European Journal of Medicinal Chemistry 50 (2012) 304–310

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Use of copper(I) catalyzed azide alkyne cycloaddition (CuAAC) for the preparation of conjugated pyrrolo[2,3-*a*]carbazole Pim kinase inhibitors

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ARTICLE INFO

Article history:
Received 20 January 2012
Received in revised form
2 February 2012
Accepted 3 February 2012
Available online 10 February 2012

Keywords:
Pyrrolo[2,3-*a*]carbazole-3-carbaldehyde
Pim kinase inhibition
CuAAC (Copper(I) Catalyzed Azide Alkyne Cycloaddition)

ABSTRACT

We have previously demonstrated that pyrrolo[2,3-*a*]carbazole-3-carbaldehydes are potent Pim kinase inhibitors with *in vitro* antiproliferative activities. In the present study, we report the synthesis of new pyrrolocarbazoles substituted at the N-10 position. When their ability to inhibit Pim kinase activities were evaluated in *in vitro* assays, we observed that this nitrogen atom can be substituted without loss of Pim-1 and Pim-3 inhibitory potencies. Moreover, when we added a fluorescent dansyl group (compound **13**), we were able to show that **13** penetrates the plasma membrane and enters the cytoplasm.

1. Introduction

The Pim kinases (Pim-1, -2, -3) form a small sub-family of three closely related mammalian serine/threonine protein kinases within the CAMK (calcium/calmodulin-dependent protein kinase) group. These enzymes are constitutively active due to a unique hinge structure within their kinase domains [1] and are known to inhibit apoptosis and promote cell cycle progression via phosphorylation of multiple substrates [2]. Normally their expression levels are tightly regulated, but both Pim-1 and Pim-2 are often found overexpressed in human haematopoietic malignancies and in prostate cancer, while elevated levels of Pim-3 have been shown to be associated with several types of solid tumours [2]. The oncogenic properties of Pim kinases were originally demonstrated in transgenic mice, where their overexpression in B and T cells resulted in development of lymphomas [3]. Similar results have also been observed from a transgenic mouse model of human prostate cancer [4].

All these data indicate that Pim kinases are important signalling proteins in tumour biology that could be targeted for the development of new antitumour drugs [5].

Recently, we described the synthesis and biological activities of pyrrolo[2,3-*a*]carbazole-3-carbaldehyde **A** [6]. When tested at 10 μM concentration towards a panel of 66 protein kinases, compound **A** was found to be a potent and selective inhibitor of the Pim kinase family members. We also revealed its crystal structure in complex with Pim-1 (Fig. 1). The structure–activity relationship study undertaken with compound **A** and its derivatives demonstrated that these compounds are potent inhibitors of Pim kinases and attractive molecules for drug development, especially to inhibit the recently demonstrated invasiveness of Pim-overexpressing cancer cells [6–8].

As shown in Fig. 1, compound **A** was found to occupy the ATP-binding site of Pim-1. However, in contrast to typical ATP-mimetic inhibitors, no hydrogen bond was formed with the hinge region. The planar pyrrolocarbazole scaffold was inserted into the ATP-binding cleft with NH groups oriented away from the hinge region. The only hydrogen bond involved the aldehyde and the conserved active site lysine, Lys67. Moreover, the C2–C3 region of the inhibitor formed an aromatic stacking interaction with the P-loop phenylalanine Phe49.

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doi:10.1016/j.ejmech.2012.02.009

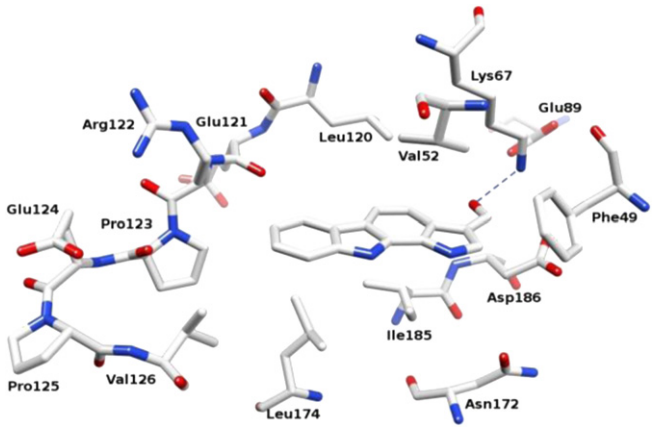


Fig. 1. Binding of pyrrolocarbazole-3-carbaldehyde A to the ATP-binding pocket of Pim-1. The hydrogen bond is indicated in broken blue line. (PDB coordinate file 3JPV). Molecular graphics images were produced using UCSF Chimera [9]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

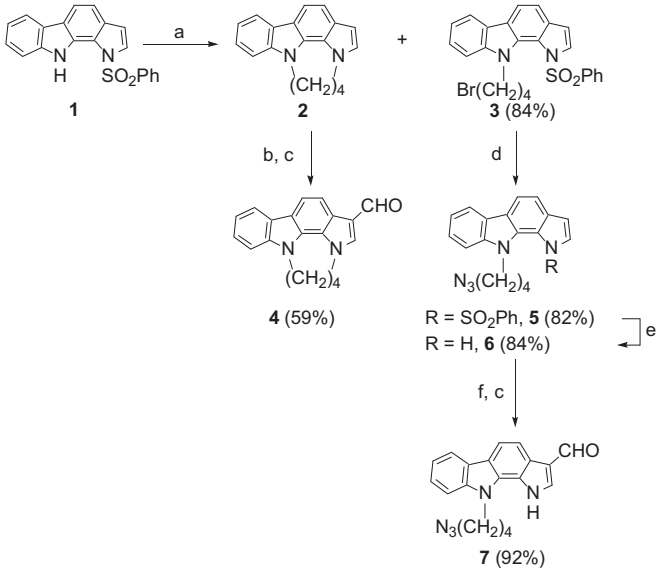
In this study, various substituents were introduced using copper (I) catalyzed azide alkyne cycloaddition (CuAAC) – a so-called "click chemistry" reaction – at the N-10 position which is not involved in the direct interaction between pyrrolo[2,3-*a*]carbazole-3-carbaldehydes and the ATP-binding site of Pim kinases. These substituents are either hydroxyl/ammonium groups to improve water solubility of these compounds or a fluorescent moiety to determine the subcellular localisation of the corresponding conjugate.

2. Chemistry

In order to synthesize the target molecules using a straightforward and modular strategy, we took advantage of CuAAC to connect either hydrophilic substituents or a dansyl fluorescent label. Consequently, we started the preparation of azide **7** (Scheme 1), which was synthesized in four steps from compound **1**. In an initial attempt, compound **1** was allowed to react with 1,4-dibromobutane in the presence of *t*BuOK. This led to a mixture of products **2** and **3**, which were isolated in 40% and 45% yields, respectively. The formation of side-product **2** was probably due to the nucleophilic attack of *t*BuOK or the generated *t*BuOH to the benzenesulfonyl group, leading to a cleavage of the N1-protecting group and subsequent cyclisation. Synthesis of compound **2** was optimized (90% yield) by reacting **1** with 1,4-dibromobutane in the presence of *t*BuONa in toluene at 100 °C. Gratefully, the replacement of alkoxydes by the non nucleophilic base NaH resulted in the production of **3** in 84% yield. To get an insight into the effect on the biological activity of the substitution of both nitrogen atoms by alkyl chains, compound **4**, the formylated analogue of **2**, was prepared in 59% yield using a Vilsmeier–Haack reaction. The azide derivative **7** was obtained by substitution of the bromine atom of **3** using sodium azide and subsequent deprotection/formylation sequence.

Having azide **7** in hands, we examined its CuAAC reaction with several alkynes bearing hydroxymethyl, choline (ammonium) and dansyl groups (Scheme 2).

Room temperature reaction of **7** with propargyl alcohol **8** catalyzed by 5 mol% of Cu(I)/THPTA (tris(hydroxypropyl)triazolylmethyl-amine)/ascorbic acid in methanol was unsuccessful [10]. Therefore, we used [CuCl(1,3-bis(2,4,6-trimethylphenyl)imidazolidin-2-ylidene)(4,7-dichloro-1,10-phenanthroline)] ([CuCl(SIMes)(4,7-Cl-Phen)]), an air stable Cu(I) complex, that belongs to the metal–NHC family. These copper(I) catalysts were introduced to efficiently catalyse CuAAC reactions on a large range of temperature

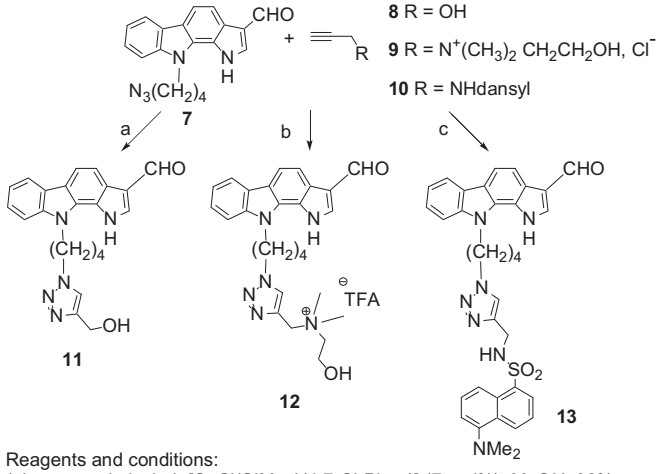


Reagents and conditions: (a) 1,4-dibromobutane, NaH (b) $POCl_3$, DMF, MW (c) NaOH (d) NaN_3, DMF (e) aq. NaOH, MeOH (f) $POCl_3$, DMF

Scheme 1. Preparation of compounds **4**, and **7**.

and solvents (including hydrophobic media required to solubilise substrates of pharmaceutical interest such **7**) without the need of reducing reagents [11,12]. We previously reported the preparation and the reactivity of several complexes related to [CuCl(SIMes)(1,10-Phen)] [11] and [CuCl(SIMes)(4,7-Cl-Phen)] [12]. Notwithstanding their general and practical interest, only a few examples of structural characterisation of such complexes were reported to date [13]. As the three-dimensional arrangement of [CuCl(SIMes)(4,7-Cl-Phen)] complex has not been described previously, and as we now succeeded in getting X-ray diffraction-suitable monocrystals after exhaustive crystallizations assays, we now report the structural characterisation of this complex (Fig. 2, Table 1).

As displayed in Table 1, the bond distances and angles fall into the range of what was found for the related catalyst [CuCl(SIMes)(1,10-Phen)]. The introduction of the two chlorine atoms on the



Reagents and conditions:
(a) propargyl alcohol, [CuCl(SIMes)(4,7-Cl-Phen)] (7 mol%), MeOH, 92%
(b) *N*-(2-hydroxyethyl)-*N*,*N*-dimethylprop-2-yn-1-aminium chloride, [CuCl(4,7-Cl-Phen)(SIMes)] (13 mol%), MeOH, 49%
(c) 5-(dimethylamino)-*N*-(2-propynyl)-1-naphthalenesulfonamide, [CuCl(4,7-Cl-Phen)(SIMes)] (6 mol%), MeOH, 92 %.

Scheme 2. Preparation of compounds **11**, **12** and **13**.

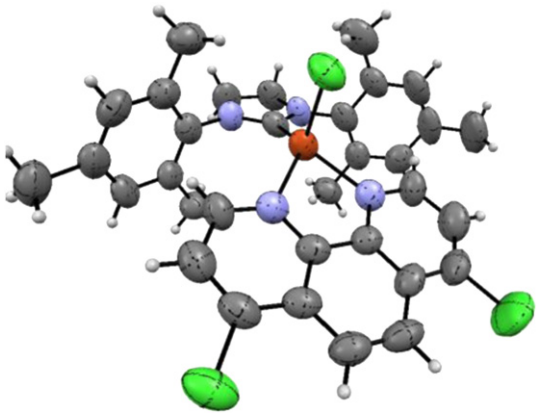


Fig. 2. ORTEP view of [CuCl(SIMes)(4,7-Cl-Phen)] (nitrogen: blue, chloride: green, copper: orange). CCDC ID: 46715. $C_{33}H_{32}Cl_3Cu_1N_4$, monoclinic, space group $C2/c$, a = 31.832(3) Å, b = 13.2828(13) Å, c = 16.6462(16) Å, β = 92.714(5)°, V = 7030.4(12) Å^3, T = 293(2) K, Z = 8, Final R ($I > 2\sigma(I)$): R_1 = 0.0492, wR_2 = 0.0463, s = 1.0978. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

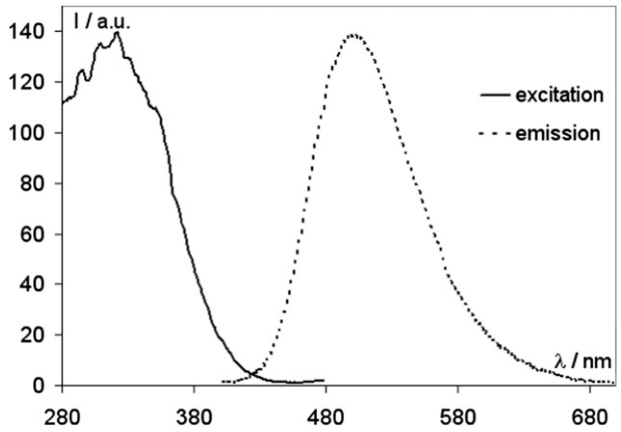


Fig. 3. Fluorescence emission and excitation spectra of dansyl conjugate **13** recorded in DMSO/0.05 mol L^{-1} pH = 7.45 phosphate buffer v/v 9:1.

phenanthroline heterocycle resulted in the shortening of Cu–Cl distance, indicating a lower electron donation of 4,7-dichloro-1,10-phenanthroline compared to that of 1,10-phenanthroline. The latter distance is still longer than the one found in [CuCl(SIMes)]. This is of importance for a CuAAC process because the Cu–Cl bond needs to be broken to enter the catalytic cycle (see discussion in ref [11]). On the other hand, the resulting lower electron density at the metal centre could explain the very high stability of [CuCl(SIMes)(4,7-Cl–Phen)] in the presence of air.

The coupling between **7** and propargyl alcohol **8** using 7 mol% of [CuCl(SIMes)(4,7-Cl–Phen)] led to complete conversion of **7** into the expected triazole **11** that was further isolated after column chromatography in 92% yield (Scheme 2). When azide **7** was used for the preparation of the choline derivative **12**, the low solubility of *N*-(2-hydroxyethyl)-*N*,*N*-dimethylprop-2-yn-1-aminium chloride **9** prompted us to perform the reaction in boiling methanol, leading to a total conversion of the starting material – thus highlighting the catalyst's robustness. Compound **12** (trifluoroacetate salt) was isolated in 49% yield after purification by reverse phase HPLC. In both cases, the aqueous solubility of compounds **11** and **12** was greatly increased as compared to **A**. Finally, pyrrolocarbazole **7** was labelled with **10** in similar conditions, leading to compound **13** in 92% yield. Compound **13** displayed a near-UV excitation band at λ_{exc} = 322 nm and, under excitation at the latter wavelength, its emission spectrum showed a maximum at λ_{em} = 501 nm (Fig. 3). These spectral features are typical of dansyl moieties in aqueous solutions [14]. No significant fluorescent emission was observed when **A** was studied in the same conditions.

3. Results and discussion

The kinase inhibitory potencies of compounds **4**, **7**, **11**, **12**, and **13** were evaluated at 10 μM concentrations in duplicate assays against all three Pim family kinases, as previously described [15] and the percentages of residual kinase activities are reported in Table 2. IC_{50} values were determined when the remaining kinase activity was less than 10% when the compounds were tested at 10 μM.

As evident from Table 2, the results obtained for compound **4** indicate that potent inhibition of Pim kinases is compatible with the substitution of both nitrogen atoms. Whereas Pim-1 inhibitory potency of compound **4** was greatly enhanced, the activities against the two other isoforms were similar to those of compound **A**. However, this feature should be verified with a larger panel of 1,10-disubstituted analogues.

Regarding compound **7**, interesting submicromolar inhibitory potencies have been measured towards both Pim-1 and Pim-3.

Compounds bearing a 1,2,3-triazole moiety **11**–**13** exhibited similar inhibitory potencies against both Pim-1 and Pim-3. Compound **11**–**13** inhibited Pim-1 in the same range as compound **A** whereas they were less active towards Pim-3. Nevertheless, the dansyl derivative **13** was slightly less potent than the non-fluorescent derivatives.

Except for compound **4**, all the other tested derivatives were less active towards Pim-2 than the reference compound **A**, suggesting that the substitution of only one of the two nitrogen atoms (N-10 position) of the pyrrolocarbazole scaffold is detrimental for Pim-2 kinase inhibition.

Altogether, these results indicate that the N-10 position of the pyrrolocarbazole scaffold can be substituted by various groups without abolishing Pim-1 inhibition, but that the potency against

Table 1
Bond lengths and angles of interest.

Bonds lengths (Å)				
	Cu–Cl	Cu–N^3	Cu–N^4	Cu–C^1
[CuCl(SIMes)(4,7-Cl–Phen)]	2.320 (2)	2.114 (4)	2.127 (4)	1.905 (4)
[CuCl(SIMes)(1,10-Phen)]	2.347 (2)	2.114 (5)	2.128 (5)	1.916 (7)
[CuCl(SIMes)]	2.099 (1)			1.882 (4)
Bond angles (°)				
	N^3–Cu–N^4	N^4–Cu–Cl	N^3–Cu–Cl	C^1–Cu–Cl
[CuCl(SIMes)(4,7-Cl–Phen)]	76.6 (2)	99.5 (1)	99.2 (1)	115.9 (2)
[CuCl(SIMes)(1,10-Phen)]	77.4 (2)	99.9 (1)	102.0 (2)	120.5 (2)
[CuCl(SIMes)]				178.5 (3)

Table 2
In vitro inhibitory potencies towards Pim kinases of compounds **A**, **4**, **7**, **11**–**13**: percentage of residual activities when tested at 10 μM (IC_{50} in μM in brackets). nd: Not determined.

Compounds	% of Pim kinase residual activities		
	Pim-1	Pim-2	Pim-3
A	2 ± 0.4 (0.12 ± 0.01)	7 ± 1 (0.51 ± 0.23)	1 ± 5 (0.01 ± 0.00)
4	7 ± 1 (0.008 ± 0.001)	6 ± 2 (0.35 ± 0.01)	1 ± 0 (0.013 ± 0.002)
7	9 ± 4 (0.105 ± 0.001)	22 ± 1 (nd)	14 ± 4 (0.108 ± 0.037)
11	8 ± 2 (0.315 ± 0.154)	36 ± 3(nd)	23 ± 9 (0.235 ± 0.023)
12	9 ± 2 (0.327 ± 0.01)	65 ± 9 (nd)	28 ± 4 (0.49 ± 0.04)
13	17 ± 3 (nd)	66 ± 8 (nd)	38 ± 6 (nd)

other Pim family members is often reduced by these substitutions. More particularly, we have demonstrated that the introduction of diversely substituted alkyl-1,2,3-triazole moieties is permitted for Pim-1 inhibition.

To analyze the ability of the fluorescent dansyl conjugate **13** to enter into the cells and to visualize its subcellular localization, PC-3 prostate cancer cells grown on coverslips were treated for 24 h with either 10 μM compound **A** or **13** or with DMSO (0,1%). When cell samples were analysed by fluorescence microscopy, both DMSO and compound **A** displayed hardly any autofluorescence, while a bright fluorescent signal was detected in cells treated with compound **13** (Fig. 4A). Interestingly, **13** localized into the cytoplasm of the cells (Fig. 4B), suggesting that it could penetrate the plasma membrane, but was unable to enter the nuclei.

In conclusion, new pyrrolocarbazole-3-carbaldehydes, substituted at the N-10 position, were synthesized and evaluated for their ability to inhibit Pim kinases in *in vitro* assays. The results obtained indicate that this nitrogen atom can be substituted without abolishing inhibitory potency towards Pim-1 and Pim-3. More particularly, Pim-1/Pim-3 inhibition is conserved when the N-10 position of the pyrrolocarbazole scaffold is substituted by alkyl-1,2,3-triazole moieties obtained by CuAAC. Furthermore, the results with the dansyl conjugate **13** indicate that fluorescent labels can be added to the compounds without significant loss of inhibitory potency against Pim-1. They can also be used to analyse the membrane permeability and subcellular localisation of the compounds. Interestingly, compound **13** was able to enter the cytoplasm, but not the nuclei, suggesting that it might be a useful research tool to distinguish between nuclear and cytoplasmic functions of Pim. Overall, we can envisage to use the CuAAC for the N-10 position functionalisation of this series in order to prepare novel Pim inhibitors.

4. Experimental

4.1. Chemistry

4.1.1. General

Starting materials were obtained from commercial suppliers and used without further purification. Solvents were distilled prior to use. IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer (in cm^{-1}). NMR spectra, performed on a Bruker AVANCE 400 (^1H: 400 MHz, ^{13}C: 100 MHz), or a Bruker AVANCE 500 (^1H: 500 MHz), are reported in ppm using the solvent residual peak as an internal standard; the following abbreviations are used: singlet (s), doublet (d), broad doublet (br d), triplet (t), quintet (quint), doublet of doublet (dd), doublet of doublet of doublet (ddd), multiplet (m), broad signal (br s). High resolution mass spectra (ESI+) were determined on a high resolution Micro Q-Tof apparatus (CRMP, Université Blaise Pascal, Clermont-Ferrand, France). Chromatographic purifications were performed by column chromatography using 40–63 μm silica gel or by preparative HPLC through a Varian HPLC system (Prepstar 218 pump, Propstar 335 PDA detector, C18 250 × 41.4 mm 8 μm column). Reactions were monitored by TLC using fluorescent silica gel plates (60 F254 from Merck). Melting points were measured on a Reichert microscope and are uncorrected. Fluorescence excitation and emission spectra were recorded on a Cary eclipse spectrophotometer (sample concentration: 10^{-5} mol L^{-1}, 5 nm emission and excitation slits). [CuCl(SIMes)(4,7-Cl–Phen)] was prepared on a 10 g scale by scaling-up a previously described method [12]. Single crystals (purple needles) were obtained by slow evaporation of a concentrated (~0.05 g mL^{-1}) solution of this compound in dichloromethane. The X-ray diffraction data were recorded on a Bruker APEX-II CCD diffractometer using MoKαradiation.

4.1.2. 4,5,6,7-Tetrahydroindolo[1,2,3-fg]pyrrolo[3,2,1-kl][1,6]benzodiazocine **2**

A mixture of compound **1** (50 mg, 0.144 mmol), *t*BuONa (69 mg, 0.72 mmol) and 1,4-dibromobutane (70 μL, 0.59 mmol) in toluene (5 mL) was stirred in an oil bath heated at 100 °C for 15 h. After cooling, water was added and the mixture was extracted with EtOAc. The assembled organic fractions were washed with brine and dried over $MgSO_4$. After evaporation, the residue was purified by column chromatography (cyclohexane/EtOAc, 95:5) to give 34 mg of compound **2** (0.131 mmol, 90%) as a beige solid. Mp = 132–134 °C; IR (ATR): 1620, 1602 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 2.01–2.07 (4H, m), 4.52–4.59 (4H, m), 6.57 (1H, d, J = 3 Hz), 7.18 (1H, ddd, J_1 = 8 Hz, J_2 = 7 Hz, J_3 = 1 Hz), 7.35–7.39 (2H, m), 7.39 (1H, d, J = 8.5 Hz), 7.64 (1H, d, J = 8 Hz), 7.80 (1H, d, J = 8.5 Hz), 8.08 (1H, d, J = 7.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 25.6, 25.8, 42.1, 45.9 (CH_2), 101.9, 109.4, 112.0, 113.0, 118.9 (2C), 123.8, 129.7 (CH_{arom}), 117.4, 122.1, 123.6, 127.8, 128.8, 140.0 (C_{arom}); HRMS (ESI+) calcd for $C_{18}H_{17}N_2$ $(M + H)^+$ 261.1392, found 261.1403.

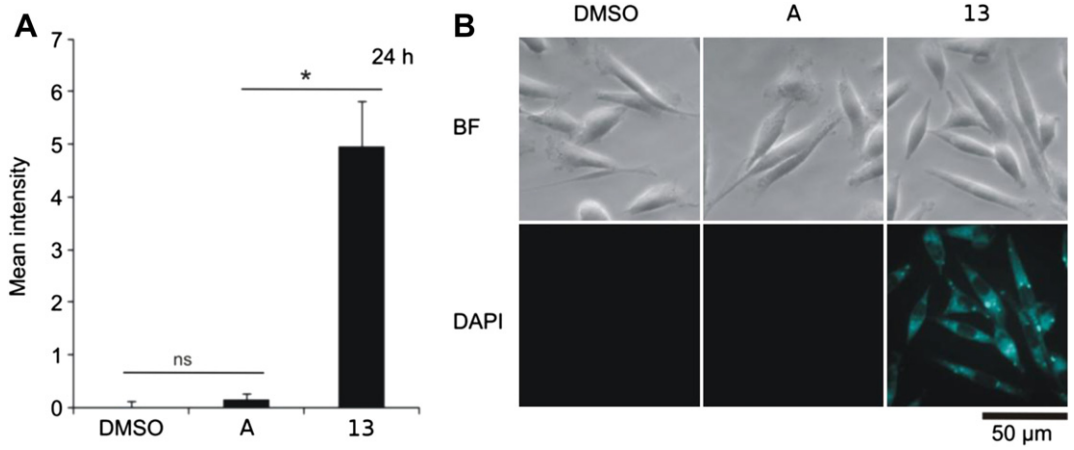


Fig. 4. (A) PC-3 prostate cancer cells grown on coverslips were treated for 24 h with either 10 μM compound **A** or **13** or with DMSO (0,1%). Bright Field (BF) and fluorescent (DAPI) pictures were taken from fixed cell samples. Mean intensity values were measured from unprocessed DAPI-filtered images. Shown are background-corrected means from three regions in two parallel samples. (B) For visualization of the subcellular localization of fluorescence, the grey-scale images were recoloured with cyan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1.3. 10-(4-Bromobutyl)-1-(phenylsulfonyl)-1,10-dihydropyrrolo [2,3-a]carbazole **3**

To a mixture of compound **1** (1.00 g, 2.89 mmol) and dibromobutane (5.4 mL) was added sodium hydride (60% in mineral oil, 460 mg, 11.5 mmol). The mixture was stirred at 100 °C for 4 h and the mixture was poured into a 1 M aqueous HCl solution (100 mL). After extraction with EtOAc (3 × 80 mL), the assembled organic fractions were dried over $MgSO_4$, evaporated and the residue was purified by column chromatography (EtOAc/cyclohexane, from 0:100 to 2:98) to give 1.16 g of compound **3** (2.41 mmol, 83%) as a grey solid, and trace amount of compound **2**. Mp = 122–124 °C; IR (ATR): 1470, 1448, 1431, 1379, 1368, 1337, 1325 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.23–1.32 (2H, m), 1.39–1.49 (2H, m), 3.25 (2H, t, J = 6.5 Hz), 4.95 (2H, t, J = 7 Hz), 6.93 (1H, d, J = 3.5 Hz), 7.17 (2H, d, J = 8 Hz), 7.22–7.31 (4H, m), 7.51 (2H, t, J = 8 Hz), 7.53 (1H, d, J = 3.5 Hz), 7.79 (1H, d, J = 8 Hz), 8.12 (1H, d, J = 8 Hz), 8.21 (1H, d, J = 7.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 26.4, 29.4, 34.1, 45.1 (CH_2), 111.7, 113.3, 118.4, 119.1, 120.0, 120.2, 125.7, 127.0 (2C), 128.5 (2C), 131.4, 134.2 (CH_{arom}), 123.0, 123.4, 124.1, 132.8, 133.4, 133.5, 141.7 (C_{arom}); HRMS (ESI+) calcd for $C_{24}H_{22}{}^{79}BrN_2O_2SBr$ $(M + H)^+$ 481.0585, found 481.0602.

4.1.4. 4,5,6,7-Tetrahydroindolo[1,2,3-fg]pyrrolo[3,2,1-kl][1,6] benzodiazocine-1-carbadehy-de **4**

$POCl_3$ (3 equiv) was slowly added to anhydrous DMF (2 mL) at 0 °C. The solution was stirred at room temperature for 45 min, and then was added to a solution of compound **2** (100 mg, 0.38 mmol) in DMF (1 mL), prepared in a 10 mL CEM reaction vessel and cooled to 0 °C. The tube was sealed and was heated at 100 °C under microwave irradiation for 20 min (P_{max} = 150 W). After cooling, the mixture was poured into a saturated aqueous $NaHCO_3$ solution (20 mL). After 30 min of stirring, the solid was filtered off and a 5% aqueous NaOH solution (20 mL) was added. The mixture was stirred at room temperature overnight. The solid was filtered off and purified by column chromatography (cyclohexane/EtOAc, from 8:2 to 4:6) to give 65 mg of compound **4** (0.23 mmol, 59%) as a brown solid. Mp = 212–214 °C (decomposition); IR (ATR): 1651, 1540, 1446, 1395 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 2.02–2.13 (4H, m), 4.59–4.63 (2H, m), 4.66–4.70 (2H, m), 7.24 (1H, t, J = 7.5 Hz), 7.44 (1H, t, J = 7.5 Hz), 7.69 (1H, d, J = 8 Hz), 8.06 (2H, s), 8.16 (1H, d, J = 7.5 Hz), 8.33 (1H, s), 9.98 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 25.2, 25.4, 42.2, 46.9 (CH_2), 109.7, 112.7, 115.4, 119.40, 119.41, 124.8, 141.3 (CH_{arom}), 117.7, 119.6, 123.0, 123.5, 125.0, 127.4, 140.5 (C_{arom}), 184.7 (C=O); HRMS (ESI+) calcd for $C_{19}H_{17}N_2O$ $(M + H)^+$ 289.1341, found 289.1339.

4.1.5. 10-(4-Azidobutyl)-1-(phenylsulfonyl)-1,10-dihydropyrrolo [2,3-a]carbazole **5**

To a solution of compound **3** (100 mg, 0.21 mmol) in DMF (4 mL) was added NaN_3 (67 mg, 1.0 mmol). The mixture was stirred at 80 °C for 5 h. After cooling, the mixture was poured into water (100 mL) and extracted with EtOAc (3 × 50 mL). The assembled organic fractions were washed with water, with brine and dried over $MgSO_4$. After evaporation under reduced pressure, the residue was purified by column chromatography (EtOAc/cyclohexane, 1:9) to give 76 mg of compound **5** (0.171, 82%) as a brown solid. Mp = 132–134 °C; IR (ATR): 2088, 1470, 1447, 1361 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.95–1.04 (2H, m), 1.32–1.42 (2H, m), 3.07 (2H, t, J = 7 Hz), 4.94 (2H, t, J = 7 Hz), 6.94 (1H, d, J = 3.5 Hz), 7.16 (2H, d, J = 8 Hz), 7.23–7.32 (4H, m), 7.51 (2H, t, J = 7.5 Hz), 7.53 (1H, d, J = 3.5 Hz), 7.79 (1H, d, J = 8 Hz), 8.12 (1H, d, J = 8 Hz), 8.21 (1H, d, J = 7.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 24.9, 25.4, 45.4, 49.9 (CH_2), 111.7, 113.3, 118.4, 119.1, 120.0, 120.2, 125.6, 127.0 (2C), 128.5 (2C), 131.3, 134.2 (CH_{arom}), 123.0, 123.4, 124.1, 132.9, 133.4, 133.5, 141.7 (C_{arom}); HRMS (ESI+) calcd for $C_{24}H_{21}N_5NaO_2S$ $(M + Na)^+$ 466.1314, found 466.1320.

4.1.6. 10-(4-Azidobutyl)-1,10-dihydropyrrolo[2,3-a]carbazole **6**

To a solution of compound **5** (45 mg, 0.101 mmol) in methanol (14 mL) was added a 5 M aqueous NaOH solution (2 mL). The mixture was refluxed for 4 h. After evaporation of methanol under reduced pressure, the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The assembled organic fractions were dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography (EtOAc/cyclohexane 2:8) to give 26 mg of compound **6** (0.086 mmol, 84%) as a beige solid.

Mp > 250 °C (decomposition); IR (ATR): 3421, 3380, 2093, 1650, 1614, 1560 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.52–1.61 (2H, m), 1.77–1.86 (2H, m), 3.29 (2H, t, J = 7 Hz), 4.75 (2H, t, J = 7 Hz), 6.63–6.65 (1H, m), 7.17 (1H, t, J = 7.5 Hz), 7.36 (1H, t, J = 7.5 Hz), 7.38 (1H, d, J = 8 Hz), 7.42 (1H, t, J = 2.5 Hz), 7.64 (1H, d, J = 8 Hz), 7.76 (1H, d, J = 8 Hz), 8.07 (1H, d, J = 7.5 Hz), 11.36 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 25.7, 27.6, 43.3, 50.4 (CH_2), 102.9, 109.3, 112.2, 112.4, 118.7, 119.1, 123.5, 124.2 (CH), 116.3, 120.9, 123.7, 127.2, 127.6, 138.7 (C); HRMS (ESI+) calcd for $C_{18}H_{17}N_5Na(M + Na)^+$ 326.1382, found 326.1395.

4.1.7. 10-(4-Azidobutyl)-1,10-dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde **7**

$POCl_3$ (1.1 mL, 11.8 mmol) was slowly added to anhydrous DMF (12 mL) at 0 °C. The solution was stirred at room temperature for 1 h and cooled to 0 °C. A solution of compound **6** (1.18 g, 3.89 mmol) in anhydrous DMF (7 mL) was added and the mixture was stirred at room temperature for 1 h. The mixture was poured into a saturated aqueous $NaHCO_3$ solution (200 mL). After 1.5 h of stirring, the solid was filtered off and a 5% aqueous NaOH solution (250 mL) was added. The mixture was stirred for 16 h at 50 °C. The solid was filtered off and then purified by column chromatography (CH_2Cl_2/MeOH, from 98:2 to 85:15) to give 1.18 g of compound **7** (3.56 mmol, 92%) as yellow-brown solid. Mp = 186–188 °C; IR (ATR): 3303, 2086, 1632 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.50–1.59 (2H, m), 1.77–1.87 (2H, m), 3.29 (2H, t, J = 7 Hz), 4.78 (2H, t, J = 7 Hz), 7.22 (1H, t, J = 7.5 Hz), 7.43 (1H, t, J = 7.5 Hz), 7.70 (1H, d, J = 8.5 Hz), 8.02 (2H, s), 8.15 (1H, d, J = 7.5 Hz), 7.39 (1H, d, J = 3 Hz), 10.07 (1H, s), 12.34 (1H, br d, J = 2.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 25.6, 27.4, 43.3, 50.4 (CH_2), 109.5, 112.4, 115.3, 119.18, 119.6, 124.5, 137.4 (CH_{arom}), 118.5, 119.17, 121.9, 123.1, 123.8, 126.7, 139.2 (C_{arom}), 185.3 (C=O); HRMS (ESI+) calcd for $C_{19}H_{18}N_5O$ $(M + H)^+$ 332.1511, found 332.1528.

4.1.8. N-(2-hydroxyethyl)-N,N-dimethylprop-2-yn-1-aminium chloride **9**

To a solution of propargyl chloride (70% w/w in toluene, 25 mL, 0.2 mol) in toluene (30 mL) was added dropwise a solution of *N*,*N*-dimethylaminoethanol (17 mL, 0.17 mol) in toluene (30 mL). The reaction was maintained at room temperature with a water bath during the addition and the reaction mixture was stirred for 3 days. The precipitate was filtered off and washed with pentane to give 23.8 g of **9** (0.15 mol, 86%) as an off-white hygroscopic solid. IR (ATR): 3254, 3216, 2124, 1458, 1441, 1376, 1149, 1085, 1026, 1002, 995, 917, 874 cm^{-1}; 1H NMR (400 MHz, CD_3OD): 3,28 (6H, s), 3.55 (1H, s), 3.61 (2H, br s), 4.03 (2H, br s), 4.46 (2H, s); ^{13}C RMN (100 MHz, CD_3OD): 52.0 ($2CH_3$), 56.5, 57.0, 66.6 (CH_2), 72.7 (CH), 83.0 (C); HRMS (ESI+) calcd for $C_7H_{14}NO$ (M^+) 128.1075, found 128.1078.

4.1.9. 10-{4-[4-(Hydroxymethyl)-1H-1,2,3-triazol-1-yl]butyl}-1,10-dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde **11**

To a solution of compound **7** (50 mg, 0.15 mmol) and propargyl alcohol (34.6 μL, 0.60 mmol) in methanol (5 mL) was

added [CuCl(SIMes)(4,7-Cl–Phen)] (7.1 mg, 0.011 mmol). The reaction mixture was stirred at room temperature for 20 h. After evaporation under reduced pressure, the residue was purified by column chromatography (EtOAc/MeOH, from 10:0 to 8:2) to give 54 mg of compound **11** (0.139 mmol, 92%) as a light brown solid. Mp = 183–185 °C; IR (ATR): 3420–3040, 1627 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.70–1.89 (4H, m), 4.29 (2H, t, J = 6.5 Hz), 4.44 (2H, d, J = 5.5 Hz), 4.77 (2H, t, J = 6.5 Hz), 5.10 (1H, t, J = 5.5 Hz), 7.22 (1H, t, J = 7.5 Hz), 7.42 (1H, t, J = 7.5 Hz), 7.69 (1H, d, J = 8 Hz), 7.83 (1H, s), 8.01 (2H, s), 8.15 (1H, d, J = 7.5 Hz), 8.38 (1H, s), 10.06 (1H, s), 12.32 (1H, br s); ^{13}C NMR (100 MHz, DMSO-d_6): 27.2, 27.3, 43.2, 48.9, 55.0 (CH_2), 109.6, 112.4, 115.3, 119.2, 119.6, 122.6, 124.5, 137.5 (CH_{arom}), 118.5, 119.2, 121.9, 123.1, 123.9, 126.7, 139.2, 147.9 (C_{arom}), 185.3 (C=O); HRMS (ESI+) calcd for $C_{22}H_{21}N_5NaO_2$ $(M + Na)^+$ 410.1593, found 410.1577.

4.1.10. N-({1-[4-(3-Formylpyrrolo[2,3-a]carbazol-10(1H)-yl)butyl]-1H-1,2,3-triazol-4-yl}methyl)-2-hydroxy-N,N-dimethylethanaminium trifluoroacetate **12**

To a solution of compound **7** (200 mg, 0.60 mmol) and *N*-(2-hydroxyethyl)-*N*,*N*-dimethylprop-2-yn-1-aminium chloride (49 mg, 0.30 mmol) in methanol (12 mL) was added [CuCl(SIMes)(4,7-Cl–Phen)] (25 mg, 0.038 mmol). The mixture was stirred at 60 °C for 20 h. After evaporation under reduced pressure, water was added and the mixture was washed with EtOAc. Water was azeotroped with ethanol and the residue was purified by preparative RPHPLC (water 0.1% TFA/acetonitrile 55:45, flow = 40 mL/min, detection at λ = 250 nm) to give 84 mg of compound **12** (0.15 mmol, 49%) as a brownish solid. Mp = 87–89 °C; IR (ATR): 3224, 1675, 1653, 1201, 1130 cm^{-1} ^1H NMR (500 MHz, DMSO-d_6): 1.71–1.80 (2H, m), 1.85–1.94 (2H, m), 2.95 (6H, s), 3.27–3.31 (2H, m), 3.83–3.89 (2H, m), 4.39 (2H, t, J = 7 Hz), 4.60 (2H, s), 4,78 (2H, t, J = 7 Hz), 5.20–5.45 (1H, br s), 7.22 (1H, t, J = 7.5 Hz), 7.41 (1H, t, J = 7.5 Hz), 7,67 (1H, d, J = 8 Hz), 8.01 (2H, s), 8.15 (1H, d, J = 7.5 Hz), 8.22 (1H, s), 8.37 (1H, d, J = 3 Hz), 10.07 (1H, s), 12.35 (1H, br d, J = 2.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 50.2 (2C) (CH_3), 26.9, 27.2, 43.2, 49.4, 55.0, 58.4, 64.6 (CH_2), 109.5, 112.4, 115.3, 119.2, 119.6, 124.5, 128.2, 137.5 (CH_{arom}), 118.5, 119.2, 121.9, 123.2, 123.9, 126.8, 135.2, 139.1 (C_{arom}), 185.4 (C=O); HRMS (ESI+) calcd for $C_{26}H_{31}N_6O_2$ (M^+) 459.2508, found 459.2492.

4.1.11. 5-(Dimethylamino)-N-({1-[4-(3-formylpyrrolo[2,3-a]carbazol-10(1H)-yl)butyl]-1H-1,2,3-triazol-4-yl}methyl)naphthalene-1-sulfonamide **13**

To a solution of compound **7** (50 mg, 0.15 mmol) and **10** [16] (45 mg, 0.156 mmol) in methanol (3 mL) was added [CuCl(SIMes)(4,7-Cl–Phen)] (6.1 mg, 0.0093 mmol). The mixture was stirred at 50 °C for 16 h. After evaporation under reduced pressure, the residue was purified by column chromatography (EtOAc/cyclohexane, from 7:3 to 10:0, then EtOAc/MeOH, from 100:0 to 95:5) to give 86 mg of compound **13** (0.139 mmol, 92%) as a yellow-brown solid. Mp = 200–202 °C; IR (ATR): 3450–3030, 1653, 1144 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.63–1.77 (4H, br s), 2.74 (6H, s), 4.02 (2H, d, J = 5.5 Hz), 4.11–4.18 (2H, m), 4.71–4.78 (2H, m), 7.14 (1H, d, J = 7.5 Hz), 7.23 (1H, t, J = 7.5 Hz), 7.39–7.51 (4H, m), 7.67 (1H, d, J = 8 Hz), 7.99–8.05 (3H, m), 8.16 (1H, d, J = 7.5 Hz), 8.21 (1H, d, J = 8.5 Hz), 8.32 (1H, d, J = 8.5 Hz), 8.37–8.42 (2H, m), 10.07 (1H, s), 12.31 (1H, br d, J = 3 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): 44.9 (2C) (CH_3), 26.9, 27.1, 37.8, 43.2, 48.8 (CH_2); 109.5, 112.4, 115.0, 115.3, 119.1, 119.2, 119.6, 122.8, 123.4, 124.5, 127.7, 128.2, 129.4, 137.4 (CH_{arom}), 118.5, 119.2, 121.9, 123.1, 123.8, 126.7, 128.9, 129.0, 135.9, 139.1, 143.4, 151.2 (C_{arom}), 185.2 (C=O). HRMS (ESI+) calcd for $C_{34}H_{33}N_7NaO_3S$ $(M + Na)^+$ 642.2263, found 642.2258.

4.2. Kinase assays

4.2.1. 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 [15].

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 c.p.m./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 orthophosphoric 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 μM. The IC_{50} values of inhibitors were determined after carrying out assays at 10 different concentrations of each compound.

4.3. Cellular localisation assays

4.3.1. Cell culture

The human PC-3 prostate cancer cell line was cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM l-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

4.3.2. Subcellular localization assay

PC-3 cells were seeded onto coverslips on 6-well plates and incubated overnight for proper attachment. Thereafter cells were treated with 10 μM compounds (A or 21) dissolved in DMSO or with 0,1% DMSO. After 24 h, cells were washed with PBS, fixed for 15 min with 4% paraformaldehyde and washed three times with PBS. Coverslip samples were mounted on glass slides with Mowiol and examined by LeicaDMRE fluorescent microscope. Pictures were taken with Display Image command by Hamamatsu C4742-95 CCD camera and HCImage software (Hamamatsu Corporation, NJ, USA). Mean intensity values were analysed from 8-bit tif files and cyan colour was added by ImageJ software (Wayne Rasband, NIH, USA). For background correction, the average mean intensity value of DMSO samples was counted and subtracted from the values of each analyzed image.

Acknowledgements

CNRS Valorisation (R A-G), Academy of Finland (PJK) and Drug Discovery Graduate School (NMS) are greatly acknowledged for financial support. The authors are grateful to Bertrand Légeret for mass spectrometry analysis, Aurélie Job for preparative RPHPLC

purification of compound **12**, and Pauline Labaume for the synthesis of [CuCl(SIMes)(4,7-Cl–Phen)].

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.02.009.

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