃ (1.1 g, 13.0 mmol, 1.2 eq), and tetrabutylammonium bisulfate (0.4 g, 1.2 mmol, 0.1 eq) were added. To this mixed solution, heptafluoroisopropyl iodide (3.8 g, 13.0 mmol, 1.2 eq) was added, drop-wise and the mixture was stirred at room temperature for 8 hours. The aqueous phase was extracted with EA and the reunited organic phases were washed with 2 N hydrochloric acid and saturated NaHCO₃. After evaporating off the solvent, the residue was purified by distillation to obtain the desired 4-(1,1,1,2,3,3,3-heptafluoropropan-2-yl)aniline (2.5 g, yield 88.6 %) as a very viscous yellow oil. ¹H NMR (CDCl₃) δ 7.48 – 7.32 (m, 2H), 6.84 – 6.68 (m, 2H), 3.92 (bs, 2H); ¹⁹F NMR (CDCl₃) δ -76.51 (d, *J* = 7.5 Hz, 6 F), -181.91 (m, 1F); HRMS: calcd. for C₉H₆F₇N 262.0467 (M+H⁺).

4-(1,1,1,2,3,3,3-Heptafluoropropan-2-yl)benzene-1-sulfonyl chloride (12c). Following the same procedure described for **12a** but starting from **15** (5.0 g, 19.0 mmol) a yellow solid (2.6 g, yield

39.7%) was obtained that was used in the following step without any further purification; ¹H NMR (200 MHz, CDCl₃) δ 8.25 – 8.15 (m, 2H), 7.90 (d, *J* = 8.5 Hz, 2H).

4-(**1**,**1**,**1**,**2**,**3**,**3**,**3**-Heptafluoropropan-2-yl)benzene-1-sulfonyl amide (**10c**). Following the same procedure described for **10a** starting from **12c** (2.5 g, 7.3 mmol) a yellow solid (0.700 g, yield 28.8%) was obtained. ¹H NMR (DMSO- d_6) δ 8.35 – 8.01 (m, 2H), 7.94 (d, *J* = 8.3 Hz, 2H), 7.61 (s, 2H); ¹³C NMR (DMSO- d_6) δ 147.7, 128.6 (d, *J* = 20.5 Hz), 127.4 (d, *J* = 1.4 Hz, 2C), 127.0 (d, *J* = 10.7 Hz, 2C), 120.52 (qd, *J* = 287.2, 27.8 Hz, 2C), 82.7 (m, 1F); ¹⁹F NMR (DMSO- d_6) δ -75.44 (d, *J* = 7.7 Hz, 6F), -182.09 (m, 1F). GC–MS m/z (% relative intensity, ion): 326 (8, M + 1), 325 (67, M⁺), 309 (23, M – NH), 261 (95, M – SO₂), 2 45 (M ⁺ – SO₂NH₂). HRMS: calcd. for C₉H₆F₇NO₂S 326.0086 (M+H⁺), found 326.0090 (M+H⁺).

4.2 Metabolism

4.2.1 Generalities

The LC-QTOF system was an Agilent 6540 UHD Accurate-Mass Quadrupole Time-of-Flight (QTOF) with a dual Jet Stream electron spray ionization source equipped with an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA). The LC consisted in a binary pump with integrated vacuum degasser, High Performance well-plate autosampler and thermostated column compartment modules. The column was a 2.1 x 50 mm ZORBAX Extended C18 RRHT, 1.8µm (Agilent Technologies, Santa Clara, CA, USA). Column temperature was set at 40 °C and injection volume was 2.0 µL. Mobile phase A: water containing 0.1 % formic acid and mobile phase B: acetonitrile with 0.1% of formic acid were utilized with a gradient from 5% B to 95% B within 6 min. The flow rate was set to 250 µL/min and the eluate was introduced into the mass spectrometer by means of an Agilent Dual Jet Stream electrospray ionization (ESI) in positive mode.

Source parameters were the following: capillary voltage was set to 4000 V, the ion source temp 300 °C and nitrogen was used as nebulizing and collision gas at 7.7 L/min and 20 psi, respectively.

Fragmentor voltage was set to 60 V and nozzle voltage at 0 V, Sheath gas flow was set to 9mL/min and Sheath Gas Temp 320 °C.

The LC-QTOF was governed by Agilent MassHunter software (B.04.00); acquisition of spectrometric data was obtained in AutoMSMS mode, performing the acquisition in MS full scan and in MS/MS scan at three fragmentation energies (20, 40, 50 V); exact protonated masses ([M+H]⁺) of precursor ions to be fragmented were isolated according to a preferred list ions generated by MetaSiteTM program (version 4.2.2, Molecular Discovery Ltd., UK [53, 54]) including the all possible metabolites predicted till the third generation. The MS/MS data were processed using Mass- MetaSiteTM (version 3.0.2) in the MetaSiteTM package [44, 45, 54]. The MetaSite-Batch Processor was set to process the MS/MS data of the compounds for metabolite identification and results were inspected using "WebMetabaseTM release-2.0.1-RC2" (Molecular Discovery Ltd, Middlesex, UK [53]).

4.2.2 Solvents and materials

DMSO, water, acetonitrile, formic acid, NADPH and methanol were purchased from Sigma -Aldrich (Milano, Italy); ammonium formate was from Agilent (Agilent Technologies, Santa Clara, CA, USA).

RPMI medium was purchased from CAMBREX (Milan, Italy). L Cytochrome isoforms and HLM UltraPool[™] HLM 150 were purchased from BD Gentest (Woburm, MA, USA). All other chemicals and solvents were of the highest grade available and obtained from common commercial sources. Bosentan was acquired from Toronto Research Chemicals.

Metabolite profiling in HLM and CYP isoforms

Human Liver microsomes (HLM) and supersomes, individual recombinant CYP isoforms (3A4, 2D6, 2C9) expressed in baculovirus-infected insect cells, were incubated according to manufacturer's recommendations (BD Biosciences) with minor modification. The final volume of incubation mixture was 250 μ l, which consisted of a final concentration of 5 μ M for the tested

compound in 100 mM potassium phosphate buffer (pH 7.4). The metabolic reaction was started by addition of NADPH at a final concentration of 1 mM. All the investigated compounds were incubated for 0, 15, 30 minutes at 37 °C in a shaking water bath and stopped by adding 250 µl of ice-cold acetonitrile (containing 0.6 µM labetalol as an internal standard). Proteins were precipitated by centrifugation at 10000 rpm for 10 min. An aliquot of 0.5 µl supernatant was injected into the LCMS. The protein concentrations and incubation times used in the assays were found to be in the linear range in preliminary experiments. Blank was prepared incubating supersomes in absence of the investigated compounds. The samples were analyzed using LC-qTOF and the metabolites detection was performed using the Mass-MetaSiteTM software (version 3.2.0, Molecular Discovery Ltd., UK [43]) The LC-MS chromatograms are reported in Supplementary material.

4.3 Biological evaluation of agonist and antagonist effects on ET_A and ET_B receptors.

4.3.1 Cell culture

Chinese hamster ovary (CHO-k1) cells were routinely cultured in DMEM F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, without phenol red, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), 50 U/ml and 50 μ g/ml penicillin-streptomycin (Sigma-Aldrich) and maintained in humidified atmosphere with 5% CO₂ at 37 °C.

4.3.2. Cell viability assay

The cytotoxic effect of compounds **1**, **5a-5c** was determined using the MTT test. Briefly, CHO-k1 cells were cultured in 96-well plates with a seeding density of 20,000 cells/well and allowed to attach overnight. Cells were incubated with either DMSO (Sigma–Aldrich) or increasing concentrations of compounds for 24 h (from 0.1 to 200 μ M). Thus, medium was replaced by a solution containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) and cells were incubated for 3 h at 37 °C. Following incubation, formazan crystals were dissolved in DMSO to measure the absorbance of the solution at 570 nm using a microplate spectrophotometer (MultiskanGo, Thermo Fisher). Results are expressed

as percentages of OD versus vehicle control cells. IC_{50} values were calculated on the best fitting of dose–response inhibition type curves using GraphPad Prism 5 software.

4.3.3. Biological activity

Final compounds **5a-c** were \geq 98% as reported in Chemistry Section 4.1. Biological evaluation of agonist and antagonist effects was performed by Cerep. Agonist and antagonist effects for ET_A were determined in Human neuroblastoma cell-line SK-N-MC incubated at 22 °C. 100 nM solution of endothelin-1 was used as reference compound (EC₅₀ = 0.67 nM) for agonist effect, solution of BQ-123 (IC₅₀ = 0.62 nM, K_B = 0.082 nM) was used as reference compound for antagonist effect [48]. Agonist and antagonist effects for ET_B were determined in human recombinant CHO-k1 cells incubated at 22 °C. 100 nM solution of endothelin-1 was used as reference compound (EC₅₀ = 0.1 nM) for agonist effect, solution of BQ-788 (IC₅₀ = 0.41 nM, K_B = 0,14 nM) was used as reference compound for antagonist effect [49]. The solutions of test compound were prepared in concentration of 1 nM, 10 nM, 100 nM and 1000 nM. Fluorimetry was used as detection method.

4.4 MetaSiteTM prediction of Site of Metabolism

Figure 3 was produced by MetaSiteTM software. MetaSiteTM is a completely automated procedure that has been developed to predict both the potential inhibition and the site of metabolism for compounds, which are known for being metabolized by human cytochromes. The method is based on flexible Molecular Interaction Fields generated by GRID force field on the CYPs 3D-structure active sites were a substrate could bind. The methodology can be used either to suggest new positions that should be protected in order to avoid certain metabolic profile or to check the suitability of a pro-drug. Moreover, this procedure can be used to determine potential interactions of virtual compounds for early toxicity filtering. The procedure is totally automatic, does not require any user assistance, and only requires the availability of the 3D structure of the enzyme. Once the 3D structure of the compound has been provided, the semi-empirical calculations of charges and

radical abstraction energy assignment, pharmacophoric recognition, descriptor handling and similarity computation are carried out automatically.

Together with Site of Metabolism, MetaSiteTM is also capable to report the docked structure of the substrate in the CYP-cavity. Bosentan was docked in the cavity of CYP2C9 and the amino acids that interact with the compound were shown. The atoms/moieties with the stronger interaction (lower energy values) were highlighted. MetaSiteTM reported that in the 2C9 cavity, bosentan orients the 2-hydroxyethoxy moiety towards Val 292 and the sulfonamide moiety towards Arg 108.

Supplementary Data

Supplementary data related to this article are provided.

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Highlights:

- Bosentan derivatives were designed and synthesized to improve metabolic stability
- Their metabolism in HLM, CYP3A4 and CYP2C9 was determined
- Cytotoxicity and activity against endothelin receptors were studied
- All compounds showed an improved metabolic stability towards CYP2C9
- Compound **5c** displayed a moderate antagonist effect towards ET_A

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