Bioorganic & Medicinal Chemistry 21 (2013) 4102–4111

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification of 1,6-dihydropyrazolo[4,3-*c*]carbazoles and 3,6-dihydropyrazolo[3,4-*c*]carbazoles as new Pim kinase inhibitors

Virginie Suchaud, Laurent Gavara, Emmanuelle Saugues, Lionel Nauton, Vincent Théry, Fabrice Anizon *, Pascale Moreau *

Clermont Université, Université Blaise Pascal, Institut de Chimie de Clermont-Ferrand, BP 10448, 63000 Clermont-Ferrand, France
CNRS, UMR 6296, ICCF, BP 80026, 63171 Aubière, France

ARTICLE INFO

Article history:
Received 10 April 2013
Revised 6 May 2013
Accepted 8 May 2013
Available online 16 May 2013

Keywords:
Pyrazolocarbazoles
Kinase inhibitors
Pim kinases
In vitro antiproliferative activities

ABSTRACT

New 1,6-dihydropyrazolo[4,3-*c*]carbazoles and 3,6-dihydropyrazolo[3,4-*c*]carbazoles were prepared and evaluated for their Pim kinase inhibitory potencies as well as their antiproliferative activities toward two prostatic cancer cell lines. Pyrazolocarbazole **15a** was found to be a potent Pim kinase modulator with inhibitory potency toward the three isoforms. Compound **6c** strongly inhibited Pim-3 with weaker effect toward Pim-1 and Pim-2, and thus could be used as an interesting molecular tool to study Pim-3 biological functions.

1. Introduction

Due to the essential biological role of Pim kinases in carcinogenesis, the identification of new Pim inhibitors appeared as a major concern during the last ten years. This led to the discovery of a large variety of chemical scaffolds that demonstrated various inhibitory potencies and selectivity toward Pim in regard to other protein kinases. Accordingly, a number of recent reviews reported the Pim kinase inhibitors published in the patent or nonpatent literature.[1–5] Early reported inhibitors were only active toward one or two but not against all Pim isoforms (Pim-1, Pim-2, Pim-3). Nowadays, several pan-Pim inhibitors have been identified and studied to evaluate their therapeutic efficiency.[6–8] Only two Pim kinase inhibitors have been submitted to clinical trials. Supergen discontinued the phase I clinical development of SGI-1776 due to cardiac toxicity linked to hERG inhibition. Astrazeneca is currently recruiting patients for a phase I clinical trial studying the pharmacokinetics and efficacy in acute myelogenous leukemia of pan-Pim inhibitor AZD1208.[9–12] Actually, despite intensive work in this field and the major interest of Pim kinases as biological targets in the development of new anticancer agents, no Pim inhibitor has reached the market to date. Thus, as part of our ongoing program targeting the identification of biologically active compounds and more particularly protein kinase modulators, we focused on the identification of new series of Pim inhibitors. Nitrogen-containing aromatic heterocyclic systems are of interest for the development of protein kinase inhibitors. For example, we showed the great potential of indole and indazole nuclei and we recently described the synthesis and Pim inhibitory potencies of diversely substituted pyrazolo[3,4-*g*]quinoxalines, pyrrolo[2,3-*a*]carbazoles and pyrrolo[2,3-*g*]indazoles[13–19] (Fig. 1). In this paper, we report the synthesis and Pim kinase inhibitory potency of new compounds showing fused indolic and indazolic moieties (Fig. 1). Moreover, as over-expression of Pim kinases has already been observed in prostatic tumors, the in vitro antiproliferative activity of the synthesized compounds was studied toward two prostatic cancer cell lines: PC3 (androgen independent cells) and LnCAP (androgen dependent cells).

2. Results and discussion

2.1. Chemistry

The synthesis of 1,6-dihydropyrazolo[4,3-*c*]carbazoles was performed from indazole derivative **2** which was previously described by our group (Scheme 1).[20] For the construction of the pyrazolo[4,3-*c*]carbazole system, we first envisaged a Suzuki cross-coupling between 7-iodoindazole **2** and 2-halophenylboronic acid

* Corresponding authors. Tel.: +33 (0) 473405364; fax: +33 (0) 473407717 (F.A.); tel.: +33 (0) 473407963; fax: +33 (0) 473407717 (P.M.).
E-mail addresses: Fabrice.ANIZON@univ-bpclermont.fr (F. Anizon), Pascale.MOREAU@univ-bpclermont.fr (P. Moreau).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.05.011

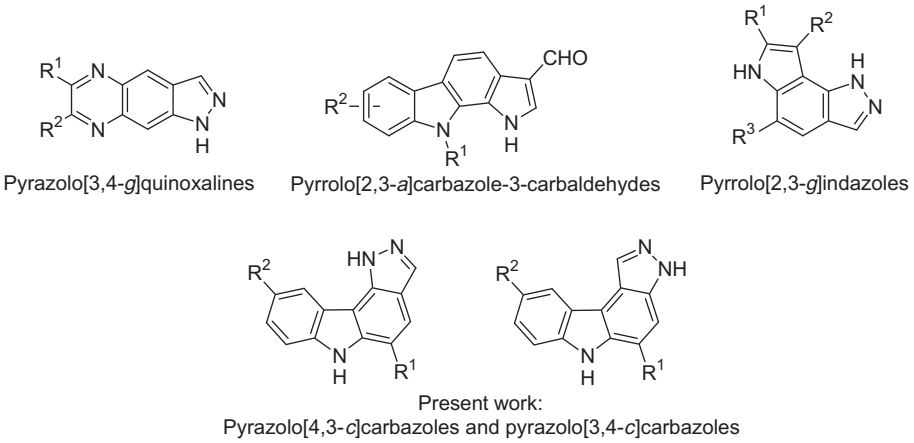
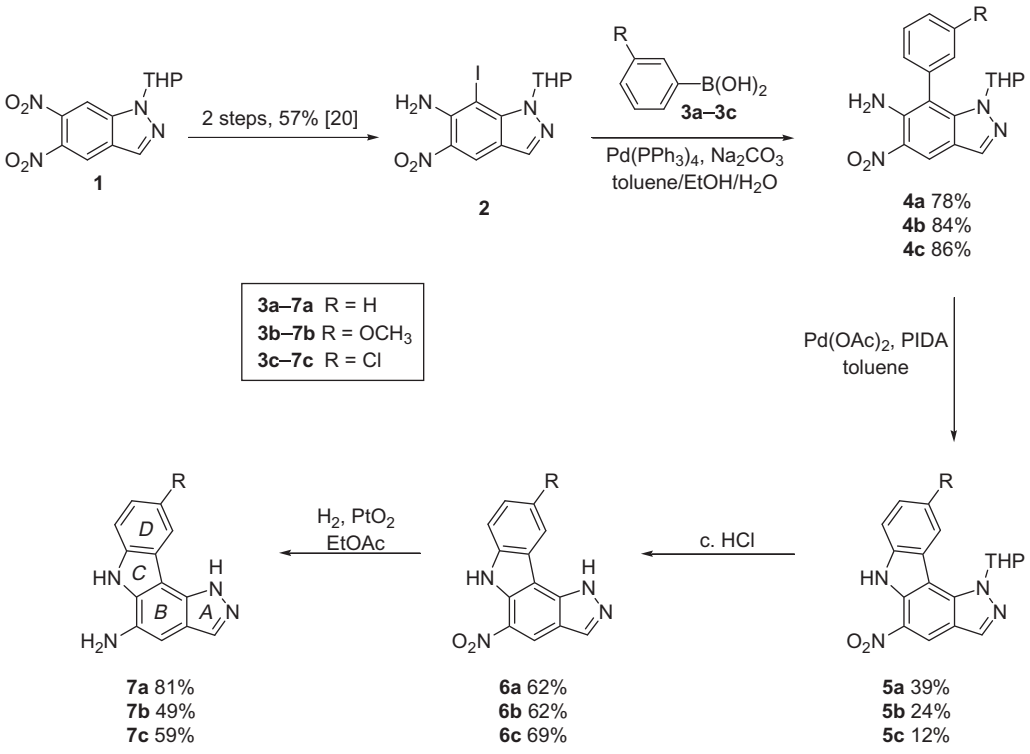


Figure 1. Structure of various Pim-kinase inhibitor scaffolds previously/presently described by our group.

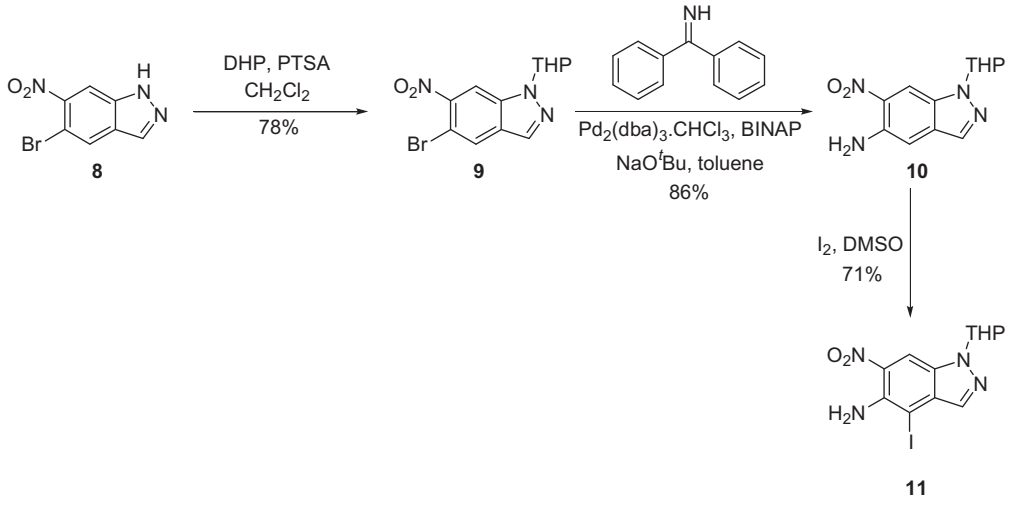


Scheme 1. Synthesis of 1,6-dihydropyrazolo[4,3-*c*]carbazoles **5**, **6** and **7**.

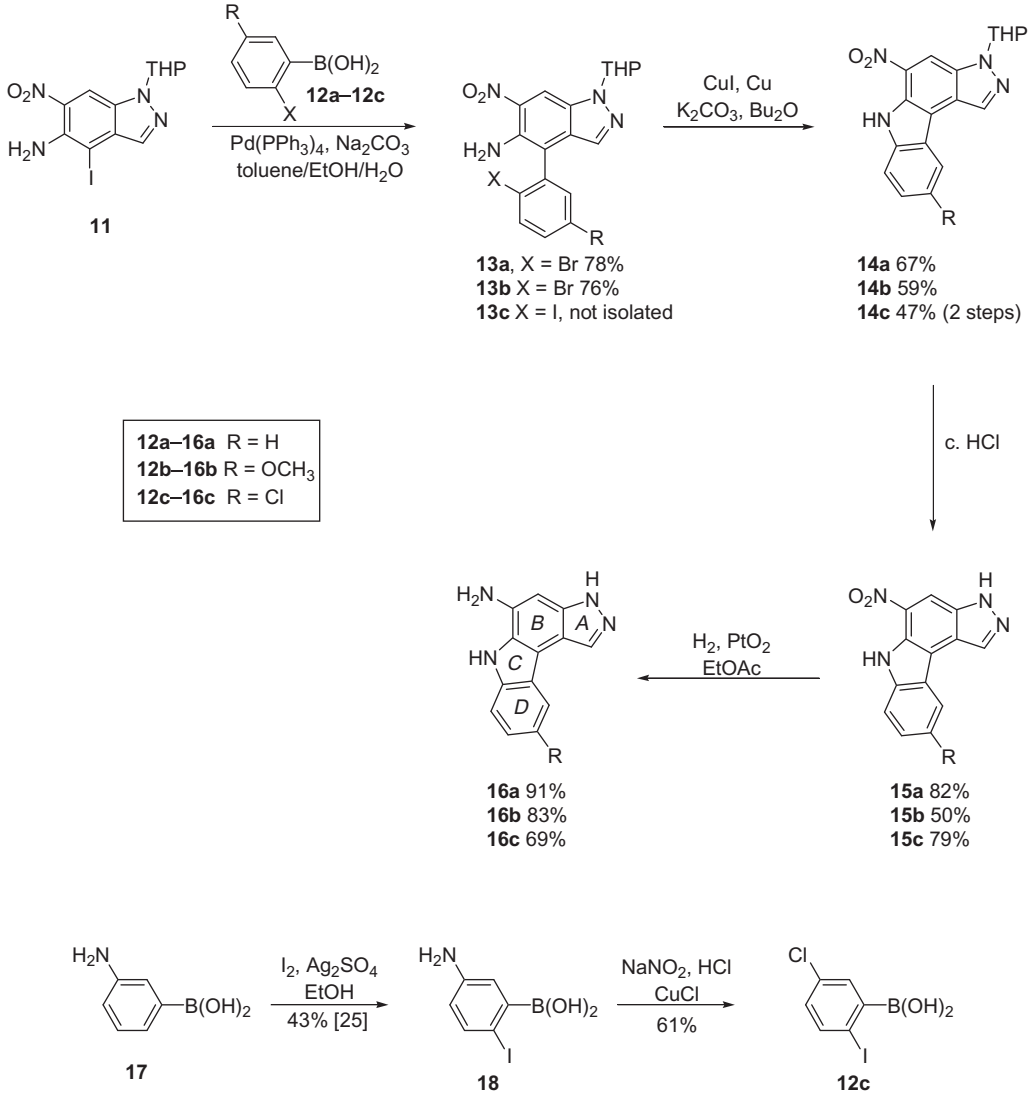
derivatives, followed by an Ullmann-type copper-mediated cyclization to form the carbazole 5-membered ring. However, none of our attempts using 2-bromophenylboronic and 2-chlorophenylboronic acids led to the desired compounds due to the formation of de-iodinated indazole product. Nevertheless, the Suzuki cross-coupling between **2** and arylboronic acids **3a**–**3c** successfully afforded aryl-substituted products **4a**–**4c** in 78–86% yield. Interestingly, the ^{1}H and ^{13}C NMR spectra of compounds **4b** and **4c** in DMSO-d_6 clearly showed the presence of two detectable conformers, probably due to the restricted rotation around the newly formed C–C bond. We next successfully performed the cyclization of **4a**–**4c** by using palladium acetate and PIDA[21] to obtain carbazole derivatives **5a**–**5c** in modest yields. Finally, THP protecting group was removed in refluxing concentrated hydrochloric acid, leading to compounds **6a**–**6c**, and a final hydrogenation of the nitro group provided compounds **7a**–**7c**.

The construction of isomeric pyrazolo[3,4-*c*]carbazole system was performed from 4-iodoindazole **11** (Scheme 2). Compound **11** was obtained in low yield during the preparation of compound **2** from **1**.[20] However, in order to facilitate the preparation of compound **11**, we developed another synthetic pathway to this compound. Therefore, compound **11** was prepared from 5-bromo-6-nitroindazole **8** which was already reported in the literature.[22] After protection of N1 with a THP group in the presence of dihydropyrane and PTSA,[23] Buchwald–Hartwig coupling at the five-position with benzophenone imine and subsequent hydrolysis,[24] **10** was obtained in 86% yield. Finally, iodination in the presence of I_2 in DMSO afforded **11** in 71% yield.

Indazole **11** was then engaged in a Suzuki cross-coupling with phenylboronic acids (Scheme 3). In this case, we were able to perform the reaction with 2-halophenylboronic acids **12a**–**12c**. Whereas **12a** is commercially available, compound **12b** was



Scheme 2. Optimized synthesis of indazole **11**.



Scheme 3. Synthesis of 3,6-dihydropyrazolo[3,4-*c*]carbazoles **14**–**16** and arylboronic acid **12c**.

prepared according to literature procedure[25] and **12c** was prepared from commercially available 3-aminophenylboronic acid **17** after iodination[25] and Sandmeyer reaction of **18** in the presence of CuCl. Compounds **13a** and **13b** were isolated in good yields. In the case

of **13c**, the product was engaged directly in the next step due to purification problems. As it was previously observed for compounds **4b** and **4c**, the ^{1}H and ^{13}C NMR spectra of **13a** and **13b** in DMSO-d_6 showed signal pairs indicating the presence of two conformers. Next, cyclization of compounds **13a–13c** was performed in the presence of CuI/Cu to give pyrazolocarbazoles **14a–14c**, which were subsequently deprotected as described above. Finally, hydrogenation of the nitro group afforded compounds **16a–16c** in good yields.

2.2. Pim kinase inhibitory potencies

The potency of compounds **6a–c**, **7a–c**, **15a–c** and **16a–c** to inhibit Pim kinases (Pim-1, Pim-2, Pim-3) was evaluated at 10 μM and 1 μM concentrations in duplicate assays by the International Centre for Kinase Profiling (Dundee, UK) as previously described.[26] The percentages of residual kinase activities are reported in Table 1. IC_{50} values were only determined when the remaining kinase activity was less than 45% when the compounds were tested at 1 μM.

As shown in Table 1, in both series nitro derivatives are more active than their amino counterparts, except for the chlorinated 3,6-dihydropyrazolo[3,4-*c*]carbazoles for which the amino analog **16c** exhibited a better activity toward Pim-3 than **15c** bearing a nitro group. Even if **15a**, the unsubstituted nitro 3,6-dihydropyrazolo [3,4-*c*]carbazole was found to be the most active derivative tested, the two regioisomer series showed similar inhibition potential, with Pim-1 and Pim-3 being the most inhibited isoforms. The Pim kinase inhibitory profile of the tested compounds is not much influenced by the nature of the substituent present at the position nine of the pyrazolocarbazole scaffold. Except **7a**, **7c** and **16a**, all the compounds tested efficiently inhibited either Pim-1 and/or Pim-3 at more than 50% when tested at 1 μM. Two of them are particularly interesting. First of all, **15a** was found to be a potent Pim kinase modulator that strongly inhibited Pim-1 and Pim-3 with IC_{50} values of 0.04 μM and 0.10 μM, respectively. Next, **6c** strongly inhibited Pim-3 with an IC_{50} value of 0.09 μM and led to more than 50% of Pim-1 and Pim-2 residual activities when tested at 10 μM. This compound could be a useful tool to study Pim-3 biological functions in regards to the ones of Pim-1 and Pim-2.

2.3. In vitro antiproliferative activities

Finally, in vitro antiproliferative activities of compounds **6a–c**, **7a–c**, **15a–c** and **16a–c** were evaluated toward two prostatic cancer cell lines (PC3 and LnCAP) using the colorimetric MTS assay by the ICSN cellular target screening platform (Gif-sur-Yvette, France).[27] All the compounds were firstly tested at 10 μM concentration. IC_{50} values were determined when the growth inhibition was found to be more than 70% with compounds tested at 10 μM (Table 1). As shown in Table 1, most of the compounds tested have been more active toward androgen independent PC3 cells. All derivatives demonstrated 40–60% of PC3 cell proliferation inhibition, except compounds **6b**, **7a** and **7b**. The percentage of growth inhibition was found to be more than 70% only for two compounds tested at 10 μM toward PC3 cells: **15a**, that is the best Pim-1 inhibitor of this series, inhibited the growth of PC3 cells with an IC_{50} value of 3 μM while **15b** demonstrated an IC_{50} value of 2.30 μM.

2.4. Molecular modeling experiments

We next carried out molecular modeling experiments in order to get an insight into the possible binding mode of these compounds in the ATP-binding pocket of Pim-1 and Pim-3 kinases. Therefore, we decided to perform the docking study using **6b** and **15a**, the best Pim-1/Pim-3 inhibitors for each pyrazolocarbazole regioisomeric series.

For these docking experiments, Pim-1 model was generated from 3JPV X-ray crystal structure available in the Protein Data Bank (PDB),[13] and Pim-3 model was constructed by homology to 1XWS Pim-1 crystal structure, with the same method we reported previouly,[19] using Modeller9V11, UCSF Chimera,[28–31] and Sybylx2.0.[32] The docking experiments were then performed with Sybylx2.0 for compounds **6b** and **15a** in either Pim-1 or Pim-3 models, and the best solution for each complex was minimized. The docking solutions found are depicted in Figure 2.

Table 1
Biological activities for compounds **6a–c**, **7a–c**, **15a–c**, **16a–c**: % of residual kinase activity at 10 μM and 1 μM, and antiproliferative activities as inhibition % of cellular proliferation at 10 μM (IC_{50} (μM) in brackets when determined)

Compd	Kinase inhibitory potencies						Antiproliferative activities	
	Pim-1		Pim-2		Pim-3		PC3	LNCaP
	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM		
6a	26 ± 1	44 ± 4 (0.4 ± 0.1)	46 ± 4	76 ± 2	16 ± 1	22 ± 5 (0.21 ± 0.08)	59 ± 4	45 ± 4
6b	39 ± 2	41 ± 9 (0.22 ± 0.05)	72 ± 0	77 ± 6	23 ± 0	28 ± 0 (0.11 ± 0.01)	29 ± 4	19 ± 1
6c	60 ± 8	69 ± 1	93 ± 16	90 ± 8	38 ± 1	50 ± 9 (0.09 ± 0.05)	51 ± 1	28 ± 3
7a	54 ± 2	98 ± 5	78 ± 12	116 ± 13	20 ± 3	63 ± 18	31 ± 3	28 ± 9
7b	43 ± 6	93 ± 6	70 ± 12	120 ± 0	14 ± 2	46 ± 17 (0.7 ± 0.6)	28 ± 1	8 ± 8
7c	29 ± 5	85 ± 2	79 ± 1	115 ± 6	14 ± 2	55 ± 1	43 ± 2	35 ± 1
15a	15 ± 1	14 ± 2 (0.04 ± 0.03)	38 ± 3	49 ± 2	17 ± 3	28 ± 5 (0.10 ± 0.06)	85 ± 1 (3 ± 1)	38 ± 3
15b	13 ± 3	29 ± 19 (0.15 ± 0.02)	35 ± 3	54 ± 1	11 ± 1	21 ± 1 (0.11 ± 0.00)	74 ± 3 (2.30 ± 0.01)	27 ± 1
15c	39 ± 2	48 ± 4 (0.2 ± 0.1)	54 ± 6	57 ± 3	19 ± 4	26 ± 2 (0.23 ± 0.03)	50 ± 3	0 ± 1
16a	16 ± 5	61 ± 2	71 ± 7	96 ± 2	20 ± 4	66 ± 3	56 ± 3	56 ± 2
16b	23 ± 3	70 ± 5	54 ± 6	123 ± 30	16 ± 7	51 ± 6 (0.6 ± 0.5)	46 ± 1	39 ± 1
16c	8 ± 1	45 ± 3 (1.1 ± 0.1)	34 ± 10	107 ± 1	7 ± 3	28 ± 0 (0.11 ± 0.06)	45 ± 2	27 ± 4

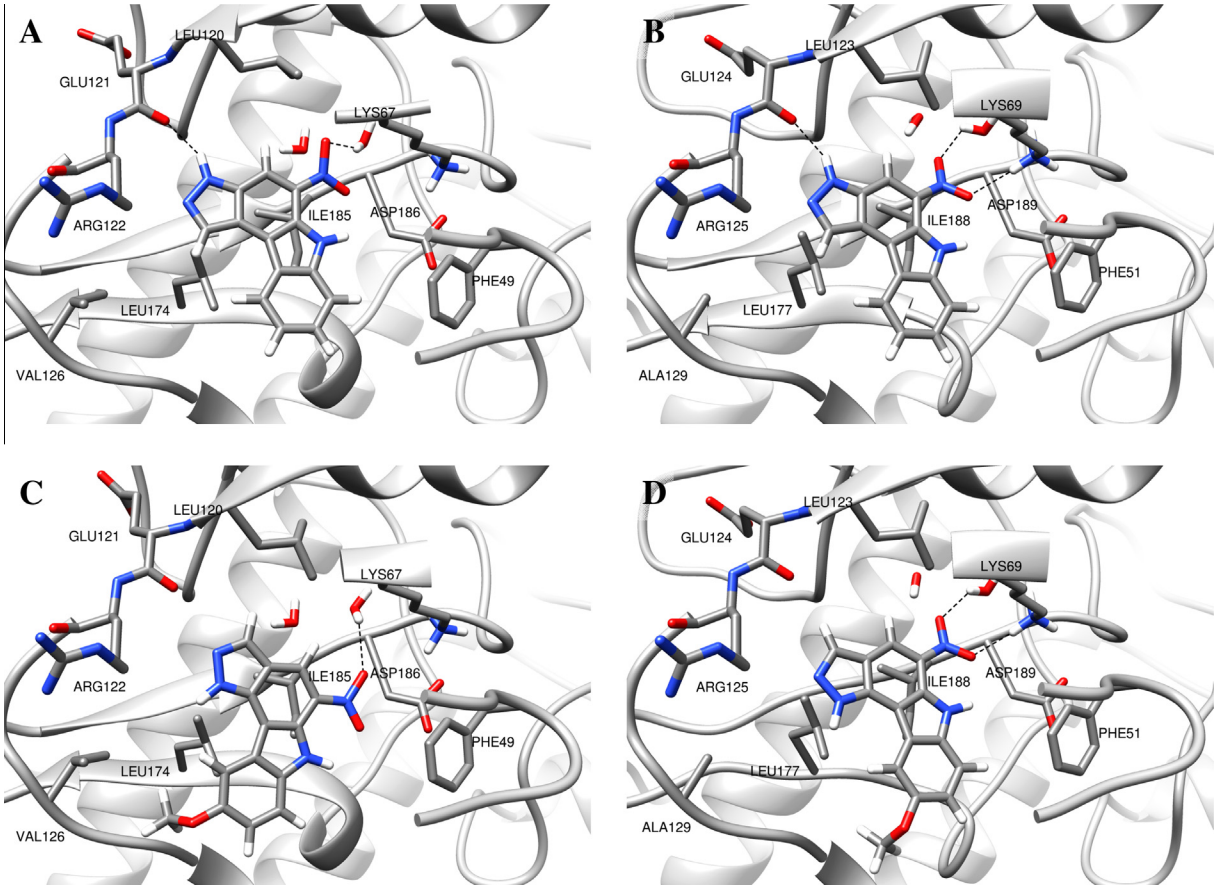


Figure 2. Docking models of (A) **15a** bound to Pim-1 ATP binding site, (B) **15a** bound to Pim-3 ATP binding site, (C) **6b** bound to Pim-1 ATP binding site, (D) **6b** bound to Pim-3 ATP binding site. Hydrogen bonds are indicated as dashed lines. Molecular graphics images were produced using UCSF Chimera.[28]

Regarding **15a**, the best inhibitor of both pyrazolocarbazole series, we found a similar binding mode with Pim-1 and Pim-3 (Fig. 2A and B). The indazole ring system of **15a** is inserted in the ATP binding cleft, with the nitro group oriented toward the hydrophilic Lys67/Lys69 region, while the pyrazole ring is placed near the hinge region, establishing a hydrogen bond between the N-3 hydrogen atom and the backbone carbonyl oxygen of Glu121 in Pim-1 (2.18 Å) or Glu124 in Pim-3 (2.09 Å). A similar orientation of the pyrazolocarbazole ring system was found in the case of compound **6b** (Fig. 2C and D). Nevertheless, as these two series are regioisomers, in the case of **6b**, the pyrazole NH is not well positioned to establish a hydrogen bond with Glu121/Glu124 hinge residues. The only polar interaction that was observed was found between the nitro group and the Lys67/Lys69 region, as it was already observed for **15a**.

For both regioisomeric series, the D ring of the pyrazolocarbazole ring system (Schemes 1 and 3) is oriented toward the outside of the ATP-binding pocket. These results might explain why, as indicated above, the Pim kinase inhibitory profile of these two dihydropyrazolocarbazole series is not very dependent on the nature of the substituent present at the position nine of the heteroaromatic scaffold.

3. Conclusions

In conclusion, 1,6-dihydropyrazolo[4,3-*c*]carbazoles and 3,6-dihydropyrazolo[3,4-*c*]carbazoles were synthesized and identified as potent Pim kinase inhibitors. In both series, except for chlorinated analogue **16c**, nitro derivatives were more potent Pim inhibitors compared to their amino counterparts. Both regioisomeric series showed similar profiles toward the three Pim isoforms, Pim-1/Pim-3 being the most inhibited kinases. The most potent compound of the series **15a** also showed interesting antiproliferative activities toward PC3 cells in the micromolar range. Finally, compound **6c**, that strongly inhibited Pim-3 with lower effect toward Pim-1 and Pim-2, could be used as an interesting molecular tool to study Pim-3 biological functions. Due to the potential interest of these compounds as Pim inhibitors, the structure–activity relationship studies performed on this series are currently widened in our group.

4. Experimental section

4.1. Chemistry

4.1.1. General

Starting materials were obtained from commercial suppliers and used without further purification. IR spectra were recorded on Shimadzu FTIR-8400S or Perkin–Elmer Spectrum 65 FT-IR spectrometers ($\bar{\nu}$ in cm^{-1}). NMR spectra, performed on a Bruker AVANCE 400 spectrometer (^1H: 400 MHz, ^{13}C: 100 MHz), or a Bruker AVANCE 500 spectrometer (^1H: 500 MHz, ^{13}C: 126 MHz), are reported in ppm using the solvent residual peak as an internal standard; the following abbreviations are used: singlet (s), doublet (d), triplet (t), doublet of doublet (dd), doublet of doublet of doublet (ddd), doublet of triplet (dt), multiplet (m), broad signal (br s); High resolution mass spectra were determined on a high-resolution Micro Q-Tof apparatus (CRMP, Université Blaise Pascal, Clermont–Ferrand, France) or on a Waters Q-Tof 2 apparatus (CRMPO, Université de Rennes, France). Chromatographic purifications were

performed by column chromatography using 40–63 μm silica gel. Reactions were monitored by TLC using fluorescent silica gel plates (60 F254 from Merck). Melting points were measured on a Reichert microscope or a Stuart SMP3 apparatus and are uncorrected.

4.1.2. General procedure for preparation of compounds 4a–4c

A mixture of indazole **2** in a 1:1:1 toluene/ethanol/H_2O mixture (0.043 mmol/mL), $Pd(PPh_3)_4$ (10 mol%), Na_2CO_3 (2.5 equiv) and boronic acid **3** (1.5–2 equiv) was refluxed for 2–4 h. Water was added and the mixture was extracted with EtOAc. The combined organic fractions were dried over $MgSO_4$ and evaporated. Column chromatography (cyclohexane/EtOAc) provided the corresponding coupling product.

4.1.3. 5-Nitro-7-phenyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-6-amine (4a)

From **2** (200 mg, 0.52 mmol) and phenylboronic acid **3a** (2 equiv) (reflux, 4 h), column chromatography (cyclohexane/EtOAc, 8:2) provided **4a** (136 mg, 0.40 mmol, 78%) as an orange powder. Mp 207–211 °C; IR (ATR): 3486, 3355, 1618, 1491, 1414, 1296, 1039, 988 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.92–1.05 (1H, m), 1.20–1.36 (2H, m), 1.61–1.68 (1H, m), 1.75–1.83 (1H, m), 2.16–2.28 (1H, m), 2.39–2.52 (1H, m), 3.58–3.64 (1H, m), 4.23 (1H, dd, J_1 = 10.5 Hz, J_2 = 2 Hz), 6.03 (2H, br s), 7.34–7.38 (1H, m), 7.40–7.44 (1H, m), 7.59–7.69 (3H, m), 8.21 (1H, s), 8.71 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 22.9, 24.3, 28.8, 66.7 (CH_2), 83.5 (CH), 120.6, 129.0, 129.6, 129.8, 130.8, 131.1, 137.0 (CH_{arom}), 107.8, 117.0, 131.0, 132.6, 140.7, 141.3 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{19}N_4O_3$ $(M+H)^+$ 339.1457, found 339.1456.

4.1.4. 7-(3-Methoxyphenyl)-5-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-6-amine (4b)

From **2** (50 mg, 0.13 mmol) and 3-methoxyboronic acid **3b** (1.5 equiv) (reflux, 2 h), column chromatography (cyclohexane/EtOAc, 8:2) provided **4b** (40 mg, 0.11 mmol, 84%) as a yellow powder. Mp 173–174 °C; IR (ATR): 3497, 3376, 1618, 1490, 1297, 1038 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.94–1.09 (1H, m), 1.21–1.40 (2H, m), 1.59–1.70 (1H, m), 1.75–1.85 (1H, m), 2.15–2.30 (1H, m), 2.45–2.59 (1H, m), 3.60–3.70 (1H, m), 3.80/3.81 (3H, 2s); 4.26/4.32 (1H, 2dd, J_1 = 10.5 Hz, J_2 = 1.5 Hz), 6.02–6.15 (2H, m), 6.88–6.99 (2H, m), 7.15–7.21 (1H, m), 7.51–7.60 (1H, m), 8.21 (1H, s), 8.70 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 55.2, 55.4 (CH_3), 22.9, 23.0, 24.31, 24.33, 28.80, 28.85, 66.7, 66.8 (CH_2), 83.62, 83.63 (CH), 115.0, 115.2, 115.5, 116.0, 120.6, 122.76, 122.83, 130.6, 130.9, 136.9, 137.0 (CH_{arom}), 107.7, 116.96, 116.99, 131.0, 133.89, 133.90, 140.59, 140.62, 141.2, 141.3, 160.2, 160.3 (C_{arom}); HRMS (ESI+) calcd for $C_{19}H_{20}N_4NaO_4$ $(M+Na)^+$ 391.1382, found 391.1400.

4.1.5. 7-(3-Chlorophenyl)-5-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-6-amine (4c)

From **2** (105 mg, 0.27 mmol) and 3-chlorophenylboronic acid **3c** (2 equiv) (reflux, 4 h), column chromatography (cyclohexane/EtOAc, 8:2) provided **4c** (87 mg, 0.23 mmol, 86%) as a yellow powder. Mp 225–227 °C; IR (ATR): 3492, 3367, 1617, 1489, 1411, 1292, 1079, 1037, 991 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6), [a]major isomer, [b]minor isomer: 0.98–1.11 ($1H^a$+$1H^b$, m), 1.21–1.40 ($2H^a$+$2H^b$, m), 1.64–1.74 ($1H^a$+$1H^b$, m), 1.76–1.86 ($1H^a$+$1H^b$, m), 2.17–2.32 ($1H^a$+$1H^b$, m), 2.40–2.56 ($1H^a$+$1H^b$, m), 3.59–3.65 ($1H^b$, m), 3.67–3.74 ($1H^a$, m), 4.23 ($1H^a$, dd, J_1 = 10.5 Hz, J_2 = 1.5 Hz), 4.28 ($1H^b$, dd, J_1 = 10.5 Hz, J_2 = 1.5 Hz), 6.19 ($2H^b$, br s), 6.20 ($2H^a$, br s), 7.31 ($1H^b$, dt, J_1 = 6.5 Hz, J_2 = 2 Hz), 7.38 ($1H^a$, t, J = 1.5 Hz), 7.41 ($1H^a$, dt, J_1 = 7 Hz, J_2 = 1.5 Hz), 7.54–7.56 ($1H^b$, m), 7.63–7.72 ($2H^a$+$2H^b$, m), 8.21 ($1H^a$+$1H^b$, s), 8.73 ($1H^a$+$1H^b$, s); ^{13}C NMR (100 MHz, DMSO-d_6): 22.8, 22.9, 24.25, 24.30, 28.7, 28.8, 66.6, 66.7 (CH_2), 83.7 (CH), 121.05, 121.08, 129.00, 129.05, 129.8, 130.1, 130.9, 131.0, 131.3, 131.5, 136.94, 136.95 (CH_{arom}), 106.31, 106.34, 116.98, 116.99, 130.96, 130.97, 133.9, 134.2, 134.8, 134.9, 140.50, 140.53, 141.51, 141.52 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{18}{}^{35}ClN_4O_3$ $(M+H)^+$ 373.1067, found 373.1056.

4.1.6. General procedure for preparation of compounds 5a–5c

A mixture of indazole **4** in toluene (0.05 mmol/mL) and $Pd(OAc)_2$ (10 mol%) was stirred at room temperature. Phenyliodonium diacetate (1.5 equiv) was then slowly added and the reaction mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography.

4.1.7. 5-Nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1,6-dihydropyrazolo[4,3-*c*]carbazole (5a)

From **4a** (30.0 mg, 0.089 mmol), column chromatography (cyclohexane/EtOAc, 9:1 then 8:2) provided **5a** (11.6 mg, 0.034 mmol, 39%) as a yellow powder. Mp 240–243 °C; IR (ATR): 3403, 1604, 1488, 1304 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.61–1.75 (2H, m), 1.78–1.90 (1H, m), 2.07–2.25 (2H, m), 2.53–2.65 (1H, m), 3.90–3.97 (2H, m), 6.54 (1H, dd, J_1= 8 Hz, J_2 = 3 Hz), 7.41 (1H, ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.53 (1H, ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.93 (1H, d, J = 8 Hz), 8.33 (1H, d, J = 8 Hz), 8.53 (1H, s), 8.94 (1H, s), 12.47 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 21.7, 24.6, 29.3, 65.5 (CH_2), 84.2 (CH), 113.1, 118.2, 120.9, 122.1, 125.7, 138.0 (CH_{arom}), 106.8, 118.2, 119.7, 129.7, 131.9, 138.1, 139.2 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{17}N_4O_3$ $(M+H)^+$ 337.1301, found 337.1319.

4.1.8. 9-Methoxy-5-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1,6-dihydropyrazolo[4,3-*c*]carbazole (5b)

From **4b** (140 mg, 0.38 mmol), column chromatography (cyclohexane/EtOAc, 9:1 then 8:2) provided **5b** (32.8 mg, 0.090 mmol, 24%) as an orange powder. Mp 246–249 °C; IR (ATR): 3430, 1610, 1488, 1393, 1326, 1301 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.59–1.89 (3H, m), 2.06–2.15 (1H, m), 2.17–2.27 (1H, m), 2.50–2.64 (1H, m), 3.87–3.97 (2H, m), 3.92 (3H, s), 6.55 (1H, dd, J_1 = 7.5 Hz, J_2 = 3 Hz), 7.23 (1H, dd, J_1 = 9 Hz, J_2 = 2 Hz), 7.82–7.86 (2H, m), 8.52 (1H, s), 8.91 (1H, s), 12.32 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 56.0 (CH_3), 21.6, 24.6, 29.3, 65.5 (CH_2), 84.0 (CH), 105.6, 113.6, 114.5, 118.2, 138.1 (CH_{arom}), 106.6, 117.8, 120.2, 129.6, 132.3, 134.2, 138.2, 154.3 (C_{arom}); HRMS (ESI+) calcd for $C_{19}H_{19}N_4O_4$ $(M+H)^+$ 367.1406, found 367.1399.

4.1.9. 9-Chloro-5-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1,6-dihydropyrazolo[4,3-*c*]carbazole (5c)

From **4c** (159 mg, 0.426 mmol), column chromatography (cyclohexane/EtOAc, 8:2) provided **5c** (18.8 mg, 0.051 mmol, 12%) as an orange powder. Mp >250 °C; IR (ATR): 3416, 1606, 1486, 1393, 1326, 1297, 1079, 1038, 994 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.61–1.89 (3H, m), 2.04–2.14 (1H, m), 2.14–2.24 (1H, m), 2.54–2.65 (1H, m), 3.89–4.04 (2H, m), 6.46 (1H, dd, J_1 = 8 Hz, J_2 = 3 Hz), 7.57 (1H, dd, J_1 = 8.5 Hz, J_2 = 2 Hz), 7.93 (1H, d, J = 8.5 Hz), 8.34 (1H, d, J = 2 Hz), 8.55 (1H, s), 8.99 (1H, s), 12.62 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 21.7, 24.6, 29.2, 65.7 (CH_2), 84.2 (CH), 114.5, 119.1, 121.3, 125.6, 137.9 (CH_{arom}), 106.0, 118.3, 120.8, 125.1, 129.7, 132.5, 137.6, 137.8 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{16}{}^{35}ClN_4O_3$ $(M+H)^+$ 371.0911, found 371.0915.

4.1.10. General procedure for preparation of compounds 6a–6c

A mixture of compound **5** in concentrated hydrochloric acid (0.1 mmol/mL) was refluxed for 3 h. An aqueous saturated $NaHCO_3$ solution was added and the product was extracted with EtOAc. The combined organic fractions were dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography.

4.1.11. 5-Nitro-1,6-dihydropyrazolo[4,3-*c*]carbazole (6a)

From **5a** (38.0 mg, 0.113 mmol), column chromatography (cyclohexane/EtOAc, 7:3) provided **6a** (17.7 mg, 0.070 mmol, 62%) as an orange powder. Mp >250 °C; IR (ATR): 3500–3000, 1646, 1524, 1473, 1337, 1297, 1243, 1192, 1157 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 7.39 (1H, ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.52 (ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.87 (1H, d, J = 8 Hz), 8.51 (1H, br s), 8.64 (1H, d, J = 8 Hz), 8.94 (1H, s), 12.32 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 112.8, 117.9, 120.7, 121.1, 125.6, 137.8 (CH_{arom}), 105.9, 116.9, 120.0, 129.5, 130.8, 136.6, 138.9 (C_{arom}); HRMS (ESI+) calcd for $C_{13}H_9N_4O_2$ $(M+H)^+$ 253.0726, found 253.0736.

4.1.12. 9-Methoxy-5-nitro-1,6-dihydropyrazolo[4,3-*c*]carbazole (6b)

From **5b** (41.0 mg, 0.112 mmol), column chromatography (cyclohexane/EtOAc, 7:3 to 5:5) provided **6b** (19.5 mg, 0.069 mmol, 62%) as an orange powder. Mp >250 °C; IR (ATR): 3500–3000, 1649, 1470, 1290, 1215, 1168 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 3.93 (3H, s), 7.14 (1H, dd, J_1 = 9 Hz, J_2 = 2.5 Hz), 7.75 (1H, d, J = 9 Hz), 8.19 (1H, d, J = 2 Hz), 8.49 (1H, s), 8.91 (1H, s), 12.16 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 55.9 (CH_3), 103.1, 113.6, 115.5, 117.8, 137.8 (CH_{arom}), 106.0, 116.5, 120.4, 129.5, 131.0, 133.6, 136.6, 154.6 (C_{arom}); HRMS (ESI+) calcd for $C_{14}H_{11}N_4O_3$ $(M+H)^+$ 283.0831, found 283.0816.

4.1.13. 9-Chloro-5-nitro-1,6-dihydropyrazolo[4,3-*c*]carbazole (6c)

From **5c** (50 mg, 0.135 mmol), column chromatography (cyclohexane/EtOAc, 8:2 then 7:3) provided **6c** (26.5 mg, 0.092 mmol, 69%) as a yellow powder. Mp >250 °C; IR (ATR): 3500–3000, 1463, 1319, 1291 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 7.54 (1H, dd, J_1 = 8.5 Hz, J_2 = 2 Hz), 7.86 (1H, d, J = 8.5 Hz), 8.51 (1H, d, J = 1.5 Hz), 8.80 (1H, d, J = 2 Hz), 8.98 (1H, s), 12.46 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 114.3, 118.9, 120.4, 125.5, 137.9 (CH_{arom}), 105.1, 116.9, 121.2, 125.2, 129.5, 131.5, 136.4, 137.3 (C_{arom}); HRMS (ESI+) calcd for $C_{13}H_8{}^{35}ClN_4O_2$ $(M+H)^+$ 287.0336, found 287.0344.

4.1.14. General procedure for preparation of compounds 7a–7c

To a solution of indazole **6** in ethyl acetate (0.01 mmol/mL) was added platinum oxide (20 mol%). The mixture was stirred for 36 h under hydrogen atmosphere (balloon), and then was evaporated under reduced pressure. The residue was purified by column chromatography.

4.1.15. 1,6-Dihydropyrazolo[4,3-*c*]carbazol-5-amine (7a)

From **6a** (36.0 mg, 0.143 mmol), column chromatography (cyclohexane/EtOAc, 2:8) provided **7a** (25.6 mg, 0.115 mmol, 81%) as a pinkish powder. Mp >250 °C; IR (ATR): 3500–2700, 1517, 1461, 1401, 1350, 1319, 1242, 1160 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 4.98 (2H, br s), 6.86 (1H, s), 7.24 (1H, ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.37 (1H, ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.59 (1H, d, J = 8 Hz), 7.89 (1H, s), 8.48 (1H, d, J = 8 Hz), 11.31 (1H, s), 13.23 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 98.0, 111.2, 119.1, 121.1, 123.8, 132.4[a] (CH_{arom}), 104.7[b], 117.7, 121.6, 129.6[b], 129.7, 130.6, 137.6 (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{13}H_{11}N_4$ $(M+H)^+$ 223.0984, found 223.0977.

4.1.16. 9-Methoxy-1,6-dihydropyrazolo[4,3-*c*]carbazol-5-amine (7b)

From **6b** (47.7 mg, 0.169 mmol), column chromatography (cyclohexane/EtOAc, 5:5 to 2:8) provided **7b** (21.0 mg, 0.083 mmol, 49%) as a pinkish powder. Mp >250 °C; IR (ATR): 3500–2700, 1481, 1309, 1216, 1146, 1030 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 3.90 (3H, s), 4.93 (2H, br s), 6.82 (1H, s), 6.99 (1H, dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz), 7.48 (1H, d, J = 8.5 Hz), 7.86 (1H, s), 8.07 (1H, s), 11.11 (1H, s), 13.25 (1H, br s); ^{13}C NMR (125 MHz, DMSO-d_6): 55.8 (CH_3), 97.7, 103.5, 111.8, 113.4, 132.4[a] (CH_{arom}), 104.6[b], 117.3, 122.0, 129.5[b], 129.7, 131.3, 132.5, 153.6 (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{14}H_{13}N_4O$ $(M+H)^+$ 253.1089, found 253.1083.

4.1.17. 9-Chloro-1,6-dihydropyrazolo[4,3-*c*]carbazol-5-amine (7c)

From **6c** (19.3 mg, 0.067 mmol), column chromatography (cyclohexane/EtOAc, 2:8) provided **7c** (10.2 mg, 0.040 mmol, 59%) as a pinkish powder. Mp >250 °C; IR (ATR): 3500–2700, 1519, 1462, 1289, 1159, 1062 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 5.00 (2H, br s), 6.90 (1H, s), 7.37 (1H, dd, J_1 = 8.5 Hz, J_2 = 2 Hz), 7.61 (1H, d, J = 8.5 Hz), 7.89 (1H, s), 8.62 (1H, s), 11.50 (1H, s), 13.27 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 98.8, 112.7, 120.3, 123.6, 132.6[a] (CH), 103.9[b], 117.8, 122.7, 123.6, 129.1[b], 129.7, 131.6, 136.1 (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{13}H_{10}{}^{35}ClN_4$ $(M+H)^+$ 257.0594, found 257.0591.

4.1.18. 5-Bromo-6-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole (9)

To a mixture of 5-bromo-6-nitroindazole **8** (1.30 g, 5.40 mmol) in anhydrous CH_2Cl_2 (40 mL) were added 3,4-dihydro-2H-pyrane (1.46 mL, 1.35 g, 16 mmol) and PTSA (monohydrate) (93 mg, 0.49 mmol). The mixture was stirred for 1 h at room temperature and then was washed with a saturated aqueous $NaHCO_3$ solution. The assembled aqueous fractions were extracted with CH_2Cl_2. The organic fractions were dried over $MgSO_4$, evaporated and the residue was purified by column chromatography (cyclohexane/EtOAc, 95:5). Pentane was added to the residue, the solid was filtered off and then was washed with a minimum of pentane/MeOH 9:1 mixture. Compound **9** (1.37 g, 4.2 mmol, 78%) was obtained as a yellow-orange solid. Mp 96–98 °C; IR (ATR): 1574, 1528, 1468, 1439, 1416 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 1.66–1.84 (3H, m), 2.09–2.18 (2H, m), 2.41–2.51 (1H, m), 3.73–3.80 (1H, m), 3.96–4.02 (1H, m), 5.75 (1H, dd, J_1 = 9.0 Hz, J_2 = 2.5 Hz), 8.05 (1H, d, J = 1.0 Hz), 8.07 (1H, s), 8.15 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): 22.0, 25.0, 29.5, 67.4 (CH_2), 86.5 (CH), 108.7, 126.9, 133.1 (CH_{arom}), 105.1, 127.1, 136.8, 148.0* (C_{arom}), *chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ES+) calcd for $C_{12}H_{12}{}^{79}BrN_3NaO_3$ $(M+Na)^+$ 347.9960, found 347.9960.

4.1.19. 6-Nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-amine (10)

A mixture of $Pd_2(dba)_3{\cdot}CHCl_3$ (5.6 mg, 5.4 μmol) and BINAP (11.4 mg, 0.018 mmol) in toluene (1 mL) degassed with argon was stirred at room temperature for 15 min, and then indazole **9** (50 mg, 0.153 mmol) and NaO^tBu (20.6 mg, 0.21 mmol) were added. To this dark-red solution, benzophenone imine (31 μL, 0.185 mmol) was added and the reaction mixture was stirred at 80 °C for 5 h. After cooling, EtOAc was added and the suspension was filtered through a pad of Celite and the filtrate was concentrated under reduced pressure. The residue was then dissolved in THF (1 mL) and a 3 M aqueous HCl solution (0.3 mL) was added. The reaction mixture was stirred at room temperature for 30 min, and then was extracted with EtOAc. The assembled organic fractions were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (cyclohexane/EtOAc, 8:2) to give **10** (34.6 mg, 0.13 mmol, 86%) as a red solid. For spectral data, see reference.[20]

4.1.20. 5-Chloro-2-iodophenylboronic acid (12c)

5-Amino-2-iodophenylboronic acid **18** (1.18 g, 4.5 mmol) was dissolved at 5 °C in concentrated HCl (2.8 mL). A solution of sodium nitrite (310 mg, 4.5 mmol) in a minimum of water was slowly added and the reaction was stirred at 5 °C for 30 min. A cold solution of CuCl (1.78 g, 18 mmol) in concentrated HCl (1 mL) was then added dropwise and the mixture was stirred at room temperature for 2 h. Water was added and the mixture was extracted with EtOAc. The combined organic fractions were dried over $MgSO_4$ and evaporated. Column chromatography (cyclohexane/EtOAc, 7:3) provided **12c** (771 mg, 2.73 mmol, 61%) as a pink powder. Mp 179–181 °C; IR (ATR): 3280, 1375, 1328, 1098, 1001 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 7.13 (1H, dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz), 7.22 (1H, d, J = 2.5 Hz), 7.74 (1H, d, J = 8.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 129.8, 132.6, 139.5 (CH_{arom}), 96.7, 132.6, 148.7* (C_{arom}), *chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI–) calcd for $C_6H_4{}^{35}Cl{}^{11}BIO_2$ $(M-H)^-$ 280.9038, found 280.9036.

4.1.21. 4-(2-Bromophenyl)-6-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-amine (13a)

Same procedure as for the preparation of compounds **4a** and **4b**. From **11** (200 mg, 0.52 mmol) and 2-bromophenylboronic acid **12a** (1.5 equiv) (reflux, 2 h), column chromatography (cyclohexane/EtOAc, 8:2) provided **13a** (168 mg, 0.40 mmol, 78%) as a red oil. IR (ATR): 3496, 3386, 1526, 1287, 1246, 1163, 1080, 1042 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.49–1.64 (2H, m), 1.67–1.81 (1H, m), 1.93–2.06 (2H, m), 2.28–2.41 (1H, m), 3.75–3.93 (2H, m), 5.77 (2H, br s), 5.96 (1H, d, J = 10 Hz), 7.37–7.51 (3H, m), 7.54–7.64 (1H, m), 7.86/7.88 (1H, 2s), 8.59/8.60 (1H, 2s); ^{13}C NMR (100 MHz, DMSO-d_6): 22.0, 22.1, 24.8, 28.7, 28.8, 66.3, 66.5 (CH_2), 83.8, 83.9 (CH), 107.9, 108.0, 128.80, 128.81, 130.8, 131.30, 131.33, 132.20, 132.24, 133.4 (CH_{arom}), 117.43, 117.45, 123.88, 123.91, 128.86, 128.91, 131.0, 134.96, 134.99, 135.7 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{17}{}^{79}BrN_4NaO_3$ $(M+Na)^+$ 439.0382, found 439.0402.

4.1.22. 4-(2-Bromo-5-methoxyphenyl)-6-nitro-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-amine (13b)

Same procedure as for the preparation of compounds **4a**–**4c**. From **11** (150 mg, 0.39 mmol) and 2-bromo-5-methoxyphenylboronic acid **12b** (2 equiv) (reflux, 4 h), column chromatography (cyclohexane/EtOAc, 9:1) provided **13b** (132 mg, 0.30 mmol, 76%) as a red powder. Mp 85–95 °C; IR (ATR): 3490, 3382, 1524, 1282, 1241, 1159, 1079, 1039 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.51–1.63 (2H, m), 1.68–1.81 (1H, m), 1.93–2.07 (2H, m), 2.29–2.41 (1H, m), 3.75–3.92 (2H, m), 3.777/3.783 (3H, 2s), 5.81 (2H, s), 5.94–5.99 (1H, m), 6.96/6.99 (1H, 2d, J = 3 Hz), 7.06 (1H, dd, J_1 = 9 Hz, J_2 = 3 Hz), 7.48 (1H, m), 7.740/7.744 (1H, 2d, J = 9 Hz), 8.58/8.60 (1H, 2d, J = 1 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 55.5, 55.6 (CH_3), 22.0, 22.1, 24.8, 28.7, 28.9, 66.4, 66.5 (CH_2), 83.8, 83.9 (CH), 107.9, 108.0, 116.9, 117.19, 117.23, 131.4, 131.5, 134.2 (CH_{arom}), 113.90, 113.93, 117.42, 117.44, 128.8, 128.9, 131.0, 134.91, 134.94, 135.71, 135.72, 135.81, 135.83, 159.30, 159.31 (C_{arom}); HRMS (ESI+) calcd for $C_{19}H_{20}{}^{79}BrN_4O_4$ $(M+H)^+$ 447.0668, found 447.0658.

4.1.23. 5-Nitro-3-(tetrahydro-2*H*-pyran-2-yl)-3,6-dihydropyrazolo[3,4-*c*]carbazole (14a)

A mixture of compound **13a** (150 mg, 0.36 mmol), Cu (68 mg), CuI (14 mg, 0.074 mmol) and K_2CO_3 (99 mg, 0.72 mmol) in dibutyl ether (5 mL) was refluxed for 24 h. Water (10 mL) was added and the mixture was extracted with EtOAc (2 × 30 mL). The combined organic fractions were dried over $MgSO_4$ and evaporated. Column chromatography (cyclohexane/EtOAc) provided **14a** (81 mg, 0.24 mmol, 67%) as a red powder. Mp >250 °C; IR (ATR): 3419, 1320, 1260, 1080, 1036, 1018 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.60–1.69 (2H, m), 1.76–1.89 (1H, m), 2.04–2.15 (2H, m), 2.44–2.57 (1H under solvent signal), 3.83–3.95 (2H, m), 6.16 (1H, d, J = 9 Hz), 7.33 (1H, t, J = 7.5 Hz), 7.50 (1H, t, J = 7.5 Hz), 7.84 (1H, d, J = 8 Hz), 8.45 (1H, d, J = 8 Hz), 8.80 (1H, s), 8.90 (1H, s), 12.12 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 21.9, 24.8, 28.9, 66.4 (CH_2), 84.3 (CH), 105.3, 112.7, 120.1, 121.3, 126.1, 131.4 (CH_{arom}), 115.3, 120.7, 121.2, 127.0, 132.7, 133.2, 139.9 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{16}N_4NaO_3$ $(M+Na)^+$ 359.1120, found 359.1121.

4.1.24. 9-Methoxy-5-nitro-3-(tetrahydro-2*H*-pyran-2-yl)-3,6-dihydropyrazolo[3,4-*c*]carbazole (14b)

Same procedure as for the preparation of compound **14a**. From **13b** (123 mg, 0.27 mmol), column chromatography (cyclohexane/EtOAc, 8:2–5:5) provided **14b** (59 mg, 0.16 mmol, 59%) as a red powder. Mp >250 °C; IR (ATR): 3419, 1516, 1445, 1436, 1313, 1285, 1239, 1221, 1207, 1153, 1037 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.58–1.68 (2H, m), 1.75–1.88 (1H, m), 2.03–2.13 (2H, m), 2.43–2.57 (1H under solvent signal), 3.86–3.93 (2H, m), 3.95 (3H, s), 6.19 (1H, dd, J_1 = 9.5 Hz, J_2 = 2 Hz), 7.18 (1H, dd, J_1 = 9 Hz, J_2 = 2.5 Hz), 7.74 (1H, d, J = 9 Hz), 7.93 (1H, d, J = 2.5 Hz), 8.81 (1H, d, J = 1 Hz), 9.06 (1H, d, J = 1 Hz), 12.03 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 55.9 (CH_3), 22.0, 24.8, 29.0, 66.5 (CH_2), 84.2 (CH), 103.3, 105.4, 113.7, 116.5, 131.8 (CH_{arom}), 115.2, 121.0, 121.2, 127.5, 132.8, 133.1, 134.9, 154.2 (C_{arom}); HRMS (ESI+) calcd for $C_{19}H_{19}N_4O_4$ $(M+H)^+$ 367.1406, found 367.1424.

4.1.25. 9-Chloro-5-nitro-3-(tetrahydro-2*H*-pyran-2-yl)-3,6-dihydropyrazolo[3,4-*c*]carbazole (14c)

Step A: same procedure as for the preparation of compounds **4a**–**4c**. From **11** (200 mg, 0.52 mmol) and 5-chloro-2-iodophenylboronic acid **12c** (2 equiv) (reflux, 4 h), **13c** was prepared and used for the next step without chromatographic purification.

Step B: same procedure as for the preparation of compound **14a**. From the coupling product obtained step A, column chromatography (cyclohexane/EtOAc, 95:5–9:1) provided **14c** (89 mg, 0.24 mmol, 47%) as an orange powder. Mp 227–228 °C; IR (ATR): 3417, 1520, 1445, 1315, 1034 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.59–1.67 (2H, m), 1.74–1.88 (1H, m), 2.04–2.12 (2H, m), 2.43–2.57 (1H under the solvent signal), 3.86–3.92 (2H, m), 6.22 (1H, dd, J_1 = 9.5 Hz, J_2 = 2 Hz), 7.55 (1H, dd, J_1 = 8.5 Hz, J_2 = 2 Hz), 7.85 (1H, d, J = 8.5 Hz), 8.63 (1H, d, J = 2 Hz), 8.90 (1H, d, J = 1 Hz), 9.10 (1H, d, J = 1 Hz), 12.33 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 22.0, 24.8, 29.0, 66.5 (CH_2), 84.1 (CH), 106.6, 114.4, 120.7, 126.3, 131.9 (CH_{arom}), 114.3, 121.0, 121.8, 124.8, 127.8, 132.9, 133.4, 138.3 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{15}{}^{35}ClN_4NaO_3$ $(M+Na)^+$ 393.0731, found 393.0730.

4.1.26. 5-Nitro-3,6-dihydropyrazolo[3,4-*c*]carbazole (15a)

Same procedure as for the preparation of compounds **6a**–**6c**. From **14a** (70 mg, 0.21 mmol) in concentrated hydrochloric acid (5 mL), column chromatography (cyclohexane/EtOAc) provided compound **15a** (43 mg, 0.17 mmol, 82%) as a red powder. Mp >250 °C; IR (ATR): 3438, 3224, 1343, 1322 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): 7.35 (1H, t, J = 7.5 Hz), 7.52 (1H, t, J = 7.5 Hz), 7.84 (1H, d, J = 8 Hz), 8.48 (1H, d, J = 8 Hz), 8.58 (1H, s), 8.92 (1H, s), 12.11 (1H, s), 13.80 (1H, br s); ^{13}C NMR (126 MHz, DMSO-d_6): 105.5, 112.8, 120.1, 121.4, 126.1, 131.8 (CH_{arom}), 114.9, 120.1, 120.9, 126.5, 132.8, 134.0, 139.7 (C_{arom}); HRMS (ESI+) calcd for $C_{13}H_8N_4NaO_2$ $(M+Na)^+$ 275.0545, found 275.0546.

4.1.27. 9-Methoxy-5-nitro-3,6-dihydropyrazolo[3,4-*c*]carbazole (15b)

Same procedure as for the preparation of compounds **6a**–**6c**. From **14b** (59 mg, 0.161 mmol), column chromatography (cyclohexane/EtOAc, 5:5) provided **15b** (22.6 mg, 0.080 mmol, 50%) as a red powder. Mp >250 °C; IR (ATR): 3436, 3241, 1472, 1445, 1307, 1288, 1208 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 3.95 (3H, s), 7.16 (1H, dd, J_1 = 9 Hz, J_2 = 2 Hz), 7.73 (1H, d, J = 9 Hz), 7.91 (1H, d, J = 1.5 Hz), 8.56 (1H, s), 9.00 (1H, s), 11.95 (1H, s), 13.77 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 55.9 (CH_3), 103.1, 105.7[a], 113.6, 116.1, 131.7[a] (CH_{arom}), 114.8, 120.0, 121.3, 126.9, 132.8, 133.8[b], 134.6, 154.1 (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{14}H_{11}N_4O_3$ $(M+H)^+$ 283.0831, found 283.0826.

4.1.28. 9-Chloro-5-nitro-3,6-dihydropyrazolo[3,4-*c*]carbazole (15c)

Same procedure as for the preparation of compounds **6a**–**6c**. From **14c** (82 mg, 0.221 mmol), column chromatography (cyclohexane/EtOAc, 5:5) provided **15c** (50 mg, 0.174 mmol, 79%) as a red powder. Mp >250 °C; IR (ATR): 3441, 3246, 1445, 1315, 1282, 1181 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 7.53 (1H, dd, J_1 = 8.5 Hz, J_2 = 2 Hz), 7.84 (1H, d, J = 8.5 Hz), 8.60 (1H, d, J = 2 Hz), 8.63 (1H, d, J = 1 Hz), 9.04 (1H, t, J = 1 Hz), 12.27 (1H, s, NH), 13.87 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 106.6, 114.3, 120.6, 126.0, 132.1 (CH_{arom}), 114.0, 119.9, 122.0, 124.6, 127.3, 132.8, 134.0, 138.1 (C_{arom}); HRMS (ESI/ASAP) calcd for $C_{13}H_8{}^{35}ClN_4O_2$ $(M+H)^+$ 287.0336, found 287.0336.

4.1.29. 3,6-Dihydropyrazolo[3,4-*c*]carbazole-5-amine (16a)

Same procedure as for the preparation of compounds **7a**–**7c**. From **15a** (10 mg, 0.040 mmol) in EtOAc (3 mL) (24 h), column chromatography (cyclohexane/EtOAc) provided **16a** (8 mg, 0,036 mmol, 91%) as a brown powder. Mp >250 °C; IR (ATR): 3500–2500, 1345, 1319, 1248, 1179, 1083, 948 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): 5.47 (2H, br s), 6.71 (1H, s), 7.19 (1H, t, J = 7.5 Hz), 7.34 (1H, t, J = 7.5 Hz), 7.57 (1H, d, J = 8 Hz), 8.21 (1H, d, J = 8 Hz), 8.33 (1H, s), 11.07 (1H, s), 12.52 (1H, br s); ^{13}C NMR (126 MHz, DMSO-d_6): 89.0, 111.3, 118.6, 120.9, 123.8, 130.7[a] (CH_{arom}), 109.1, 111.5, 122.7, 126.1, 134.8, 137.9, 138.1[b] (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{13}H_{11}N_4$ $(M+H)^+$ 223.0984, found 223.0993.

4.1.30. 9-Methoxy-3,6-dihydropyrazolo[3,4-*c*]carbazol-5-amine (16b)

Same procedure as for the preparation of compounds **7a**–**7c**. From **15b** (25.0 mg, 0.089 mmol), column chromatography (cyclohexane/EtOAc, 5:5–0:10) provided **16b** (18.6 mg, 0.074 mmol, 83%) as a pinkish powder. Mp 254–255 °C; IR (ATR): 3500–2700, 1299, 1214, 1144, 1032, 934 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 3.90 (3H, s), 5.43 (2H, br s), 6.68 (1H, d, J = 1 Hz), 6.98 (1H, dd, J_1 = 9 Hz, J_2 = 2.5 Hz), 7.47 (1H, d, J = 9 Hz), 7.67 (1H, d, J = 2.5 Hz), 8.38 (1H, d, J = 0.5 Hz), 10.87 (1H, s), 12.48 (1H, br s); ^{13}C NMR (125 MHz, DMSO-d_6): 55.7 (CH_3), 88.9, 103.1, 112.0, 113.6, 130.8[a] (CH_{arom}), 109.0, 111.5, 122.9, 126.9, 133.0, 134.8, 137.9[b], 153.1 (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{14}H_{13}N_4O$ $(M+H)^+$ 253.1089, found 253.1096.

4.1.31. 9-Chloro-3,6-dihydropyrazolo[3,4-*c*]carbazol-5-amine (16c)

Same procedure as for the preparation of compounds **7a**–**7c**. From **15c** (60 mg, 0.209 mmol), column chromatography (cyclohexane/EtOAc, 1:9) provided **16c** (37.0 mg, 0.144 mmol, 69%) as a beige powder. Mp >250 °C; IR (ATR): 3450–2800, 1467, 1291, 1066, 935 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 5.50 (2H, br s), 6.74 (1H, d, J = 1 Hz), 7.34 (1H, dd, J_1 = 8.5 Hz, J_2 = 2 Hz), 7.59 (1H, d, J = 8.5 Hz), 8.28 (1H, d, J = 2 Hz), 8.42 (1H, s), 11.28 (1H, s), 12.57 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 89.8, 112.8, 120.1, 123.7 130.9[a] (CH_{arom}), 108.8, 110.8, 123.0, 123.7, 127.2, 134.8, 136.3, 138.2[b] (C_{arom}), [a]chemical shift measured from a HSQC ^1H–^{13}C experiment, [b]chemical shift measured from a HMBC ^1H–^{13}C experiment; HRMS (ESI+) calcd for $C_{13}H_{10}{}^{35}ClN_4$ $(M+H)^+$ 257.0605, found 257.0594.

4.2. In vitro kinase inhibition assays

The procedures for the in vitro protein kinase assays and for the expression and activation of the protein kinases have been described previously.[26]

Source and purification of kinases: All protein kinases were of human origin and encoded full-length proteins. All proteins were either expressed as GST (glutathione transferase) fusion proteins in *Escherichia coli* or as hexahistidine (His_6)-tagged proteins in Sf21 (*Spodoptera frugiperda* 21) insect cells. GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose, and His_6-tagged proteins on nickel/nitrilotriacetate–agarose.

Protein kinase assays: All assays (25.5 μL volume) were carried out robotically at room temperature (21 °C) and were linear with respect to time and enzyme concentration under the conditions used. Assays were performed for 30 min using Multidrop Micro reagent dispensers (Thermo Electron Corporation, Waltham, MA, U.S.A.) in a 96-well format. The concentration of magnesium acetate in the assays was 10 mM and [γ-^{33}P]ATP (800 cpm/pmol) was used at 5 μM for Pim-2 and 20 μM for Pim-1 and Pim-3, in order to be at or below the K_m for ATP for each enzyme.

The assays were initiated with MgATP, stopped by the addition of 5 μL of 0.5 M *ortho*phosphoric acid and spotted on to P81 filter plates using a unifilter harvester (PerkinElmer, Boston, MA, U.S.A.). Kinase substrate was RSRHSSYPAGT (300 μM) for Pim-1, Pim-2 and Pim-3. The enzymes were diluted in a buffer consisting of 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 1 mg/mL BSA and 0.1% 2-mercaptoethanol and assayed in a buffer comprising 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1% 2-mercaptoethanol.

The inhibition profile of the tested compounds was expressed as the percentage of the residual kinase activity for an inhibitor concentration of 10 or 1 μM. The IC_{50} values of inhibitors were determined after carrying out assays at 10 different concentrations of each compound.

4.3. In vitro antiproliferative activity assays

Prostate adenocarcinoma PC3 or LNCap cells were grown in RPMI 1640 + L-Glutamine medium supplemented with 10% (v/v) fetal calf serum, 100 UI penicillin, 100 μg/mL streptomycin and 1.5 μg/mL fungizone and kept under 5% CO_2 atmosphere at 37 °C.

96 well plates were seeded with 2500 PC3 cells or 5000 LNCap cells per well in 200 μL medium.

Twenty four hours later, tested drugs dissolved in DMSO were added for 72 h at a final concentration in a fixed volume of DMSO (1% final concentration). Controls received an equal volume of DMSO. The number of viable cells was measured at 490 nm with the MTS reagent (Promega, Madison, WI) and IC_{50} was calculated as the concentration of compound eliciting a 50% inhibition of cell proliferation.

4.4. Molecular modeling experiments

Pim-1 3JPV crystal structure from the Protein Data Bank (PDB) was used to generate Pim-1 model. After removing 1DR ligand,

protein structure was prepared and all hydrogen atoms were added using Sybylx[32] with its biopolymer module.

Regarding Pim-3, Modeller9V11 software[28] from the module included in UCSF Chimera molecular modeling system[29–31] was used to generate the 3D model from Pim-1 1XWS crystal structure. The Pim-3 model was generated taking into account water molecules and hydrogen bonds existing in the model structure. The Pim-3 model having the best RMSD in comparison with 1XWS was processed with Sybylx2.0. Protein structure was prepared and all hydrogen atoms were added using Sybylx2.0 with its biopolymer module.

For Pim-1 and Pim-3 models, the position of hydrogen atoms and amino-acid residue side chains were then optimized by performing an energy minimization calculation (Tripos force field, MMF94 charges, dielectric constant set to 4.0, conjugate gradient method), before minimization without any constraint.

Docking experiments were performed using Sybylx2.0. After superimposition of Pim-1 or Pim-3 models with 3JPV structure, a protomol was created around 1DR ligand (threshold and bloat values set to 0.05 and 10, respectively). The Surflex-Dock Geom X method was used, with the option allowing H and heavy atom movements. The best docking solutions were selected from the analysis of the molecular database generated by Sybylx. In particular, Polar versus Total_Score, Crash versus Total_Score and Strain versus Total_Score 2D-graphs were produced and analyzed. The best docking solution for each ligand was then minimized (Tripos force field, MMF94 charges, dielectric constant set to 78, conjugate gradient method).

Acknowledgments

The authors are grateful for the financial support of ANR (ANR-08-JCJC-0131-CSD 3) and the French Ministère de l'Enseignement Supérieur et de la Recherche (ES).

References and notes

1. Anizon, F.; Shtil, A. A.; Danilenko, V. N.; Moreau, P. *Curr. Med. Chem.* **2010**, *17*, 4114.
2. Morwick, T. *Exp. Opin. Ther. Patents* **2010**, *20*, 193.
3. Schenone, S.; Tintori, C.; Botta, M. *Curr. Pharm. Des.* **2010**, *16*, 3964.
4. Brault, L.; Gasser, C.; Bracher, F.; Huber, K.; Knapp, S.; Schwaller, J. *Haematologica* **2010**, *95*, 1004.
5. Isaac, M.; Siu, A.; Jongstra, J. *Drug Resist. Update* **2011**, *14*, 203.
6. Tsuhako, A. L.; Brown, D. S.; Koltun, E. S.; Aay, N.; Arcalas, A.; Chan, V.; Du, H.; Engst, S.; Franzini, M.; Galan, A.; Huang, P.; Johnston, S.; Kane, B.; Kim, M. H.; Laird, A. D.; Lin, R.; Mock, L.; Ngan, I.; Pack, M.; Stott, G.; Stout, T. J.; Yu, P.; Zaharia, C.; Zhang, W.; Zhou, P.; Nuss, J. M.; Kearney, P. C.; Xu, W. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3732.
7. Dakin, L. A.; Block, M. H.; Chen, H.; Code, E.; Dowling, J. E.; Feng, X.; Ferguson, A. D.; Green, I.; Hird, A. W.; Howard, T.; Keeton, E. K.; Lamb, M. L.; Lyne, P. D.; Pollard, H.; Read, J.; Wu, A. J.; Zhang, T.; Zheng, X. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4599.
8. Haddach, M.; Michaux, J.; Schwaebe, M. K.; Pierre, F.; O'Brien, S. E.; Borsan, C.; Tran, J.; Raffaele, N.; Ravula, S.; Drygin, D.; Siddiqui-Jain, A.; Darjania, L.; Stansfield, R.; Proffitt, C.; Macalino, D.; Streiner, N.; Bliesath, J.; Omori, M.; Whitten, J. P.; Anderes, K.; Rice, W. G.; Ryckman, D. M. *ACS Med. Chem. Lett.* **2012**, *3*, 135.
9. Drygin, D.; Haddach, M.; Pierre, F.; Ryckman, D. M. *J. Med. Chem.* **2012**, *55*, 8199.
10. Chen, L. S.; Redkar, S.; Bearss, D.; Wierda, W. G.; Gandhi, V. *Blood* **2009**, *114*, 4150.
11. http://clinicaltrials.gov/ct2/show/NCT01239108
12. http://clinicaltrials.gov/ct2/show/NCT01489722
13. Akué-Gédu, R.; Rossignol, E.; Azzaro, S.; Knapp, S.; Filippakopoulos, P.; Bullock, A. N.; Bain, J.; Cohen, P.; Prudhomme, M.; Anizon, F.; Moreau, P. *J. Med. Chem.* **2009**, *52*, 6369.
14. Akué-Gédu, R.; Nauton, L.; Théry, V.; Bain, J.; Cohen, P.; Anizon, F.; Moreau, P. *Bioorg. Med. Chem.* **2010**, *18*, 6865.
15. Gavara, L.; Saugues, E.; Alves, G.; Debiton, E.; Anizon, F.; Moreau, P. *Eur. J. Med. Chem.* **2010**, *45*, 5520.
16. Letribot, B.; Akué-Gédu, R.; Santio, N. M.; El-Ghozzi, M.; Avignant, D.; Cisnetti, F.; Koskinen, P. J.; Gautier, A.; Anizon, F.; Moreau, P. *Eur. J. Med. Chem.* **2012**, *50*, 304.
17. Akué-Gédu, R.; Letribot, B.; Saugues, E.; Debiton, E.; Anizon, F.; Moreau, P. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3807.
18. Giraud, F.; Akué-Gédu, R.; Nauton, L.; Candelon, N.; Debiton, E.; Théry, V.; Anizon, F.; Moreau, P. *Eur. J. Med. Chem.* **2012**, *56*, 225.
19. Gavara, L.; Suchaud, V.; Nauton, L.; Théry, V.; Anizon, F.; Moreau, P. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 2298.
20. Gavara, L.; Anizon, F.; Moreau, P. *Tetrahedron* **2011**, *67*, 7330.
21. Jordan-Hore, J. A.; Johansson, C. C. C.; Gulias, M.; Beck, E. M.; Gaunt, M. J. *J. Am. Chem. Soc.* **2008**, *130*, 16184.
22. Zhu, G.-D.; Gong, J.; Gandhi, V. B.; Woods, K.; Luo, Y.; Liu, X.; Guan, R.; Klinghofer, V.; Johnson, E. F.; Stoll, V. S.; Mamo, M.; Li, Q.; Rosenberg, S. H.; Giranda, V. L. *Bioorg. Med. Chem.* **2007**, *15*, 2441.
23. Slade, D. J.; Pelz, N. F.; Bodnar, W.; Lampe, J. W.; Watson, P. S. *J. Org. Chem.* **2009**, *74*, 6331.
24. Rotzler, J.; Vonlanthen, D.; Barsella, A.; Boeglin, A.; Fort, A.; Mayor, M. *Eur. J. Org. Chem.* **2010**, 1096.
25. Al-Zoubi, R. M.; Hall, D. G. *Org. Lett.* **2010**, *12*, 2480.
26. Bain, J.; Plater, L.; Elliott, M.; Shpiro, N.; Hastie, J.; McLauchlan, H.; Klervernic, I.; Arthur, S. C.; Alessi, D. R.; Cohen, P. *Biochem. J.* **2007**, *408*, 297.
27. http://www.icsn.cnrs-gif.fr.
28. Sali, A.; Blundell, T. L. *J. Mol. Biol.* **1993**, *234*, 779.
29. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. *J. Comput. Chem.* **2004**, *25*, 1605.
30. Meng, E. C.; Pettersen, E. F.; Couch, G. S.; Huang, C. C.; Ferrin, T. E. *BMC Bioinformatics* **2006**, *7*, 339.
31. Yang, Z.; Lasker, K.; Schneidman-Duhovny, D.; Webb, B.; Huang, C. C.; Pettersen, E. F.; Goddard, T. D.; Meng, E. C.; Sali, A.; Ferrin, T. E. *J. Struct. Biol.* **2012**, *179*, 269.
32. Sybylx2.0, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.