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Original article

A small-molecule cell-based screen led to the identification of biphenylimidazoazines with highly potent and broad-spectrum anti-apicomplexan activity

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Plasmodium falciparum

ABSTRACT

An *in vitro* screening of the anti-apicomplexan activity of 51 compounds, stemming from our chemical library and from chemical synthesis, was performed. As a study model, we used *Toxoplasma gondii* (*T. gondii*), expressing β-galactosidase for the colorimetric assessment of drug activity on parasites cultivated *in vitro*. This approach allowed the validation of a new series of molecules with a biphenylimidazoazine scaffold as inhibitors of *T. gondii* growth *in vitro*. Hence, 8 molecules significantly inhibited intracellular replication of *T. gondii in vitro*, with EC_{50} < 1 μM, while being non-toxic for human fibroblasts at these concentrations. Most attractive candidates were then selected for further biological investigations on other apicomplexan parasites (*Neospora caninum*, *Besnoitia besnoiti*, *Eimeria tenella* and *Plasmodium falciparum*). Finally, two compounds were able to inhibit growth of four different apicomplexans with EC_{50} in the submicromolar to nanomolar range, for each parasite. These data, including the broad anti-parasite spectrum of these inhibitors, define a new generation of potential anti-parasite compounds of wide interest, including for veterinary application. Studies realized on *E. tenella* suggest that these molecules act during the intracellular development steps of the parasite. Further experiments should be done to identify the molecular target(s) of these compounds.

1. Introduction

Apicomplexan protozoa constitute a family of unicellular parasites, which have a worldwide considerable medical, veterinary and economic impact.

In human medicine, two diseases are of major health concern: malaria, generated by *Plasmodium* spp., and toxoplasmosis, caused by *Toxoplasma gondii* (*T. gondii*). Malaria remains one of the most important infectious diseases of the developing world resulting in an estimated 800,000 deaths annually, secondary to the acute failure of one or several major functions (e.g. cerebral injuries) [1]. Toxoplasmosis is a wide-spread ubiquitous zoonosis (nearly one-

Abbreviations: EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; SAR, structure–activity relationship; HFF, human foreskin fibroblasts; *T. g.*, *Toxoplasma gondii*; *N. c.*, *Neospora caninum*; *B. b.*, *Besnoitia besnoiti*; *E. t.*, *Eimeria tenella*; *P. f.*, *Plasmodium falciparum*; MDBK, Madin–Darby bovine kidney.

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third of humanity has been exposed to this parasite) [2]. This infection is, most of the time, asymptomatic but can generate severe disorders in immunocompromised hosts and during pregnancy, leading to serious birth defects (e.g. neurologic and ocular manifestations) [3]. Immunodepression can indeed trigger latent infection reactivation, entailing cerebral toxoplasmosis, which could result in encephalitis, compromising vital prognosis [4].

Infections due to apicomplexan parasites (*T. gondii*, *Neospora caninum*, *Besnoitia* spp., *Eimeria spp.*) are also frequent in livestock of many species (e.g. sheep, cattle and poultry). New-born animals are particularly concerned, with diarrhoea, malformations or abortion. In adults, decreases of meat and milk production, reduced fertility, and death can be observed. These parasites are thus responsible for huge economic losses each year in the world [5–8].

Since the complexity of apicomplexan parasites impaired the development of vaccines, the control and fight against parasite infections mainly rely on chemotherapy. However, the therapeutic arsenal is particularly restricted and, in the case of veterinary applications, mainly consisted of non parasite-specific drugs, which frequently display limited efficiency and high levels of toxicity. Furthermore, the emergence of multi-resistant parasites, especially in *Eimeria* spp. and *Plasmodium* spp., is a major concern [5,7,9–11]. In this context, there is an urgent need for new therapeutic alternatives.

As part of our drug discovery program, the aim of this study was to identify new hit compounds, acting on parasite-specific targets. In this purpose, we first performed a cell-based screening of our chemical library on *T. gondii* growth *in vitro*, which allowed the identification of a new series of biphenylimidazoazine compounds. To obtain molecules with improved anti-parasite activity, we then initiated structure–activity relationship (SAR) studies. With the most attractive candidates, we extended biological tests to other apicomplexans, such as *N. caninum*, *Besnoitia besnoiti*, *Eimeria tenella* and *Plasmodium falciparum*, which have an important impact on animal or human health.

2. Chemistry

The synthetic pathway used to prepare the 3-biphenylimidazoazines unsubstituted on the terminal phenyl was recently reported [12]. Access to compounds **2a**–**m** was thus performed *via* a Suzuki cross-coupling reaction in position 3 of the previously described 3-iodoimidazo[1,2-*b*]pyridazines **1a**, **1c** and **1e** or 3-iodoimidazo[1,2-*a*]pyridines **1b**, **1d**, **1f** and **1g** [12], in classical conditions (1.2 eq of boronic acid, 2 mol% of $Pd(PPh_3)_4$, 2 eq of Na_2CO_3 in DME/H_2O), leading respectively to imidazo[1,2-*b*] pyridazines **2a**–**b**, **2e**–**f** and **2i**–**j** and imidazo[1,2-*a*]pyridines **2c**–**d**, **2g**–**h**, **2k**–**l** and **2m** (Scheme 1, for structures see Tables 2 and 4). Micro-wave irradiation heating was used for derivatives **2c**, **2e** and **2i**.

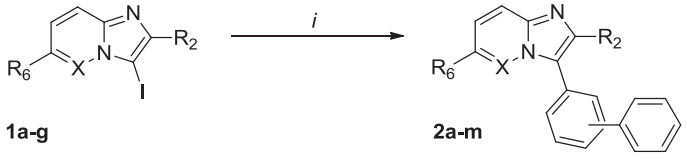
Pharmacomodulation of the biphenyl group in position 3 required another synthetic route (Scheme 2), starting from the 3-(3-bromophenyl)imidazo[1,2-*b*]pyridazines **7a**–**b** and **7d** or the 3-(3-bromophenyl)imidazo[1,2-*a*]pyridines **7c** and **7e**–**g** to give imidazoazines **8a**–**o** by a Suzuki cross-coupling reaction. Synthesis of synthons **7a**–**g** was achieved by condensation between the suitable aminopyridazine **6a** [12] or aminopyridines **6b**–**d** [12,13] and the appropriate α-halogenocarbonyl compounds **5a**–**c** [12]. Intermediates **5a**–**c** were obtained by bromination of the corresponding ketones **4a**–**c** using bromine and hydrobromic acid in chloroform.

Ketones **4a** and **4c** were prepared by action of the conveniently substituted Weinreb benzamides **3a** and **3b**, respectively, on 3-bromobenzylmagnesium bromide in anhydrous diethyl ether to give the corresponding ketones [14]. For the preparation of ketone **4b**, the *N*-methoxy-*N*-methylpivalamide (Weinreb amide of pivalic acid) did not give the expected ketone. Pivalaldedyde was thus used as starting material, to give an alcohol intermediate, which was then oxidised under Swern conditions to obtain the desired ketone.

Finally, compounds **12**, **17** and **21** were synthesized in order to complete the pharmacomodulation studies in position 6 of the imidazoheteroaryle scaffold (Schemes 3–5). Preparation of the imidazo[1,2-*b*]pyridazine **12** was achieved by condensation between 6-phenylpyridazin-3-amine **9** [15] and 1-chloropinacolone in refluxing ethanol, leading to imidazo[1–2-*b*]pyridazine **10**, which, after iodination (using *N*-iodosuccinimide in acetonitrile), followed by a Suzuki cross-coupling reaction in position 3 gave the desired derivative **12** (Scheme 3).

Compound **17** was obtained in 4 steps (Scheme 4): condensation between 6-iodopyridazin-3-amine **13** [16] and 2-bromo-4′-fluoroacetophenone in refluxing *n*-butanol gave the 6-iodoimidazo [1,2-*b*]pyridazine **14** which underwent a Sonogashira cross-coupling reaction, in position 6, in classical conditions (2.55 eq of ethynyltrimethylsilane, 6 mol% of $Pd(PPh_3)_4$, 10 mol% of CuI, 1.16 eq of iPr_2NH in THF), followed by an iodination and a Suzuki cross-coupling (1.4 eq of biphen-3-ylboronic acid, 2.8 mol% of $Pd(PPh_3)_4$, 2 eq of Na_2CO_3 in Toluene/EtOH) in position 3 to give the deprotected compound **17**.

To conclude, access to molecule **21** was achieved, starting from previously described methyl 2-(2-methoxyphenyl)imidazo[1,2-*a*] pyridine-6-carboxylate **18** [17]. As previously, an iodination and a Suzuki cross-coupling reaction in position 3 gave the compound **20** which was then reduced with diisobutylaluminium hydride (DIBAL-H) in THF, leading to derivative **21** (Scheme 5).

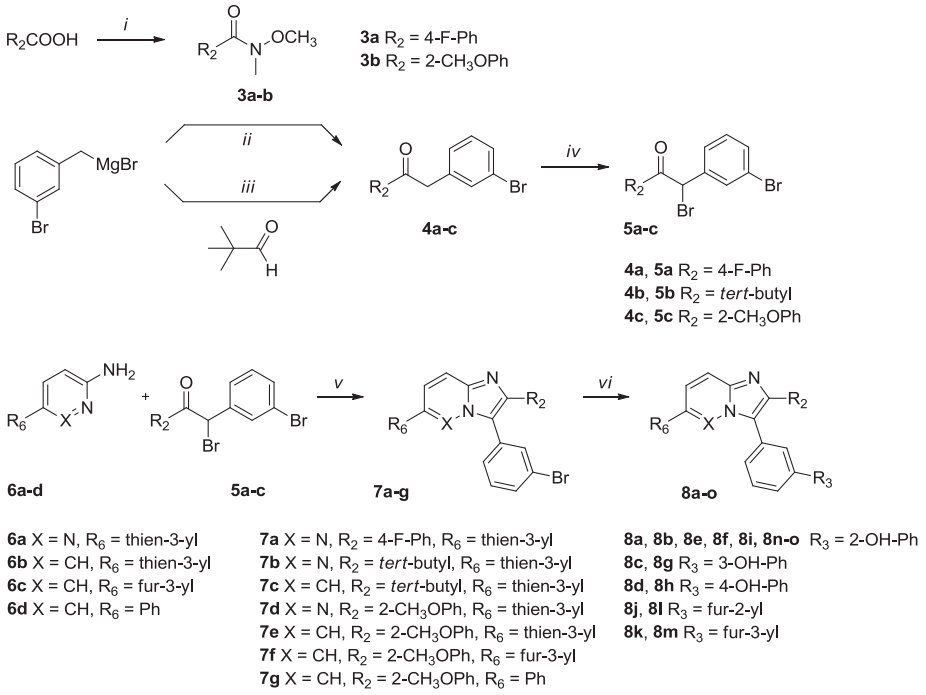


1a-g **2a-m**

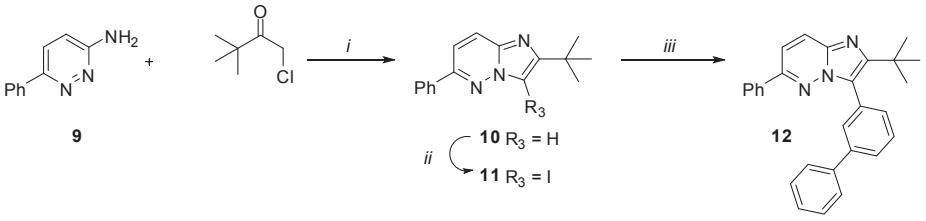
a X = N, R_2 = 4-F-Ph, R_6 = thien-3-yl
b X = CH, R_2 = 4-F-Ph, R_6 = thien-3-yl
c X = N, R_2 = *tert*-butyl, R_6 = thien-3-yl
d X = CH, R_2 = *tert*-butyl, R_6 = thien-3-yl
e X = N, R_2 = 2-CH_3OPh, R_6 = thien-3-yl
f X = CH, R_2 = 2-CH_3OPh, R_6 = thien-3-yl
g X = CH, R_2 = 4-F-Ph, R_6 = SPh

2a-b X = N, R_2 = 4-F-Ph, R_6 = thien-3-yl
2c-d X = CH, R_2 = 4-F-Ph, R_6 = thien-3-yl
2e-f X = N, R_2 = *tert*-butyl, R_6 = thien-3-yl
2g-h X = CH, R_2 = *tert*-butyl, R_6 = thien-3-yl
2i-j X = N, R_2 = 2-CH_3OPh, R_6 = thien-3-yl
2k-l X = CH, R_2 = 2-CH_3OPh, R_6 = thien-3-yl
2m X = CH, R_2 = 4-F-Ph, R_6 = SPh

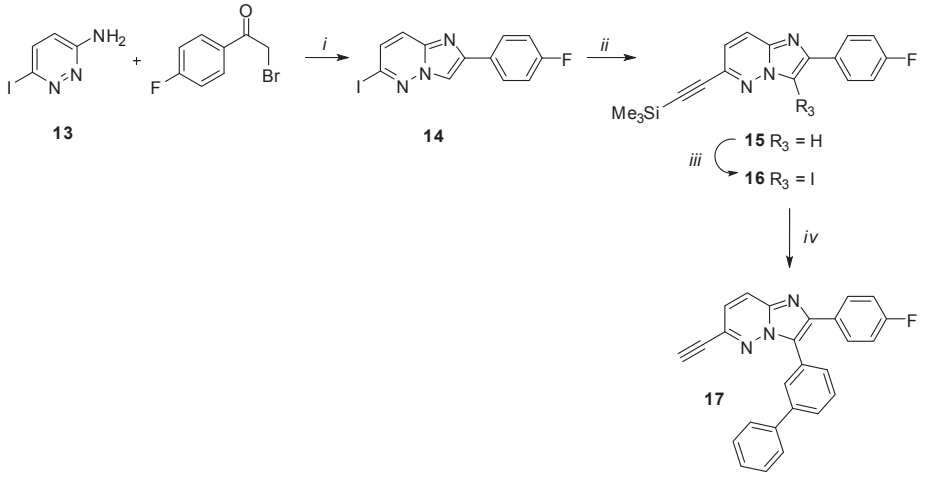
Scheme 1. Reagents and conditions: (*i*) biphenylboronic acids (1.2 eq), $Pd(PPh_3)_4$ (2 mol%), Na_2CO_3 (2 eq), DME/H_2O.



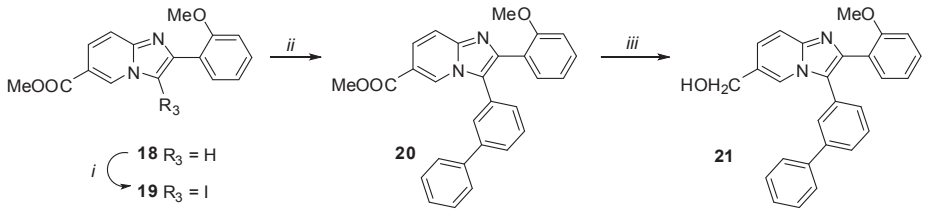
Scheme 2. Reagents and conditions: (*i*) a) $ClCOOCH_2CH_3$ (1 eq), Et_3N (1 eq), CH_2Cl_2; b) $CH_3NHOCH_3.HCl$ (1 eq), Et_3N (1 eq); (*ii*) **3a**–**b** (0.4 eq), Et_2O; (*iii*) a) pivaldehyde (0.85 eq), Et_2O; b) $(COCl)_2$ (1.1 eq), DMSO (2.2 eq), Et_3N (5 eq), CH_2Cl_2; (*iv*) Br_2 (1 eq), HBr (0.1 eq), $CHCl_3$; (*v*) a) DME, rt; b) EtOH, reflux; (*vi*) $R_3B(OH)_2$ (1.2 eq), $Pd(PPh_3)_4$ (2 mol%), Na_2CO_3 (2 eq), DME/H_2O.



Scheme 3. Reagents and conditions: (*i*) EtOH, reflux; (*ii*) NIS (1.7 eq), CH_3CN; (*iii*) biphen-3-ylboronic acid (1.2 eq), $Pd(PPh_3)_4$ (2 mol%), Na_2CO_3 (2 eq), DME/H_2O.



Scheme 4. Reagents and conditions: (*i*) *n*-butanol, reflux; (*ii*) ethynyltrimethylsilane (2.55 eq), $Pd(PPh_3)_4$ (6 mol%), CuI (10 mol%), iPr_2NH (1.16 eq), THF; (*iii*) NIS (1.7 eq), CH_3CN; (*iv*) biphen-3-ylboronic acid (1.4 eq), $Pd(PPh_3)_4$ (2.8 mol%), Na_2CO_3 (2 eq), Toluene/EtOH.



Scheme 5. Reagents and conditions: (*i*) NIS (1.7 eq), CH_3CN; (*ii*) biphen-3-ylboronic acid (3 eq), $Pd(PPh_3)_4$ (2 mol%), Na_2CO_3 (2 eq), DME/H_2O; (*iii*) DIBAL-H (8 eq), THF.

3. Results and discussion

3.1. In vitro screen of T. gondii growth inhibition and unspecific cytotoxicity

The *in vitro T. gondii* growth inhibition assay was performed *via* a focused cell-based screen. The advantage of this type of approach, compared to target-based ones, is that the potential cellular targets are in their native environment. The compounds tested were selected from our chemical library composed of more than 500 compounds containing heterocyclic scaffolds of pharmacological interest and displaying drug-like properties. This library includes molecules that were originally designed to target a variety of kinases [18–20] and receptors [21], but also compounds of unknown targets with anti-viral properties [12,22,23]. We first screened 21 compounds, selected according to toxicity and diversity criteria but also pharmacokinetic and solubility parameters (Table 1). These molecules comprised imidazo[1,2-*a*]pyridines and imidazo[1,2-*b*]pyridazines mainly substituted in position 2, 3 and 6 but also imidazo[2,1-*b*]thiazoles and imidazo[2,1-*b*][1,3,4]thiadiazoles diversely functionalized in position 2, 5 and 6. The screening was based on the *in vitro* culture of a recombinant *T. gondii* expressing bacterial β-galactosidase and a colorimetric microtiter assay, previously described for the high-throughput assessment of anti-*T. gondii* activity [24]. Compounds that displayed *T. gondii* growth

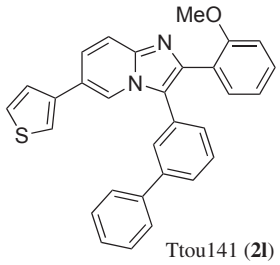
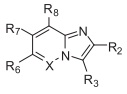
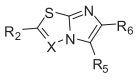


Fig. 1. Chemical structure of the Ttou141 compound.

Table 1
In vitro T. gondii growth inhibition and unspecific cytotoxicity on HFF of first screened imidazoazines.



Name	*X*	R_2	R_3	R_6	R_7	R_8	*T. gondii* EC_{50} (μM)[a]	HFF CC_{50} (μM)[a]
Ca2	CH	4-F-Ph	Pyrazin-2-yl	H	H	H	>10	ND
Cs43	CH	4-F-Ph	Carbamoyl	*N-Tert*-butylcarbamoyl	H	H	>10	ND
Mel284	N	4-F-Ph	Carbamoyl	*N-Tert*-butylcarbamoyl	H	H	>10	ND
Tct1	CH	Ph	4*H*-1,2,4-triazol-4-yl	H	H	H	>10	ND
Tct59	CH	4-F-Ph	Pyridin-4-yl	$-CH_3$	H	4-CH_3OPh	>10	ND
Tct114	N	Pyridin-3-yl	Pyridin-4-yl	*N*-Methylpiperazin-1-yl	H	H	8.26 ± 0.76	ND
Ttou8	N	4-F-Ph	H	4-Benzylpiperidin-1-yl	H	H	>10	ND
Ttou141	CH	2-CH_3OPh	Biphen-3-yl	Thien-3-yl	H	H	0.63 ± 0.02	42.2 ± 5.8
Ttou205	N	*tert*-butyl	I	Ph	H	H	>10	ND
Ttou211	N	4-F-Ph	I	Ph	H	H	>10	ND
Ttou220	CH	4-F-Ph	H	3-Methoxybenzamido	H	H	>10	ND
Ttou232	CH	4-F-Ph	H	H	thien-3-yl	H	>10	ND
Ttou258	CH	4-F-Ph	Cl	3-Methoxybenzamido	H	H	>10	ND
Ttou259	CH	4-F-Ph	Pyridin-4-yl	3-Methoxybenzamido	H	H	>10	ND
Ttou263	CH	4-F-Ph	H	3-Methoxybenzylcarbamoyl	H	H	>10	ND
Ttou264	N	4-F-Ph	H	3-Methoxybenzamido	H	H	>10	ND
Ttou269	CH	*Tert*-butyl	H	3-Methoxybenzamido	H	H	>10	ND



Name	*X*	R_2	R_5	R_6	*T. gondii* EC_{50} (μM)[a]	HFF CC_{50} (μM)[a]
Cs44	CH	*N-Tert*-butylcarbamoyl	Carbamoyl	4-F-Ph	>10	ND
Tct28	CH	H	Pyridin-4-yl	4-F-Ph	0.89 ± 0.11	5.1 ± 0.1
Tct134	N	(3a*R*,6a*S*)-Hexahydropyrrolo[3,4-*c*]pyrrol-2(1*H*)-yl	2-Methylpyridin-4-yl	4-F-Ph	0.62 ± 0.01	5.0 ± 0.0
Tct140	N	4-(Pyrrolidin-1-yl)piperidin-1-yl	2-Methylpyridin-4-yl	4-F-Ph	1.24 ± 0.06	ND

HFF: human foreskin fibroblasts.
ND: not determined.
[a] Values are a mean of $n \geq 3$ independent experiments.

inhibition with EC_{50} < 10 μM were selected. Three of them (Ttou141, Tct28 and Tct134) had an EC_{50} < 1 μM and underwent further toxicity evaluation on human foreskin fibroblasts (HFF) host cells. Cytotoxicity effects were determined by microscopic inspection of HFF treated with the compounds and confirmed by an Alamar Blue reduction assay. While Ttou141 did not inhibit HFF growth at concentrations at which it exerts its antiparasitic activity (EC_{50} = 0.63 ± 0.02 μM, CC_{50} = 42.2 ± 5.8 μM), Tct28 and Tct134 were disqualified due to toxicity against HFF. The imidazo[1,2-*a*]pyridine Ttou141 was consequently identified as a new promising anti-parasite hit compound (Fig. 1).

This molecule was interestingly part of a drug discovery program concerning new antiviral compounds with a biphenylimidazoazine scaffold [12]. To confirm the interest of this series and to initiate SAR analysis, seventeen Ttou141-analogues from our chemical library and thirteen newly synthesized molecules were tested, leading to the identification of 24 products with significant anti-parasite activity (EC_{50} < 10 μM) (Tables 2–4).

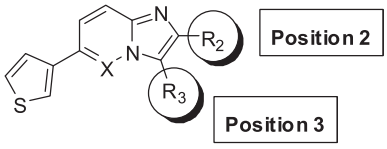
3.2. SAR study on the biphenylimidazoazine series

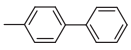
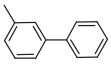
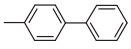
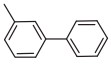
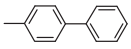
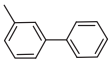
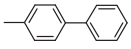
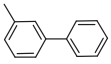
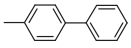
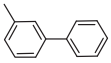
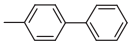
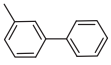
The influence of the substituents located on position 2, 3 and 6 of the imidazoazine ring was investigated and each newly synthesized compound was compared to both the imidazo[1,2-*a*]pyridine and imidazo[1,2-*b*]pyridazine series (Fig. 2).

3.2.1. Initial SAR study on positions 2 and 3 of the imidazo[1,2-a] pyridine and imidazo[1,2-b]pyridazine series (Table 2)

We first studied influence of the type of substituent in position 2 (4-F-Ph, *tert*-butyl or 2-CH_3OPh) according to the nature of the biphenyl group in position 3 (biphen-3-yl or biphen-4-yl) in both imidazo[1,2-*a*]pyridine and imidazo[1,2-*b*]pyridazine series. One of the striking observations made was that minor modification of the biphenyl position, affecting spatial occupation at this site, had significant repercussions on compounds activity. Indeed, replacement of the biphen-3-yl group by a biphen-4-yl moiety, led to a loss of activity in nearly every case (Table 2, entries 1, 3, 5, 7 and 11). Thus, biphen-4-yl compounds **2a**, **2g** and **2k** (EC_{50} of 5.17 ± 0.09 μM, 4.53 ± 0.13 μM and 2.24 ± 0.02 μM, respectively) were less potent

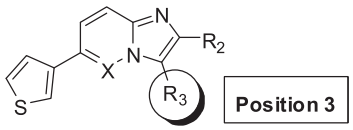
Table 2
SAR study on positions 2 and 3 of the imidazo[1,2-*a*]pyridine and imidazo[1,2-*b*]pyridazine series.

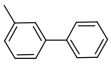
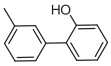
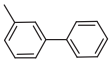
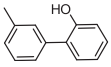
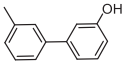
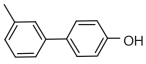
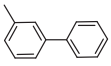
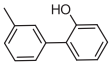
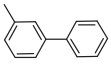
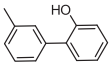
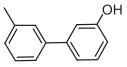
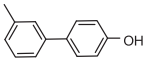
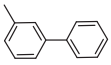
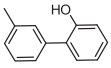
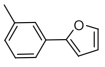
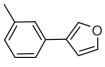
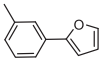
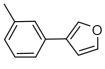


Entry	Compound	X	R_2	R_3	*T. gondii* EC_{50} (μM)[a]	HFF CC_{50} (μM)[a]
1	**2a**	N	4-F-Ph		5.17 ± 0.09	24.1 ± 1.7
2	**2b**	N	4-F-Ph		1.23 ± 0.14	12.5 ± 0.8
3	**2c**	CH	4-F-Ph		>10	ND
4	**2d**	CH	4-F-Ph		2.21 ± 0.03	>50
5	**2e**	N	*Tert*-butyl		>10	ND
6	**2f**	N	*Tert*-butyl		2.23 ± 0.03	17.5 ± 0.0
7	**2g**	CH	*Tert*-butyl		4.53 ± 0.13	>50
8	**2h**	CH	*Tert*-butyl		1.47 ± 0.01	17.5 ± 0.0
9	**2i**	N	2-CH_3OPh		4.80 ± 0.30	>50
10	**2j**	N	2-CH_3OPh		>10	ND
11	**2k**	CH	2-CH_3OPh		2.24 ± 0.02	>50
12	**2l**	CH	2-CH_3OPh		0.63 ± 0.02	42.2 ± 5.8
13	**Pyrimethamine**				0.34 ± 0.02	>50

HFF: human foreskin fibroblasts.
ND: not determined.
[a] Values are a mean of $n \geq 3$ independent experiments.

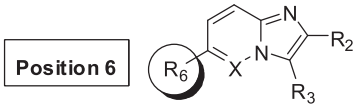
Table 3
Pharmacomodulation of the biphenyl group in position 3 of the imidazo[1,2-*a*]pyridine and imidazo[1,2-*b*]pyridazine series.

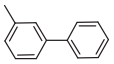
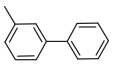
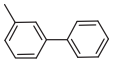
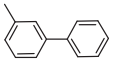
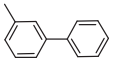
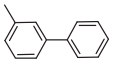
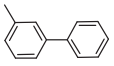
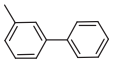
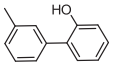
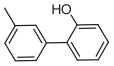
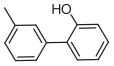


Entry	Compound	*X*	R_2	R_3	*T. gondii* EC_{50} (μM)[a]	HFF CC_{50} (μM)[a]
1	**2b**	N	4-F-Ph		1.23 ± 0.14	12.5 ± 0.8
2	**8a**	N	4-F-Ph		0.61 ± 0.02	>50
3	**2f**	N	*Tert*-butyl		2.23 ± 0.03	17.5 ± 0.0
4	**8b**	N	*Tert*-butyl		0.90 ± 0.05	5.9 ± 0.4
5	**8c**	N	*tert*-butyl		2.21 ± 0.02	5.0 ± 0.0
6	**8d**	N	*Tert*-butyl		1.69 ± 0.14	9.5 ± 2.1
7	**2h**	CH	*Tert*-butyl		1.47 ± 0.01	17.5 ± 0.0
8	**8e**	CH	*Tert*-butyl		0.08 ± 0.01	>50
9	**2j**	N	2-CH_3OPh		>10	ND
10	**8f**	N	2-CH_3OPh		0.36 ± 0.04	31.9 ± 7.1
11	**8g**	N	2-CH_3OPh		0.79 ± 0.11	21.4 ± 9.9
12	**8h**	N	2-CH_3OPh		0.40 ± 0.05	31.8 ± 4.0
13	**2l**	CH	2-CH_3OPh		0.63 ± 0.02	42.2 ± 5.8
14	**8i**	CH	2-CH_3OPh		0.27 ± 0.03	>50
15	**8j**	N	*Tert*-butyl		>10	ND
16	**8k**	N	*Tert*-butyl		1.58 ± 0.38	8.1 ± 3.1
17	**8l**	N	2-CH_3OPh		>10	ND
18	**8m**	N	2-CH_3OPh		>10	ND
19	**Pyrimethamine**				0.34 ± 0.02	>50

HFF: human foreskin fibroblasts.
ND: not determined.
[a] Values are a mean of $n \geq 3$ independent experiments.

Table 4
SAR studies on position 6 of biphenylimidazoazines.



Entry	Compound	X	R_2	R_3	R_6	*T. gondii* EC_{50} (μM)[a]	HFF CC_{50} (μM)[a]
1	**2f**	N	*Tert*-butyl			2.23 ± 0.03	17.5 ± 0.0
2	**12**	N	*Tert*-butyl			1.66 ± 0.04	17.6 ± 0.1
3	**2d**	CH	4-F-Ph			2.21 ± 0.03	>50
4	**2m**	CH	4-F-Ph			4.88 ± 0.21	>50
5	**2b**	N	4-F-Ph			1.23 ± 0.14	12.5 ± 0.8
6	**17**	N	4-F-Ph			2.21 ± 0.16	17.5 ± 0.0
7	**2l**	CH	2-CH_3OPh			0.63 ± 0.02	42.2 ± 5.8
8	**21**	CH	2-CH_3OPh			1.37 ± 0.23	>50
9	**8i**	CH	2-CH_3OPh			0.27 ± 0.03	>50
10	**8n**	CH	2-CH_3OPh			2.04 ± 0.20	>50
11	**8o**	CH	2-CH_3OPh			0.73 ± 0.03	>50
12	**Pyrimethamine**					0.34 ± 0.02	>50

HFF: human foreskin fibroblasts.
[a] Values are a mean of $n \geq 3$ independent experiments.

than their biphen-3-yl isomers **2b**, **2h** and **2l** (4-fold, 3-fold and 3.5-fold, respectively). In the same manner, compounds **2c** and **2e**, bearing a biphen-4-yl moiety, were inactive while their biphen-3-yl analogues (**2d** and **2f**, respectively) displayed micromolar potency against *T. gondii* (EC_{50} of 2.21 ± 0.03 μM and 2.23 ± 0.03 μM, respectively). In only one case, the opposite phenomenon has nevertheless been observed: **2j** was inactive whereas its "biphen-4-yl" analogue, **2i**, exhibited a moderate activity (EC_{50} of 4.80 ± 0.30 μM).

All the active derivatives bearing a non-substituted biphen-3-yl group in position 3 demonstrated similar EC_{50} values (~1–2 μM) suggesting a low influence of the nature of the imidazoheteroaryle

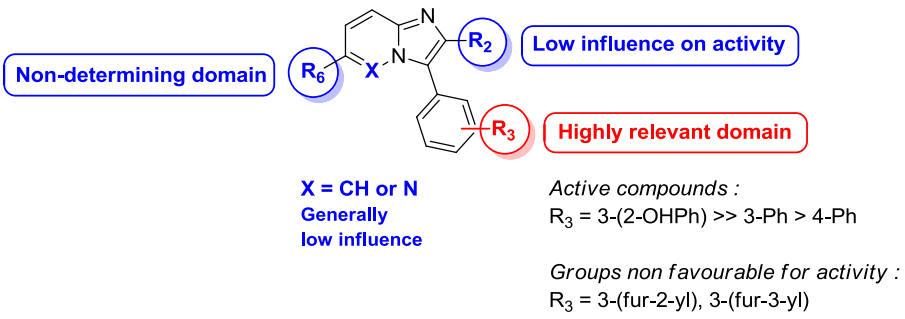
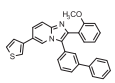
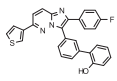
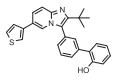
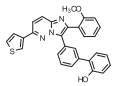
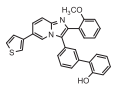


Fig. 2. Summary of major SAR.

Table 5
Structure, *in vitro* cytotoxicity on HFF and antiparasitic potency of most attractive compounds.

		EC_{50} (μM)[a]					CC_{50} (μM)[a]
		Toxoplasma gondii	*Neospora caninum*	*Besnoitia besnoiti*	*Eimeria tenella*[b]	*Plasmodium falciparum*	HFF
2l		0.63 ± 0.02	0.39 ± 0.02	0.25 ± 0.12	Inv.: >10 Growth: 0.84 ± 0.07	7.70 ± 1.90	42.2 ± 5.8
8a		0.61 ± 0.02	0.77 ± 0.04	0.37 ± 0.07	Inv.: >10 Growth: 3.5 ± 0.24	3.42 ± 0.41	>50
8e		0.08 ± 0.01	0.25 ± 0.03	0.37 ± 0.07	Inv.: >10 Growth: 0.43 ± 0.01	1.01 ± 0.12	>50
8f		0.36 ± 0.04	0.35 ± 0.14	0.07 ± 0.003	Inv.: >10 Growth: 6.90 ± 0.24	2.70 ± 0.30	31.9 ± 7.1
8i		0.27 ± 0.03	0.59 ± 0.02	0.32 ± 0.12	Inv.: >10 Growth: 1.7 ± 0.08	3.06 ± 0.55	>50
Pyrimethamine		0.34 ± 0.02	0.68 ± 0.04	1.26 ± 0.33	Inv.: >10 Growth: >10	ND	>50
Chloroquine		ND	ND	ND	ND	0.0139 ± 0.0016	ND

HFF: human foreskin fibroblasts.
ND: not determined.
[a] Values are a mean of $n \geq 3$ independent experiments.
[b] Inv.: invasion assay; Growth: growth assay.

ring and of the group in position 2 on the antiparasitic activity of this series.

3.2.2. *Pharmacomodulation of the biphenyl group in position 3 (Table 3)*

The influence of substituting different positions of the terminal phenyl of the biphen-3-yl moiety was investigated. Remarkably, the addition of a hydroxyl group at the position 2′ in the terminal phenyl of the biphen-3-yl substituent considerably improved the anti-*T. gondii* activity (Table 3, entries 2, 4, 8, 10 and 14). Thus, the five analogues, bearing a 2′-hydroxybiphen-3-yl moiety in position 3 (**8a**, **8b**, **8e**, **8f** and **8i**), showed submicromolar potency against *T. gondii* growth (EC_{50} of 0.61 ± 0.02, 0.90 ± 0.05, 0.08 ± 0.01, 0.36 ± 0.04 and 0.27 ± 0.03 μM, respectively) and were significantly more potent than their non-substituted analogues (**2b**, **2f**, **2h**, **2j** and **2l**, respectively). Two of these compounds (**8e** and **8i**) exerted a higher anti-*T. gondii* activity than the reference drug pyrimethamine. Moreover, **8e** showed particularly exceptional *in vitro* potency against *T. gondii* growth with an EC_{50} of 80 ± 10 nM. These derivatives, with the exception of compound **8b**, were non-toxic against HFF cells (Table 3). The modification of the position of the hydroxyl group in the benzene ring in compounds **8b** and **8f**, from position 2′ to 3′ or 4′, had low influence on activity and cytotoxicity of the derivatives (Table 3, entries 5, 6, 11 and 12).

To explore the role of the terminal phenyl of the biphen-3-yl side group in the antiparasitic activity, we then replaced it by a furyl moiety. This pharmacomodulation mainly produced inactive compounds **8j**, **8l** and **8m**, with the exception of molecule **8k** (EC_{50} of 1.58 ± 0.38 μM), which demonstrated the same level of activity as its "phenyl" analogue (**2f**), but displayed cytotoxicity against HFF cells.

Comparison of efficiency of derivatives **8a** (R_2 = 4-F-Ph), **8b** (R_2 = *tert*-butyl), and **8f** (R_2 = 2-CH_3OPh), confirmed the low influence of the position 2 substituent on antiparasitic activity (Table 3, entries 2, 4 and 10).

Finally, the imidazo[1,2-*a*]pyridine compounds (**8e** and **8i**) were more attractive, in terms of anti-*T. gondii* efficiency and toxicity against HFF cells, than their imidazo[1,2-*b*]pyridazine analogues (**8b** and **8f**, respectively).

All these findings indicate that the position 3 of the imidazo-heteroaryle scaffold represents a highly relevant domain for the anti-parasite activity of these compounds, while the position 2 appearsto have virtually no influence.

3.2.3. *SAR study on position 6 of biphenylimidazoazines*

Several derivatives with heterogenous steric and electronic physicochemical properties were tested to assess the importance of the thien-3-yl group in position 6. As shown in Table 4, the replacement of the thien-3-yl group by phenyl (**12**, EC_{50} = 1.66 ± 0.04 μM) or thiophenyl (**2m**, EC_{50} = 4.88 ± 0.21 μM) groups maintained the anti-parasite activity with EC_{50} in the micromolar range. Moreover, the substitution by diverse alkyl group, like ethynyl (**17**, EC_{50} = 2.21 ± 0.16 μM) or hydroxymethyl (**21**, EC_{50} = 1.37 ± 0.23 μM), did not affect either the potency of these compounds. These data demonstrated that a very broad spectrum of substitutions could be introduced in position 6 without affecting the anti-*T. gondii* activity, highlighting the less decisive role of this site for this application. Finally, the replacement of the thien-3-yl group of the very active compound **8i** (EC_{50} = 0.27 ± 0.03 μM) by a fur-3-yl (**8n**) or a phenyl (**8o**) substituent did not improve its anti-parasite activity (EC_{50} of 2.04 ± 0.20 μM and 0.73 ± 0.03 μM, respectively).

3.3. *Effect of compounds on the growth of other apicomplexans*

The development of broad-spectrum molecules represents a main challenge in veterinary medicine for economic but also toxicity considerations, allowing reduction of the number of drugs administered to the animals. In addition, apicomplexans share significant homologies in terms of metabolic pathways or life cycle properties and could therefore be equally sensitive to compounds

shown to be active on *T. gondii*. For these reasons, we tested our best candidates in term of therapeutic index (**2l**, **8a**, **8e**, **8f** and **8i**) on other parasites that belong to the Apicomplexa phylum (Table 5).

We first focused our attention on two parasites closely related to *T. gondii*, namely *N. caninum* and *B. besnoiti* (causal agents of neosporosis and besnoitiosis, respectively), and drugs were evaluated for growth inhibition of respective tachyzoites. Compounds **2l**, **8a**, **8e**, **8f** and **8i** displayed EC_{50} values in the submicromolar to nanomolar range (Table 5) on *N. caninum* and *B. besnoiti* indicating that antiparasitic activity was conserved on these closely related parasites. Moreover, **2l**, **8e**, **8f** and **8i** were more potent on *N. caninum* than the reference drug pyrimethamine. Concerning *B. besnoiti*, all evaluated drugs exhibited submicromolar activity. Compound **8f** was particularly attractive with an EC_{50} of 70 ± 3 nM on this parasite intracellular replication.

Then, these molecules were tested on the invasion and development steps of *E. tenella*, the etiologic agent of avian coccidiosis. Although the five tested compounds did not show any effect on *E. tenella* invasion, they all displayed an activity on parasite growth. Compounds **8a**, **8f** and **8i** showed moderate potency (EC_{50} > 1 μM) whereas derivatives **2l** and **8e** demonstrated submicromolar activity.

In addition, these compounds were also tested *in vitro* on *P. falciparum* intra-erythrocytic asexual cycle, which forms are responsible for the majority of the mortality and morbidity of malaria in humans. They demonstrated a moderate activity on this parasite, with EC_{50} values >1 μM (Table 5). The most potent compound (molecule **8e**) displayed however an EC_{50} of 1.01 ± 0.12 μM.

The slight decrease of activity observed on *E. tenella* or *P. falciparum* could be explained by biological and structural differences between these parasites and the previously tested ones, which were more closely related, as they were all members of Sarcocystidae family (Coccidia class), while *E. tenella* belongs to the Eimeriidae family (Coccidia class) and *P. falciparum*, to the Haemosporida class. Differences in cellular model (HFF, for *T. gondii*, *N. caninum* and *B. besnoiti versus* MDBK, for *E. tenella* and human erythrocytes, for *P. falciparum*) and, especially, in drug exposure time (96 h for *T. gondii*, *N. caninum* and *B. besnoiti versus* 4 h, for *E. tenella* and 48 h for *P. falciparum*) have also to be taken into account to explain these differences.

4. Conclusion

In this study, we reported the *in vitro* anti-*T. gondii* activity of 51 compounds. We have shown that molecules with a biphenylimidazoazine scaffold display significant inhibition of the *in vitro* growth of the *T. gondii* parasite. Indeed, 8 of these compounds displayed highly potent activity against *T. gondii* growth *in vitro*, with EC_{50} below 1 μM, without demonstrating cytotoxic effects on human fibroblastic cell at equivalent concentrations.

SAR studies in positions 2, 3 and 6 of the imidazoazine scaffold demonstrated the decisive role of the position 3 for the antiparasite activity of these compounds. Moreover, the highest inhibitory activities were observed for the derivatives bearing in position 3 a biphen-3-yl moiety non-substituted or substituted with a hydroxyl group in position 2′ of the terminal phenyl.

Candidates with the best therapeutic indexes were then evaluated on other apicomplexans of veterinary and human health interest. Finally, the most potent imidazo[1,2-*a*]pyridine compound (**8e**) inhibited growth of *T. gondii*, *N. caninum*, *B. besnoiti* and *E. tenella* with EC_{50} in the nanomolar range. This broad-spectrum anti-parasite pattern offers promising perspectives, particularly for veterinary applications. Considering the lack of efficient treatment against besnoitiosis [25,26], the imidazo[1,2-*b*]pyridazine **8f** is particularly attractive in regard to its exceptional potency against *B. Besnoiti in vitro* growth. These compounds could therefore represent new attractive leads in the field of anti-apicomplexan drug research.

Studies realized on *E. tenella* revealed that these compounds did not seem to block the invasion process of host cells, but were more susceptible to act on the parasite intracellular development. Further work will be necessary to identify the parasite target(s) of this new class of compounds. The biotinylation of the most potent compounds, for instance on the position 6 of the imidazoazine scaffold, which seems to be weakly involved in the anti-parasite activity, could represent an appropriate approach to identify the native parasite target (s) [27]. Finally, these compounds have to be evaluated in animal models so as to confirm their activity *in vivo*.

5. Experimental section

5.1. General remarks

All solvents were anhydrous reagents from commercial sources. Unless otherwise noted, all chemicals and reagents were obtained commercially and used without purification. Microwave heating was carried out with a single-mode Discover (CEM) unit. Melting points (Mp) were determined on a Stuart capillary apparatus and are uncorrected. NMR spectra were recorded at 300 MHz (^{1}H) or 75 MHz (^{13}C) on a Bruker Avance (300 MHz) spectrometer or a Bruker DPX instrument (200 MHz). The chemical shifts are reported in parts per million (ppm, δ) relative to residual deuterated solvent peaks. The abbreviations s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet and br s = broad signal were used throughout. For the peak assignation, the following abbreviations were used: Ph = Phenyl, F-Ph = fluorophenyl, Br-Ph = bromophenyl, CH_3OPh = methoxyphenyl, BiPh = biphenyl, Th = thienyl, Fur = furyl, Pyr = pyridinyl; the position of the considered proton on the corresponding heterocycle was indicated by its number after a dash; proton numbering was assigned according to IUPAC nomenclature. The possible inversion of two values in the NMR spectra is expressed by an asterisk. Known compounds were prepared according to literature procedures: bromoketones **5a**–**c** [12], amines **6a** and **6b** [12], amine **6d** [13], amine **9** [15], amine **13** [16], imidazoazines **1a**–**g**, **2a**, **2b**, **2d**, **2f**, 2**h**, **2j**, **2l**, **2m**, **7b**, **7d**, **8b**–**d**, **8f**–**h** and **8j**–**m** [12] and imidazo[1,2-*a*]pyridine **18** [17].

5.2. Chemistry

5.2.1. 3-(Biphen-4-yl)-2-(4-fluorophenyl)-6-(thien-3-yl)imidazo[1,2-a]pyridine (**2c**)

Method A: into a microwave vial were introduced, under argon, the 3-iodoimidazo[1,2-*a*]pyridine **1b** (210 mg, 0.5 mmol), biphen-4-ylboronic acid (119 mg, 0.6 mmol), *tetrakis*(triphenylphosphine) palladium (11.5 mg, 2 mol%), and Na_2CO_3 (106 mg, 1.0 mmol) in 1,2-dimethoxyethane (2 mL) and water (1 mL). The reaction mixture was stirred magnetically at 120 °C for 1 h in a CEM Discover microwave apparatus. After cooling, the suspension was taken up in water, extracted with CH_2Cl_2 three times. The organic layer was dried with $MgSO_4$, and evaporated to dryness. The residue was chromatographed on silica gel eluting with CH_2Cl_2 to give 100% of **2c**. Mp 222 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (br s, 1H, H-5), 7.85–7.27 (m, 16H), 7.03 (*t*, 2H, J = 8.7 Hz, F-Ph-3,5). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.5 (J = 246 Hz), 143.8, 141.9, 141.7, 140.0, 138.1, 131.0 (2 × C), 130.0 (J = 3 Hz), 129.8 (2 × C, J = 8 Hz), 129.0 (2 × C), 128.4 (2 × C), 128.1, 127.9, 127.1 (3 × C), 125.8, 125.6, 122.3, 121.0, 120.9, 119.9, 117.3, 115.4 (2 × C, J = 21 Hz). Anal. Calcd for $C_{29}H_{19}FN_2S$: C, 78.00; H, 4.29; N, 6.27. Found: C, 77.95; H, 4.21; N, 6.19.

*5.2.2. 3-(Biphen-4-yl)-2-tert-butyl-6-(thien-3-yl)imidazo[1,2-b] pyridazine (**2e**)*

Compound **2e** was synthesized following the general method A, using **1c** as starting material (99% yield).

Mp 307 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (d, 1H, J = 9.0 Hz, H-8), 7.78–7.71 (m, 5H), 7.57–7.38 (m, 7H), 7.34 (dd, 1H, J = 5.1, 3.0 Hz, Th-5), 1.43 (s, 9H, 3 × CH_3).^{13}C NMR (75 MHz, $CDCl_3$) δ 147.7, 141.5, 140.5, 137.9, 135.7, 132.3 (2 × C), 129.0, 128.9 (3 × C), 127.7, 127.2 (2 × C), 126.9 (3 × C), 126.1, 124.8, 124.7, 124.3, 116.4, 34.0, 31.1 (3 × C). Anal. Calcd for $C_{26}H_{23}N_3S$: C, 76.25; H, 5.66; N, 10.26. Found: C, 76.29; H, 5.70; N, 10.19.

*5.2.3. 3-(Biphen-4-yl)-2-tert-butyl-6-(thien-3-yl)imidazo[1,2-a] pyridine (**2g**)*

Method B: a screw-cap test tube was charged, under argon, with the 3-iodoimidazo[1,2-*a*]pyridine **1d** (191 mg, 0.5 mmol), biphen-4-ylboronic acid (119 mg, 0.6 mmol), tetrakis(triphenylphosphine) palladium (11.5 mg, 2 mol%), and Na_2CO_3 (106 mg, 1.0 mmol) in 1,2-dimethoxyethane (2 mL) and water (1 mL). The screw-cap test tube was sealed with a cap and the reaction mixture was stirred magnetically at 100 °C for 4h. After cooling, the suspension was taken up in water and extracted with CH_2Cl_2 three times. The organic layer was dried with $MgSO_4$, and evaporated to dryness. The residue was chromatographed on neutral alumina eluting with CH_2Cl_2 to give 89% of **2g**.

Mp 264–266 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.80–7.71 (m, 6H), 7.55–7.32 (m, 8H), 7.19 (dd, 1H, J = 4.8, 1.5 Hz, Th-4), 1.38 (s, 9H, 3 × CH_3).^{13}C NMR (75 MHz, $CDCl_3$) δ 152.5, 142.2, 141.9, 140.2, 138.5, 132.5 (2 × C), 129.9, 129.0 (2 × C), 127.8, 127.7 (2 × C), 127.1 (2 × C), 126.8, 125.9, 124.5, 121.6, 120.6, 120.0, 120.0, 116.7, 33.7, 31.3 (3 × C). Anal. Calcd for $C_{27}H_{24}N_2S$: C, 79.37; H, 5.92; N, 6.86. Found: C, 79.30; H, 5.94; N, 6.81.

*5.2.4. 3-(Biphen-4-yl)-2-(2-methoxyphenyl)-6-(thien-3-yl)imidazo [1,2-b]pyridazine (**2i**)*

Compound **2i** was synthesized following the general method A, using **1e** as starting material (75% yield).

Mp 243 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.08 (d, 1H, J = 9.6 Hz, H-8), 7.89 (dd, 1H, J = 3.0, 1.2 Hz, Th-2), 7.80–7.65 (m, 8H), 7.50–7.34 (m, 6H), 7.08 (td, 1H, J = 7.5, 0.9 Hz, CH_3OPh-5), 6.91 (d, 1H, J = 8.1 Hz, CH_3OPh-3), 3.45 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.9, 147.4, 141.4, 140.7, 140.2, 138.3, 137.9, 131.9, 129.9, 129.1 (2 × C), 128.9 (2 × C), 128.8, 127.4, 127.0 (2 × C), 126.9, 126.6 (2 × C), 126.2, 126.1, 125.4, 124.7, 123.6, 120.9, 115.8, 111.3, 55.0. Anal. Calcd for $C_{29}H_{21}N_3OS$: C, 75.79; H, 4.61; N, 9.14. Found: C, 75.72; H, 4.70; N, 9.19.

*5.2.5. 3-(Biphen-4-yl)-2-(2-methoxyphenyl)-6-(thien-3-yl)imidazo [1,2-a]pyridine (**2k**)*

Compound **2k** was synthesized following the general method B, using **1f** as starting material (76% yield).

Mp 250–252 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.48 (dd, 1H, J = 1.8, 0.9, H-5), 7.82–7.30 (m, 16H), 7.03 (td, 1H, J = 7.5, 0.9 Hz, CH_3OPh-5), 6.86 (d, 1H, J = 8.4 Hz, CH_3OPh-3), 3.42 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.7, 143.9, 141.0, 140.7, 140.3, 138.5, 132.1, 129.5, 129.5 (3 × C), 128.9 (2 × C), 127.6, 127.6 (2 × C), 127.0 (3 × C), 125.9, 124.9, 123.2, 122.8, 122.1, 120.8, 120.7, 119.8, 117.7, 111.0, 54.8. Anal. Calcd for $C_{30}H_{22}N_2OS$: C, 78.57; H, 4.84; N, 6.11. Found: C, 78.54; H, 4.87; N, 6.08.

*5.2.6. 4-Fluoro-N-methoxy-N-methylbenzamide (**3a**)*

Method C: a solution of ethyl chloroformate (3.40 mL, 35.68 mmol) in anhydrous dichloromethane (6 mL) was added dropwise at 10 °C to a solution of 4-fluorobenzoic acid (5.00 g, 35.68 mmol) and triethylamine (5 mL, 35.68 mmol) in anhydrous dichloromethane (50 mL). This reaction mixture was stirred at 10 °C for 50 min, and then *N*,*O*-dimethylhydroxylamine hydrochloride (3.48 g, 35.68 mmol) and triethylamine (5 mL, 35.68 mmol) were added. The resulting mixture was stirred for 1 h at 10 °C and the suspension was taken up in water (150 mL) and extracted with CH_2Cl_2 (150 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was finally chromatographed on silica gel eluting with ethyl acetate:petroleum ether (gradient from 0/100 to 20/80 v/v) to give 77% of **3a** as clear oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (m, 2H, F-Ph-2,6), 7.02 (m, 2H, F-Ph-3,5), 3.47 (d, 3H, J = 1.2 Hz, OCH_3), 3.29 (d, 3H, J = 1.2 Hz, NCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.7, 164.0 (J = 249 Hz), 130.8 (2 × C, J = 9 Hz), 129.8 (J = 3 Hz), 115.0 (2 × C, J = 22 Hz), 61.0, 33.6.

*5.2.7. N,2-Dimethoxy-N-methylbenzamide (**3b**)*

Compound **3b** was synthesized following the general method C, using 2-methoxybenzoic acid as starting material (84% yield).

Mp 49 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (ddd, 1H, J = 9.3, 7.5, 1.8 Hz, H-4), 7.27 (dd, 1H, J = 7.5, 1.8 Hz, H-6), 6.97 (td, 1H, J = 7.5, 0.9 Hz, H-5), 6.93 (dd, 1H, J = 9.3, 0.9 Hz, H-3), 3.84 (s, 3H, OCH_3), 3.49 (br s, 3H, N–OCH_3), 3.33 (br s, 3H, NCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.8, 130.6, 127.7, 125.2, 120.5, 111.1 (2 × C), 61.0, 55.7 (2 × C).

*5.2.8. 2-(3-Bromophenyl)-1-(4-fluorophenyl)ethanone (**4a**)*

Method D: a solution of 4-fluoro-*N*-methoxy-*N*-methylbenzamide **3a** (1.47 g, 8.00 mmol) in anhydrous diethyl ether (28 mL) was added dropwise at 0 °C to a solution of 3-bromobenzylmagnesium bromide in anhydrous diethyl ether (20 mL, 20.01 mmol). The reaction mixture was allowed to warm to room temperature and then stirred overnight. A saturated solution of ammonium chloride (21 mL) was added followed by extraction with ethyl acetate (50 mL). The organic layer was washed with a 1 N citric acid solution (50 mL), a $NaHCO_3$ saturated solution (50 mL) and brine (50 mL). The final organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was finally chromatographed on silica gel eluting with dichloromethane/petroleum ether (20/80 v/v) to give 55% of **4a**.

Mp 64–65 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (m, 2H, F-Ph-2,6), 7.42 (m, 2H, Br-Ph-2,4), 7,23–7.12 (m, 4H, Br-Ph-5,6, F-Ph-3,5), 4.24 (s, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.3, 165.9 (J = 254 Hz), 136.7, 132.8 (J = 4 Hz), 132.6, 131.2 (2 × C, J = 9 Hz), 130.2, 130.1, 128.4, 122.7, 115.9 (2 × C, J = 22 Hz), 44.7.

*5.2.9. 1-(3-Bromophenyl)-3,3-dimethylbutan-2-one (**4b**)*

Pivaldehyde (2.92 g, 34.00 mmol), in anhydrous diethyl ether (34 mL) was added dropwise at 0 °C to a solution of 3-bromobenzylmagnesium bromide in anhydrous diethyl ether (84 mL, 40.02 mmol). The reaction mixture was allowed to warm to room temperature and then stirred overnight. The suspension was, then, taken up in water (100 mL) and extracted with diethyl ether (100 mL) and the organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure to give (4 g, 15.63 mmol) of the crude alcohol, which was then dissolved in anhydrous CH_2Cl_2 and cooled at −40 °C. In parallel, dimethyl sulfoxide (2.68 g, 34.36 mmol) in anhydrous CH_2Cl_2 previously cooled at −50 °C was added into a three-neck round-bottom flask to a solution of oxalyl chloride (2.18 g, 17.17 mmol) in CH_2Cl_2 cooled at −50 °C. The alcohol solution and, then, triethylamine were added dropwise to the mixture. The reaction was allowed to warm to room temperature and stirred overnight. After that, water was added and the organic layer was washed with 1% hydrochloric acid solution (100 mL), Na_2CO_3 saturated solution (100 mL) and brine (100 mL). The final organic layer was dried over $MgSO_4$, filtered and concentrated

under reduced pressure. The crude product was finally chromatographed on silica gel eluting with dichloromethane/petroleum ether (20/80 v/v) to give 46% of **4b** as colorless oil.

^{1}H NMR (200 MHz, $CDCl_3$) δ 7.40 (d, 1H, J = 7.6 Hz, Ph-4), 7.36 (m, 1H, Ph-2), 7.21 (*t*, 1H, J = 7.6 Hz, Ph-5), 7.13 (d, 1H, J = 7.6 Hz, Ph-6), 3.80 (s, 2H, CH_2), 1.24 (s, 9H, 3 × CH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ 211.8, 137.2, 132.5, 129.7, 129.6, 128.3, 122.1, 44.5, 42.5, 26.2 (3 × C).

*5.2.10. 2-(3-Bromophenyl)-1-(2-methoxyphenyl)ethanone (**4c**)*

Compound **4c** was synthesized following the general method D, using *N*,2-dimethoxy-*N*-methylbenzamide **3b** as starting material. The crude residue was chromatographed on silica gel eluting with petroleum ether/ethyl acetate (gradient from 100/0 to 70/30 v/v) to give 71% of **4c** as yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (dd, 1H, J = 7.8, 1.8 Hz, CH_3OPh-6), 7.46 (ddd, 1H, J = 9.0, 7.2, 1.8 Hz, CH_3OPh-4), 7.40–7.33 (m, 2H, Br-Ph-2,5), 7.16 (m, 2H, Br-Ph-4,6), 7.02–6.94 (m, 2H, CH_3OPh-3,5), 4.27 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3).

*5.2.11. 5-(Fur-3-yl)pyridin-2-amine (**6c**)*

A mixture of 5-iodopyridin-2-amine (200 mg, 0.91 mmol), fur-3-ylboronic acid (203 mg, 1.82 mmol), Na_2CO_3 (204 mg, 1.93 mmol) and *tetrakis*(triphenylphosphine)palladium (52 mg, 0.045 mmol) in 1,2-dimethoxyethane (4 mL) and water (2 mL) was stirred magnetically at 120 °C for 30 min in a CEM Discover microwave apparatus. After cooling, the suspension was taken up in water, extracted three times with CH_2Cl_2. The organic layer was dried with $MgSO_4$, and evaporated to dryness. The residue was chromatographed on silica gel eluting with CH_2Cl_2/CH_3OH (100/1 v/v) to give 68% of **6c**.

Mp 139–141 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (d, 1H, J = 2.4 Hz, Pyr-6), 7.60 (br s, 1H, Fur-2), 7.47 (dd, 1H, J = 8.4, 2.4 Hz, Pyr-4), 7.43 (dd, 1H, J = 1.8, 1.5 Hz, Fur-5), 6.58 (dd, 1H, J = 1.8, 0.9 Hz, Fur-4), 6.48 (d, 1H, J = 8.4 Hz, Pyr-3), 4.69 (br s, 2H, NH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.7, 145.1, 143.7, 137.4, 135.6, 123.6, 118.7, 108.8, 108.5.

*5.2.12. 3-(3-Bromophenyl)-2-(4-fluorophenyl)-6-(thien-3-yl) imidazo[1,2-b]pyridazine (**7a**)*

Method E: a mixture of 6-(thien-3-yl)pyridazin-3-amine **6a** (21 mg, 0.12 mmol), 2-bromo-2-(3-bromophenyl)-1-(4-fluorophenyl)ethanone **5a** (53 mg, 0.14 mmol), and 1,2-dimethoxyethane (330 μL) was stirred overnight at room temperature. The solvent was removed under reduced pressure, the resulting residue was then dissolved in ethanol (330 μL) and refluxed overnight. After cooling and concentration, the residue was suspended in water, made alkaline with Na_2CO_3 and extracted three times with CH_2Cl_2. After drying with $MgSO_4$, the organic layers were evaporated to dryness. The residue was chromatographed on silica gel eluting with CH_2Cl_2 to give **7a** in quantitative yield.

Mp 161–164 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (d, 1H, J = 9.6 Hz, H-8), 7.94 (dd, 1H, J = 1.8 Hz, Br-Ph-2), 7.86 (dd, 1H, J = 3.0, 1.5 Hz, Th-2), 7.72–7.64 (m, 3H, Th-4, F-Ph-2,6), 7.60–7.52 (m, 2H, Br-Ph-4,6), 7.50 (d, 1H, J = 9.6 Hz, H-7), 7.44 (dd, 1H, J = 5.1, 3.0 Hz, Th-5), 7.35 (dd, 1H, J = 8.1 Hz, Br-Ph-5), 7.07 (m, 2H, F-Ph-3,5). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.8 (J = 247 Hz), 147.6, 143.1, 138.2, 137.9, 133.2, 131.4, 130.8, 130.2 (2 × C, J = 8 Hz), 130.1, 129.9 (J = 3 Hz), 129.0, 127.1, 126.0, 125.4, 124.8, 123.4, 122.4, 116.7, 115.6 (2 × C, J = 21 Hz).

*5.2.13. 3-(3-Bromophenyl)-2-tert-butyl-6-(thien-3-yl)imidazo[1,2-a]pyridine (**7c**)*

Compound **7c** was synthesized following the general method E, using 5-(thien-3-yl)pyridin-2-amine **6b** and 1-bromo-1-(3-bromophenyl)-3,3-dimethylbutan-2-one **5b** as starting materials. The crude residue was chromatographed on neutral alumina eluting with CH_2Cl_2 to give **7c** in 50% yield.

Mp 193 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.67–7.62 (m, 3H), 7.58 (*t*, 1H, J = 1.5 Hz, Br-Ph-2), 7.42–7.33 (m, 4H), 7.31 (dd, 1H, J = 3.0, 1.5 Hz, Th-2), 7.17 (dd, 1H, J = 5.1, 1.5 Hz, Th-4), 1.32 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.1, 142.4, 138.3, 135.0, 133.5, 132.4, 130.8, 130.6, 126.9, 125.9, 124.6, 123.0, 121.7, 120.7, 119.7, 118.6, 116.9, 33.7, 31.3 (3 × C).

*5.2.14. 3-(3-Bromophenyl)-2-(2-methoxyphenyl)-6-(thien-3-yl) imidazo[1,2-a]pyridine (**7e**)*

Compound **7e** was synthesized following the general method E, using 5-(thien-3-yl)pyridin-2-amine **6b** and 2-bromo-2-(3-bromophenyl)-1-(2-methoxyphenyl)ethanone **5c** as starting materials. The crude residue was chromatographed on neutral alumina eluting with CH_2Cl_2 to give **7e** in 16% yield.

Mp 241 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (br s, 1H, H-5), 7.72 (dd, 1H, J = 9.3, 0.6 Hz, H-8), 7.62 (dd, 1H, J = 7.5, 1.8 Hz, CH_3OPh-6), 7.59 (br s, 1H, Br-Ph-2), 7.53–28 (m, 8H), 7.03 (td, 1H, J = 7.5, 0.9 Hz, CH_3OPh-5), 6.84 (d, 1H, J = 7.8 Hz, CH_3OPh-3), 3.40 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.5, 143.7, 140.8, 138.1, 132.5, 131.9 (2 × C), 131.2, 130.5, 129.9, 127.9, 127.2, 125.9, 125.5, 122.9, 122.6, 122.2, 121.5, 121.1, 120.8, 119.5, 117.6, 111.0, 54.8.

*5.2.15. 3-(3-Bromophenyl)-6-(fur-3-yl)-2-(2-methoxyphenyl) imidazo[1,2-a]pyridine (**7f**)*

Compound **7f** was synthesized following the general method E, using 5-(fur-3-yl)pyridin-2-amine **6c** and 2-bromo-2-(3-bromophenyl)-1-(2-methoxyphenyl)ethanone **5c** as starting materials. The crude residue was chromatographed on neutral alumina eluting with CH_2Cl_2 to give **7f** in 18% yield.

Mp 99–100 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (dd, 1H, J = 1.5, 0.9 Hz, H-5), 7.76–7.72 (m, 2H), 7.63–7.50 (m, 4H), 7.40–7.30 (m, 4H), 7.03 (td, 1H, J = 7.5, 1.2 Hz, CH_3OPh-5), 6.84 (d, 1H, J = 8.4 Hz, CH_3OPh-3), 6.62 (dd, 1H, J = 1.8, 0.9, Fur-4), 3.41 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.5, 144.2, 143.5, 138.9, 132.3, 131.9, 131.9, 131.3, 130.6, 130.0, 129.2, 127.9, 127.1, 125.1, 122.9, 122.8, 121.4, 120.8, 119.2, 118.8, 117.6, 111.0, 108.5, 54.8.

*5.2.16. 3-(3-Bromophenyl)-2-(2-methoxyphenyl)-6-phenylimidazo [1,2-a]pyridine (**7g**)*

Compound **7g** was synthesized following the general method E, using 5-phenylpyridin-2-amine **6d** and 2-bromo-2-(3-bromophenyl)-1-(2-methoxyphenyl)ethanone **5c** as starting materials. The crude residue was chromatographed on neutral alumina eluting with CH_2Cl_2 to give **7g** in 47% yield.

Mp 255–256 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (dd, 1H, J = 1.8, 0.9 Hz, H-5), 7.77 (dd, 1H, J = 9.3, 0.9 Hz, H-8), 7.64 (dd, 1H, J = 7.5, 1.8 Hz, CH_3OPh-6), 7.61–7.29 (m, 11H), 7.04 (td, 1H, J = 7.5, 1.2 Hz, CH_3OPh-5), 6.84 (d, 1H, J = 7.5 Hz, CH_3OPh-3), 3.41 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.5, 144.1, 141.5, 137.5, 132.8, 132.0, 131.9, 131.0, 130.5, 129.7, 129.1 (2 × C), 127.9, 127.9, 127.3, 127.1 (2 × C), 125.6, 122.9, 122.8, 121.6, 120.8, 120.2, 117.7, 111.0, 54.7.

*5.2.17. 2-(4-Fluorophenyl)-3-(2′-hydroxybiphen-3-yl)-6-(thien-3-yl)imidazo[1,2-b]pyridazine (**8a**)*

Compound **8a** was synthesized following the general method A, using imidazo[1,2-*b*]pyridazine **7a** and (2-hydroxyphenyl)boronic acid as starting materials (100% yield).

Mp 233–235 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.06 (d, 1H, J = 9.3 Hz, H-8), 7.87 (dd, 1H, J = 2.7, 1.2 Hz, Th-2), 7.82 (br s, 1H, BiPh-2), 7.76–7.40 (m, 10H), 7.29–7.22 (m, 1H), 7.09–6.96 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.0 (J = 248 Hz), 152.8, 148.4, 138.1, 137.4 (2 × C), 132.2, 132.0 (3 × C), 131.1, 130.4, 130.3 (J = 3 Hz), 129.7,

129.5 (2 × C, J = 9 Hz), 129.3, 128.6, 128.5, 127.5, 127.2, 126.0, 125.4, 124.8, 120.8, 116.2, 115.8 (2 × C, J = 21 Hz). Anal. Calcd for $C_{28}H_{18}FN_3OS$: C, 72.55; H, 3.91; N, 9.07. Found: C, 72.50; H, 3.97; N, 9.08.

5.2.18. 3-(2′-Hydroxybiphen-3-yl)-2-tert-butyl-6-(thien-3-yl) imidazo[1,2-a]pyridine (**8e**)

Compound **8e** was synthesized following the general method A, using imidazo[1,2-*a*]pyridine **7c** and (2-hydroxyphenyl)boronic acid as starting materials (81% yield).

Mp 260–261 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 (br s, 1H), 7.75–71 (m, 2H), 7.67–7.59 (m, 3H), 7.42–7.15 (m, 7H), 7.09–6.99 (m, 2H), 1.34 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.8, 152.5, 141.9, 139.5, 138.3, 132.9, 130.8, 130.6, 130.3, 130.0, 129.8, 129.3, 128.1, 127.4, 126.2, 124.2, 121.7, 121.0, 120.4, 120.1, 119.8, 117.1, 116.7, 33.9, 31.6 (3 × C). Anal. Calcd for $C_{27}H_{24}N_2OS$: C, 76.38; H, 5.70; N, 6.60. Found: C, 76.30; H, 5.73; N, 6.56.

5.2.19. 3-(2′-Hydroxybiphen-3-yl)-2-(2-methoxyphenyl)-6-(thien-3-yl)imidazo[1,2-a]pyridine (**8i**)

Compound **8i** was synthesized following the general method B, using imidazo[1,2-*a*]pyridine **7e** and (2-hydroxyphenyl)boronic acid as starting materials (42% yield).

Mp 231–235 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.58 (s, 1H, OH), 8.62 (s, 1H, H-5), 7.94–6.81 (m, 17H), 3.28 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 157.0, 154.8, 143.6, 141.3, 139.5, 138.2, 132.0, 130.7, 130.0, 129.9, 129.7, 129.2, 129.1, 129.0, 128.2, 127.7, 127.3, 126.3, 124.9, 124.2, 123.3, 122.0, 121.7, 120.8, 120.0, 119.8, 117.7, 116.7, 112.0, 55.0. Anal. Calcd for $C_{30}H_{22}N_2O_2S$: C, 75.93; H, 4.67; N, 5.90. Found: C, 75.89; H, 4.68; N, 5.86.

5.2.20. 6-(Fur-3-yl)-3-(2′-hydroxybiphen-3-yl)-2-(2-methoxyphenyl)imidazo[1,2-a]pyridine (**8n**)

Compound **8n** was synthesized following the general method A, using imidazo[1,2-*a*]pyridine **7f** and (2-hydroxyphenyl)boronic acid as starting materials. Three equivalents of (2-hydroxyphenyl) boronic acid were used and the mixture was stirred magnetically at 120 °C for 30 min. The crude residue was chromatographed on silica gel eluting with CH_2Cl_2 to give compound **8n** in 62% yield.

Mp 159–160 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.01 (br s, 1H, OH), 8.46 (s, 1H, H-5), 7.81 (s, 1H, BiPh-2), 7.64–6.75 (m, 15H), 6.54 (s, 1H, Fur-2), 3.34 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.8, 154.5, 143.8, 143.5, 140.0, 139.3, 138.7, 132.0, 130.3, 130.2, 129.6 (2 × C), 129.2, 128.9, 128.8, 127.9, 127.7, 124.5, 123.3, 122.7, 122.5, 120.5, 119.9, 119.3, 118.7, 116.9, 116.8, 111.0, 108.4, 54.7. Anal. Calcd for $C_{30}H_{22}N_2O_3$: C, 78.59; H, 4.84; N, 6.11. Found: C, 78.63; H, 4.85; N, 6.07.

5.2.21. 3-(2′-Hydroxybiphen-3-yl)-2-(2-methoxyphenyl)-6-phenylimidazo[1,2-a]pyridine (**8o**)

Compound **8o** was synthesized following the general method A, using imidazo[1,2-*a*]pyridine **7g** and (2-hydroxyphenyl)boronic acid as starting materials. Three equivalents of (2-hydroxyphenyl) boronic acid were used. The crude residue was obtained by recrystallization in diethyl ether to give **8o** in 22% yield.

Mp 215–216 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (s, 1H, OH), 8.14 (br s, 1H, H-5), 7.80–6.78 (m, 19H), 3.50 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.8, 154.2, 139.9, 132.2, 132.0 (2 × C), 131.5, 131.2, 130.7, 130.1, 130.0, 129.9, 129.8, 129.4 (3 × C), 128.6 (2 × C), 128.5, 128.0, 127.0 (2 × C), 126.7, 123.2, 120.9, 120.7, 120.2, 117.3, 115.5, 115.0, 111.4, 55.2. Anal. Calcd for $C_{32}H_{24}N_2O_2$: C, 82.03; H, 5.16; N, 5.98. Found: C, 82.01; H, 5.19; N, 5.95.

5.2.22. 2-Tert-butyl-6-phenylimidazo[1,2-b]pyridazine (**10**)

A mixture of 6-phenylpyridazin-3-amine (500 mg, 2.92 mmol) and 1-chloropinacolone (0.8 mL, 5.13 mmol) in ethanol (10 mL) was refluxed for 24 h under magnetic stirring. The solvent was then removed under reduced pressure and the resulting residue was suspended in water, made alkaline with Na_2CO_3 and extracted with CH_2Cl_2. After drying with $MgSO_4$, the organic layers were evaporated to dryness. The residue was purified by flash chromatography on silica gel eluting with CH_2Cl_2/petroleum ether (50/50 v/v) to give **10** in 81% yield.

Mp 98–100 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (d, 1H, J = 9.6 Hz, H-8), 7.92 (m, 2H, Ph-2,6), 7.81 (s, 1H, H-3), 7.54–7.43 (m, 4H, H-7, Ph-3,4,5), 1.45 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.3, 151.4, 137.7, 135.7, 129.9, 129.0 (2 × C), 126.9 (2 × C), 124.7, 116.0, 111.9, 32.6, 30.1 (3 × C).

5.2.23. 2-Tert-butyl-3-iodo-6-phenylimidazo[1,2-b]pyridazine (**11**)

Method F: a mixture of imidazo[1,2-*b*]pyridazine **10** (101 mg, 0.40 mmol) and *N*-iodosuccinimide (153 mg, 0.68 mmol) in acetonitrile (1 mL) was stirred overnight at room temperature. The resulting solid was filtered off, washed with 10 mL of petroleum ether and chromatographed on silica gel eluting with CH_2Cl_2 to give **11** in 70% yield.

Mp 160–161 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.12–8.07 (m, 2H, Ph-2,6), 7.96 (d, 1H, J = 9.4 Hz, H-8), 7.63–7.53 (m, 4H, H-7, Ph-3,4,5), 1.63 (s, 9H, 3 × CH_3).

5.2.24. 3-(Biphen-3-yl)-2-tert-butyl-6-phenylimidazo[1,2-b] pyridazine (**12**)

Compound **12** was synthesized following the general method B, using imidazo[1,2-*b*]pyridazine **11** and biphen-3-ylboronic acid as starting materials. The mixture was stirred overnight and the crude residue was purified by flash chromatography on silica gel eluting with CH_2Cl_2/MeOH (60/40 v/v) to obtain **12** in 64% yield.

Mp 145–146 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (dd, 1H, J = 9.3, 1.5 Hz, BiPh-4), 7.84–7.36 (m, 15H), 1.42 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.0, 150.9, 141.0, 140.7, 136.0, 135.8, 131.2, 130.8, 130.7, 129.7, 128.9 (4 × C), 128.7, 127.5, 127.4, 127.2 (2 × C), 126.9 (2 × C), 124.9, 124.6, 115.6, 34.1, 31.2 (3 × C). Anal. Calcd for $C_{28}H_{25}N_3$: C, 83.34; H, 6.24; N, 10.41. Found: C, 83.39; H, 6.20; N, 10.40.

5.2.25. 2-(4-Fluorophenyl)-6-iodoimidazo[1,2-b]pyridazine (**14**)

A mixture of 6-iodopyridazin-3-amine (15.2 g, 68.80 mmol) and 2-bromo-4′-fluoroacetophenone (17.9 g, 82.56 mmol) in *n*-butanol (220 mL) was refluxed overnight under magnetic stirring. The solvent was then removed under reduced pressure and the resulting residue was suspended in $CHCl_3$ and basified with a saturated Na_2CO_3 solution. After drying with $MgSO_4$, the organic layers were evaporated to dryness. The resulting solid was washed with diethyl ether to give **14** in 66% yield.

Mp 214 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (s, 1H, H-3), 7.93 (m, 2H, F-Ph-2,6), 7.64 (dd, 1H, J = 9.3, 0.6 Hz, H-8), 7.33 (d, 1H, J = 9.3 Hz, H-7), 7.15 (m, 2H, F-Ph-3,5). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.2 (J = 247 Hz), 145.0, 137.8, 128.8, 127.9 (2 × C, J = 8 Hz), 127.4, 125.2, 116.0 (2 × C, J = 22 Hz), 112.4, 110.6.

5.2.26. 2-(4-Fluorophenyl)-6-((trimethylsilyl)ethynyl)imidazo[1,2-b]pyridazine (**15**)

Into a dry single-neck round bottom flask was introduced compound **14** (500 mg, 1.47 mmol), *tetrakis*(triphenylphosphine) palladium (88.2 mg, 6 mol%) and copper(I) iodide (147 mg, 10 mol %). The flask was evacuated and backfilled with argon and diisopropylamine (2.4 mL, 1.70 mmol), ethynyltrimethylsilane (367 mg, 3.75 mmol) and THF (4 mL) were added. This suspension was

energetically stirred at room temperature for 5 h. After this time, was added a saturated ammonium chloride solution (25 mL) and the suspension was extracted three times with CH_2Cl_2. The organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The resulting solid was finally washed with diethyl ether to give compound **15** in 99% yield as a yellow solid.

Mp 210 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (s, 1H, H-3), 7.94 (m, 2H, F-Ph-2,6), 7.89 (d, 1H, J = 9.3 Hz, H-8), 7.19–7.12 (m, 3H, H-7, F-Ph-3,5), 0.32 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.2 (J = 247 Hz), 145.9, 138.3, 138.1, 129.1 (J = 3 Hz), 127.9 (2 × C, J = 8 Hz), 124.4, 121.0, 115.9 (2 × C, J = 22 Hz), 112.5, 99.5, 99.3, −0.4 (3 × C).

5.2.27. 2-(4-Fluorophenyl)-3-iodo-6-((trimethylsilyl)ethynyl) imidazo[1,2-b]pyridazine (**16**)

Compound **16** was synthesized following the general method F, using imidazo[1,2-*b*]pyridazine **15** as starting material (81% yield).

Mp 199 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (m, 2H, F-Ph-2,6), 7.84 (d, 1H, J = 9.3 Hz, H-8), 7.25–7.17 (m, 3H, H-7, F-Ph-3,5), 0.33 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.2 (J = 247 Hz), 147.6, 140.8, 138.8, 130.3 (2 × C, J = 8 Hz), 129.1 (J = 3 Hz), 124.3, 122.2, 115.5 (2 × C, J = 22 Hz), 100.2, 99.5, 67.4, −0.4 (3 × C).

5.2.28. 3-(Biphen-3-yl)-6-(ethynyl)-2-(4-fluorophenyl)imidazo [1,2-b]pyridazine (**17**)

A screw-cap test tube was charged, under argon, with the compound **16** (100 mg, 0.23 mmol), biphen-3-ylboronic acid (65 mg, 0.33 mmol), tetrakis(triphenylphosphine)palladium (7.4 mg, 2.8 mol %) and 2 N Na_2CO_3 (0.46 mL, 0.46 mmol) in toluene (1.4 mL) and ethanol (0.5 mL). The screw-cap test tube was sealed with a cap and the reaction mixture was stirred magnetically at 88 °C for 6 h. The solvent was then removed under reduced pressure and the resulting residue was taken up in water and extracted with CH_2Cl_2 three times. The organic layer was dried with $MgSO_4$, evaporated to dryness and chromatographed on silica gel eluting with CH_2Cl_2/MeOH (100/1 v/v) to give 22% of **17**.

Mp 137–142 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97 (d, 1H, J = 9.3 Hz, H-8), 7.81–7.33 (m, 11H, F-Ph-2,6, biPh), 7.19 (d, 1H, J = 9.3 Hz, H-7), 7.07 (dd, 2H, J = 8.7 Hz, F-Ph-3,5), 3,27 (s, 1H, H ethynyl). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.9 (J = 247 Hz), 143.5, 141.8, 140.6, 137.7, 137.3, 130.8, 130.3 (2 × C, J = 8 Hz), 129.8 (J = 3 Hz), 129.4, 129.3, 128.9 (2 × C), 128.6, 127.8, 127.6, 127.2 (2 × C), 125.2, 124.8, 120.9, 115.6 (2 × C, J = 21 Hz), 80.5, 79.5. Anal. Calcd for $C_{26}H_{16}FN_3$: C, 80.19; H, 4.14; N, 10.79. Found: C, 80.07; H, 4.24; N, 10.75.

5.2.29. Methyl 3-iodo-2-(2-methoxyphenyl)imidazo[1,2-a] pyridine-6-carboxylate (**19**)

Compound **19** was synthesized following the general method F, using imidazo[1,2-*a*]pyridine **18** as starting material (99% yield).

Mp 182–183 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.93 (dd, 1H, J = 1.5, 0.9 Hz, H-5), 7.94 (dd, 1H, J = 9.3, 1.5 Hz, H-7), 7.88 (dd, 1H, J = 9.3, 0.9 Hz, H-8), 7.49 (ddd, 1H, J = 7.5, 1.8 Hz, CH_3OPh-4), 7.42 (dd, 1H, J = 7.5, 1.8 Hz, CH_3OPh-6), 7.13–7.04 (m, 2H, CH_3OPh-3,5), 4.02 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.4, 164.5, 157.2, 147.1, 132.4, 131.5, 130.7, 127.0, 120.5, 119.8, 118.5, 116.9, 111.4, 66.3, 55.5, 52.9.

5.2.30. Methyl 3-(biphenyl-3-yl)-2-(2-methoxyphenyl)imidazo[1,2-a]pyridine-6-carboxylate (**20**)

Compound **20** was synthesized following the general method B, using imidazo[1,2-*a*]pyridazine **19** and biphen-3-ylboronic acid as starting materials. Three equivalents of biphen-3-ylboronic acid were used and the mixture was stirred magnetically at 100 °C for 30 min. The crude residue was chromatographed on neutral alumina eluting with CH_2Cl_2 to give compound **20** in 66% yield.

Mp 100–102 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.05 (br s, 1H, H-5), 7.80–7.31 (m, 13H), 7.04 (dd, 1H, J = 7.2 Hz, CH_3OPh-5), 6.85 (d, 1H, J = 8.4 Hz, CH_3OPh-3), 3.91 (s, 3H, OCH_3), 3.37 (s, 3H, OCH_3).

5.2.31. 3-((Biphen-3-yl)-2-(2-methoxyphenyl)imidazo[1,2-a] pyridin-6-yl)methanol (**21**)

To a solution of methyl ester **20** (200 mg, 0.46 mmol) in anhydrous THF (5 mL) was added dropwise at 0 °C, under argon atmosphere, a solution of diisobutylaluminium hydride (3 mL, 3.68 mmol) in hexane. The reaction mixture was allowed to warm to room temperature and then stirred 1 h. After this time, water was added to the mixture and the suspension was extracted three times with CH_2Cl_2. The organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude residue was finally purified by silica gel column chromatography eluting with CH_2Cl_2/MeOH (100/1 v/v) to give 70% of compound **21**.

Mp 115–117 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (br s, 1H, H-5), 7.61–7.27 (m, 12H), 7.14 (d, 1H, J = 9.0 Hz, H-7), 6.97 (dd, 1H, J = 7.5 Hz, CH_3OPh-5), 6.79 (d, 1H, J = 8.1 Hz, CH_3OPh-3), 5.31 (br s, 1H, OH), 4.60 (s, 2H, CH_2), 3.33 (s, 3H, CH_3). NMR (75 MHz, $CDCl_3$) δ 156.6, 144.1, 141.8, 140.5, 132.0, 130.8, 129.6, 129.3, 128.9 (2 × C), 127.8, 127.6, 127.6, 127.0 (3 × C), 126.7, 126.6, 125.1, 123.0, 122.9, 120.8, 120.7, 117.1, 111.0, 62.3, 54.7. Anal. Calcd for $C_{27}H_{22}N_2O_2$: C, 79.78; H, 5.46; N, 6.89. Found: C, 79.69; H, 5.47; N, 6.84.

5.3. Biology

5.3.1. Parasites

T. gondii RH-β-gal is the RH strain carrying the *Escherichia coli lacZ* gene (coding for β-galactosidase) under the control of the *T. gondii sag1* promoter. *T. gondii* RH-β-gal, *N. caninum* (NC-1 strain), and *B. besnoiti* (isolate collected in the South of France) were maintained in confluent human foreskin fibroblasts (HFF) in Dulbecco's modified Eagle's medium (DMEM, Pan Biotech GmbH) with 5% fetal calf serum (FCS) and 2 mM glutamine at 37 °C with 5% CO_2. *E. tenella* YFP + parasites, carrying YFP gene under the control of *E. tenella mic1* promotor were produced by infecting PA12 chickens of 4–6 weeks of age with sporulated oocysts (10^4 per bird). Seven days post inoculation, unsporulated oocysts were harvested from infected caeca and purified as previously described [28]. To trigger sporulation, oocysts were suspended in water containing 2% (w/v) potassium dichromate and incubated at 26 °C for 72 h. Oocysts walls were broken with glass beads and sporocysts were incubated for about 1 h at 41 °C in excystation medium [0.25% (w/v) trypsin, 0.5% (w/v) biliary salts, in 10 mM PBS, pH 7.4]. Sporozoites were then purified on both cotton and 5 μm polycarbonate filters (Whatman, USA). *P. falciparum* reference clone 3D7 was cultured *in vitro* in RPMI 1640 medium containing L-glutamine, 25 mM HEPES (Invitrogen) supplemented with 10% decomplemented human serum (AB+), 100 μM hypoxanthine (c.c.pro GmbH), and 50 μg/mL gentamycin (Sigma). Parasites were cultured at 37 °C in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere.

5.3.2. In vitro inhibition of T. gondii growth

HFF were seeded in 96-well plates at 2 × 10^4 cells per well in 100 μL DMEM (without phenol red) with 1% FCS and 2 mM glutamine (Pan Biotech GmbH) at 37 °C in 5% CO_2 atmosphere. After 24 h, tachyzoites of the *T. gondii* RH-β-gal strain (100/50 μL per well) were then added followed by the molecules to be tested (stock solution at 4 mM in DMSO) diluted at different concentrations in 50 μL medium [maximum 0.1% (v/v) DMSO]. Pyrimethamine (Dr. Ehrenstorfer GmbH, stock solution at 8 mM in DMSO) was used as reference for *T. gondii* growth inhibition, and the non-

infected cells were used as negative control. All conditions were tested in 4 wells. The plates were then incubated at 37 °C in 5% CO_2 atmosphere for 96 h. At this time, lysis solution (Triton X-100 at 0.1%, Sigma) was added to release the β-galactosidase. After that, β-galactosidase substrate solution (chlorophenol red-β-D-galactopyranoside at 1 mM, Sigma) in 100 mM HEPES pH 8 (Pan Biotech GmbH) was added. The plates were afterward incubated at 37 °C until appearance of a red coloration. Finally, optical density (OD) was measured at 565 nm on a microtiter plate reader (BioTek Instruments, Inc.). OD was proportionally correlated to the parasite load of the well. β-galactosidase activity obtained in the presence of the molecules was compared to the activity of non-treated parasites. When a dose-dependent activity was observed, a regression line was obtained and EC_{50} was then calculated. Data were collected from three independent experiments and the values reported are means ± standard errors of the mean. Compounds with EC_{50} values of greater than 10 μM were considered inactive.

5.3.3. In vitro inhibition of N. caninum and B. besnoiti growth

HFF were seeded in 6-well plates at 3×10^5 cells per well in 3200 μL DMEM with 1% FCS and 2 mM glutamine at 37 °C in 5% CO_2 atmosphere. After 24 h, tachyzoites (1600 per well in 400 μL) and the drugs (stock solution at 4 mM in DMSO) at different concentrations (400 μL per well) were added. All conditions were tested in 3 wells. The plates were then incubated at 37 °C in 5% CO_2 atmosphere for 96 h. At this time, tachyzoites were freed by scraping HFF and enumerated microscopically with a Malassez chamber [29]. When a dose-dependent activity was observed, a regression line was obtained and EC_{50} was then calculated. Data were collected from three independent experiments and the values reported are means ± standard errors of the mean.

5.3.4. In vitro inhibition of E. tenella cell invasion and growth

Madin–Darby bovine kidney (MDBK) cells were dispensed into 24-well plates at a density of 2×10^5 cells per well in 500 μL Ham's F12 medium containing 10% (v/v) fetal bovine serum (FCS). The plates were incubated 3 h (invasion test) or overnight (development test) at 41 °C in a 5% CO_2 incubator during which time confluent monolayers developed. Freshly excysted purified YFP + sporozoites (3×10^5 per well in 500 μL) were treated for 1 h at room temperature with Ham's F12 medium containing the molecules to be tested [maximum 0.1% (v/v) DMSO]. Culture medium was removed from the confluent monolayers of MDBK cells and the treated sporozoites were immediately added to the cells. Plates were incubated at 41 °C in a 5% CO_2 incubator. For the invasion test, cells were washed twice in PBS 4 h post infection, fixed with 2.7% (w/v) PFA at 4 °C for 20 min and washed again in PBS. Cover slips were mounted in Vectashield containing DAPI to label the nuclei. Randomly selected fields were imaged using fluorescent microscopy (Zeiss Axiovert 200 microscope, Carl Zeiss, Germany) to determine parasite invasion. The percentage of intracellular parasites was determined as the number of intracellular YFP + parasites for 100 MDBK cells (blue nuclei). More than 300 cells were counted for each condition in three independent experiments with two wells per condition, and the values reported are means ± standard errors of the mean. For the growth test, cells were washed twice in PBS 4 h post infection, supplied with fresh medium containing 1% (v/v) FCS and incubated overnight. The medium was discarded and renewed twice per day with Ham's F12 medium containing 1% (v/v) FCS. After 72 h of infection, the infected cells were washed twice in PBS and fixed. Parasite development was assessed as follows. The percentage of developed stages (schizonts) was determined as the number of schizonts divided by the total number of sporozoites and schizonts. More than 300 cells were counted for each condition in three independent experiments with two wells per condition, and the values reported are means ± standard errors of the mean. When a dose-dependent activity was observed, a regression line was obtained and EC_{50} was then calculated.

5.3.5. In vitro inhibition of P. falciparum growth

Quantitative assessment of the antimalarial activity of the selected compounds was performed as previously described [30,31] on an asynchronous culture with 0.5% parasitemia and 1% hematocrit, and with 10 μM hypoxanthine in culture medium. EC_{50} on the parasite growth was determined by following nonlinear regression analysis with HN-NonLin V1.1 software (http://malaria.farch.net).

5.3.6. Cytotoxicity assay

HFF (2×10^4 cells) were cultivated in 96-well plates in DMEM (without phenol red) with 5% FCS, 2 mM glutamine and the different compounds to be evaluated at different concentrations in triplicate. Non-treated cells were used as negative control. Plates were afterward incubated at 37 °C in 5% CO_2 atmosphere for 96 h and 50 μL of medium were then removed from each well. UptiBlue™ (10 μL, Interchim) was then added and the plates were incubated in the dark at 37 °C with 5% CO_2 for 4 h. The appearance of a pink color showed a non-cytotoxic effect. OD was measured at 565 nm and 630 nm to calculate the reduction of the oxidation-reduction indicator. When a dose-dependent activity was observed, a regression line was obtained and CC_{50} was then calculated. Data were collected from three independent experiments and the values reported are means ± standard errors of the mean. Compounds with CC_{50} values of less than 10 μM were considered cytotoxic.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.10.057.

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