Bioorganic & Medicinal Chemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Design and synthesis of potent and orally active GPR4 antagonists with modulatory effects on nociception, inflammation, and angiogenesis

Wolfgang Miltz [a,*], Juraj Velicky [a], Janet Dawson [b], Amanda Littlewood-Evans [b], Marie-Gabrielle Ludwig [c], Klaus Seuwen [c], Roland Feifel [d], Berndt Oberhauser [a], Arndt Meyer [a], Daniela Gabriel [c], Mark Nash [e], Pius Loetscher [b]

[a] Global Discovery Chemistry, Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland
[b] Autoimmunity, Transplantation and Inflammation, Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland
[c] Chemical Biology and Therapeutics, Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland
[d] PK Sciences, Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland
[e] Muscoloskeletal Diseases, Novartis Institutes for BioMedical Research, CH-4002 Basel, Switzerland

ARTICLE INFO

Article history:
Received 9 May 2017
Revised 26 June 2017
Accepted 28 June 2017
Available online xxxx

Keywords:
GPR4
Amino-pyrimidine derivatives
Imidazo-pyridine derivatives
Angiogenesis
Inflammation
Pain

ABSTRACT

GPR4, a G-protein coupled receptor, functions as a proton sensor being activated by extracellular acidic pH and has been implicated in playing a key role in acidosis associated with a variety of inflammatory conditions. An orally active GPR4 antagonist **39c** was developed, starting from a high throughput screening hit **1**. The compound shows potent cellular activity and is efficacious in animal models of angiogenesis, inflammation and pain.

1. Introduction

GPR4 is a G-protein coupled receptor (GPCR) and belongs to the family of proton sensing receptors comprising also ovarian cancer G protein-coupled receptor 1 (OGR1; GPR68), and T-cell death-associated gene 8 (TDAG8; GPR65).[1] The sensing of extracellular protons and the stimulation of intracellular secondary messengers upon exposure to acidic pH have been reported.[2] The pH-dependent activation of GPR4 involves the protonation of histidine residues of the receptor located at the cellular surface leading to a conformational change of the receptor and formation of cAMP via Gαs. GPR4 is expressed in a wide range of tissues including vasculature, lung, kidney, heart and liver.[3–5]

GPR4 has been linked to pH sensor function in a number of tissues and organs under physiological and pathological conditions. For instance, extracellular acidification occurs at sites of inflammation.[6] It has been shown that activation of GPR4 by acidic pH increases endothelial cell adhesion with leukocytes and up-regulates the expression of the adhesion molecules VCAM-1 and ICAM-1 in association with cAMP accumulation.[4] Furthermore, GPR4 is suggested to be involved in acidic pH-induced expression of a number of inflammatory genes, including chemokines, cytokines, NF-κB pathway genes, COX-2 and stress response genes.[7] An acidic milieu is also characteristic for the tumor microenvironment. It has been reported that GPR4-deficient mice show strongly reduced pathological angiogenesis and tumor growth.[3] An involvement of GPR4 in the modulation of glucose homeostasis has been demonstrated. GPR4 deficiency improves glucose tolerance and insulin sensitivity.[8] Moreover, GPR4 has very recently been implicated in the regulation of breathing by CO_2.[9] Recently, it has been reported that blocking GPR4 reduced the myocardial infarction-induced injury in mice which is characterized by decreased tissue pH.[10] Furthermore, the pro-inflammatory properties of GPR4 within inflamed intestinal tissues have been demonstrated by studies with GPR4-deficient mice.[11] Also, GPR4 has been reported as a novel mediator for endoplasmic reticulum (ER) stress in response to acidosis in endothelial cells.[12]

Here we report the development of small molecules targeting GPR4 that are expected to offer a new treatment option for

* Corresponding author.
E-mail address: wolfgang.miltz@novartis.com (W. Miltz).

http://dx.doi.org/10.1016/j.bmc.2017.06.050
0968-0896/

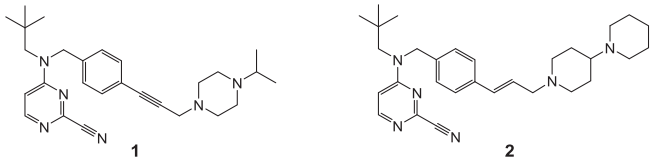


Fig. 1. GPR4 antagonists identified by HTS.

inflammatory disorders, starting from the HTS-hits **1** and **2** (Fig. 1, Table 1). In addition, we have just published the further development and optimization of the compounds.[13]

2. Results and discussion

2.1. Chemistry

The aminopyrimidine derivatives with a propargyl linker (compounds **7a–c**, Table 2a) were synthesized as outlined in Scheme 1. Starting from the corresponding 4-chloro pyrimidine (**3**), the chlorine was substituted by neopentyl amine. The amino group in **4** was further alkylated with 4-iodobenzylbromide to give compound **5**. The 2-methoxy group in **5b** was introduced by nucleophilic substitution of chlorine atom in the 2-chloro derivative **5a** with sodium methylate.[14] In the next step, the compounds **6a-c** were synthesized from **5a-c** by reaction with propargylalcohol under Sonogashira coupling conditions.[15] The final products **7a–c** were prepared by mesylation of the alcohol **6** and subsequent reaction with *N*-isopropylpiperazine.

Derivatives with the allyl linker (compounds **11a–f**, Table 2b; and compound **12**, Table 3) were prepared according to Scheme 2. *N*-alkylation of intermediates **4a** and **4c–e** with 4-bromo-benzylbromide led to the corresponding bromointermediates **8a** and **8c–e**. The methoxy derivative **8b** was again prepared by reaction of chloro-derivative **8a** with sodium methoxide, while dimethylamino-analog **8f** was prepared from **8a** by reaction with dimethylamine. Heck reaction of products **8a–f** with methyl acrylate[16] and subsequent reduction of the ester group in **9a–f** led to allyl alcohols **10a–f**. Introduction of the basic head groups was achieved by direct reaction of alcohols **10a–f** with the Zaragoza reagent (cyanomethyl-trimethylphosphonium iodide)[17] leading to compounds **11a–f** and **12**.

Amide derivative **15** (Table 3) was synthesized starting from the common *N*-neopentyl intermediate **4c** by its alkylation with ethyl *p*-bromomethyl benzoate, followed by saponification of the ester and coupling of the formed acid with 4-dimethylaminobutylamine (Scheme 3).

For the synthesis of the corresponding inverse amide **18** (Table 3), bromo intermediate **8c** was transferred into aniline **16** by the Buchwald amination using diphenylmethanimine as an ammonia equivalent.[18] Amide coupling with 5-chloropentanoic acid gave chloride **17** which was converted into the dimethylamino derivative **18** by its reaction with dimethylamine (Scheme 4).

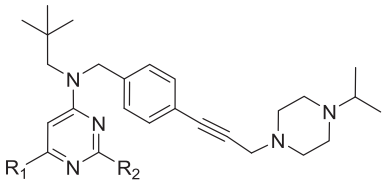
Dimethylpyrimidine derivative with triazole linker (**21**, Table 3) was also synthesized via the common bromo-intermediate **8c** being first converted into the azide **19** by copper mediated coupling with NaN_3.[19] Subsequent Huisgen 1,3-dipolar cycloaddition (Click-chemistry)[20] with 1-bromo-3-butyne gave the bromoethyltriazole **20** which could further be converted by reaction with 4-hydroxypiperidine to derivative **21** (Scheme 5).

Pyrrolopyrimidine derivative **29** (Table 4) was accessed via intermediate **26** (Scheme 6). This compound was prepared starting from acetylacetate **22** by its alkylation with 1-bromo-2-butanone,[21] followed by cyclization with acetamidine,[22] conversion of the formed pyrimidinone **24** to the chloropyrimidine **25** and its final cyclization with 4-bromo benzylamine.[23] It was then submitted to Heck reaction with methyl acrylate leading to methyl cinnamate **27**. The ester in **27** was then reduced with DIBAL to allyl alcohol **28** which was transformed to the analog **29** by its reaction with *N*-isopropylpiperazine using Zaragoza reagent.[17]

Synthesis of the imidazopyridine derivatives **39** (Table 4) was performed through the common intermediates **35a–f** which could be prepared by two different routes depending on the 2-substituent (Scheme 7). Method A was employed for synthesis of derivatives **39b**,**c**,**e** and the key-intermediates **35b**,**c**,**e** were synthesized from imidazolone **32** obtained from malonamidine by its cyclization with acetylacetone[24] followed by Hoffmann rearrangement/cyclization.[25] Reaction of **32** with the corresponding carboxylic anhydride in the presence of $MgCl_2$ gave intermediates **35b**,**c**,**e**.[26] A different route (method B) was used for analogs **35a**, **d**,**f**. This method is based on cyclization of the pyridinediamines **34a**,**b**, obtained from 3-nitro-2-aminopyridines **33a**,**b** by nitro-group reduction, with the corresponding carboxylic acid under dehydration conditions.[27]

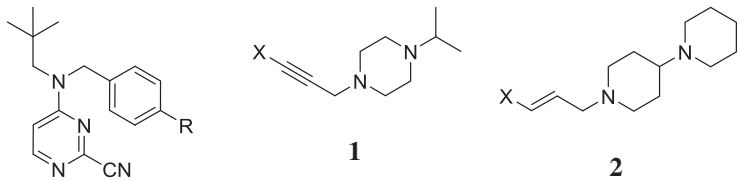
Benzyl acrylates **37a–f** could be reached from intermediates **35a–f** either by a two-steps procedure (alkylation with *p*-bromobenzylbromide followed by the Heck reaction with methylacry-

Table 2a
Propargylamine analogues without cyano group.

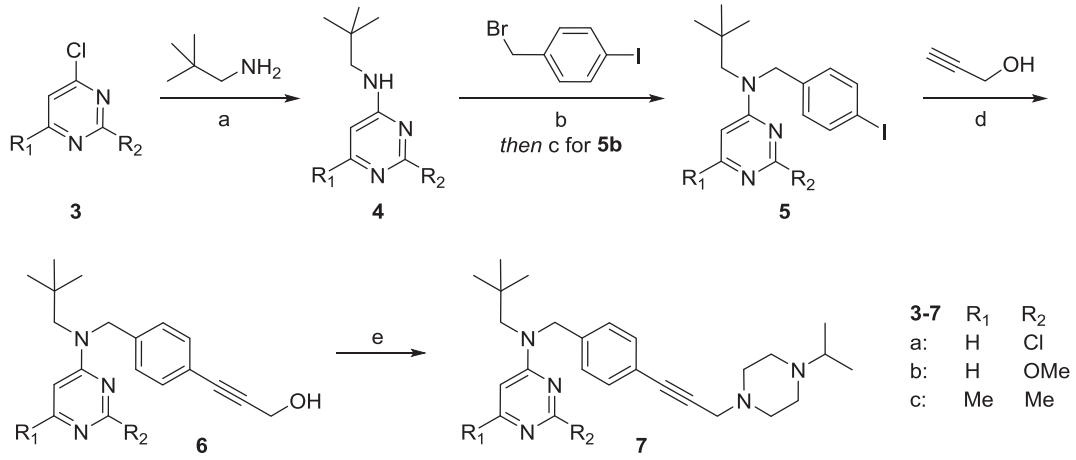


R_1	R_2	**7**	cAMP (HeLa) IC_{50} [μM]
H	Cl	**a**	0.30 ± 0.09 (n = 10)
H	OMe	**b**	0.14 ± 0.02 (n = 6)
Me	Me	**c**	0.80 ± 0.04 (n = 3)

Table 1
HTS hits.



	1	**2**
cAMP (HeLa); IC_{50} [μM]	1.93 ± 0.63 (n = 4)	0.49 ± 0.03 (n = 112)
human TDAG8; IC_{50} [μM]	>30 (n = 1)	>23 (n = 1)
h Cat K; IC_{50} [μM]	0.003 (n = 1)	0.001 (n = 1)



Scheme 1. Synthesis of the propargyl derivatives **7a**–**c**. Reagents and conditions: a) K_2CO_3, DMF, 100 °C, 2 h (45–93%); (b) NaH, DMF, rt, 10 min (48–83%); (c) NaH, MeOH, 60 C, 16 h (100%); (d) CuI, $PdCl_2(PPh_3)_2$, Et_3N, DMF, 100 °C, 1.5 h (39–90%); (e) MsCl, Et_3 N, rt, 5 min; then *N*-isopropylpiperazine, K_2CO_3, DMF, 80 °C, 15 min (30–59%).

late) or by a direct one-step synthesis for derivative **37b** using (*E*)-methyl 3-(4-(bromomethyl) phenyl) acrylate. The final products **39a**–**f** were synthesized by reduction of **37a**–**f** with DIBAL followed by Zaragoza-type amination of the obtained allyl alcohol **38a**–**f**.

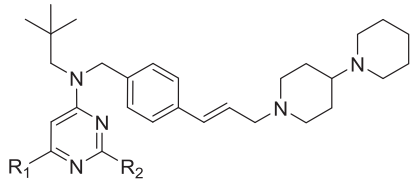
2.2. Lead optimization

2.2.1. Aminopyrimidines

Two 4-aminopyrimidine derivatives **1** and **2** were identified by HTS as potential GPR4 inhibitors showing potency in a pH-dependent cAMP release assay (HeLa cells) in a micromolar range. Interestingly, such compounds displayed a selectivity against close pH sensing receptors such as TDAG8 (Table 1). Both compounds are highly potent Cathepsin K inhibitors with the cyano group being the active principle.[28]

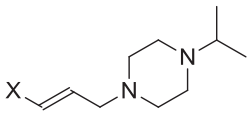
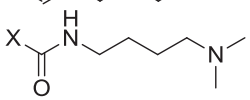
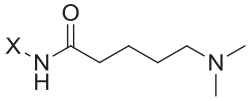
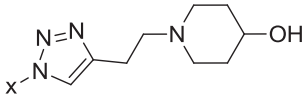
Therefore, removal of the cyano group in 2-position, being crucial for the activity on cysteine proteases such as Cathepsin K, was

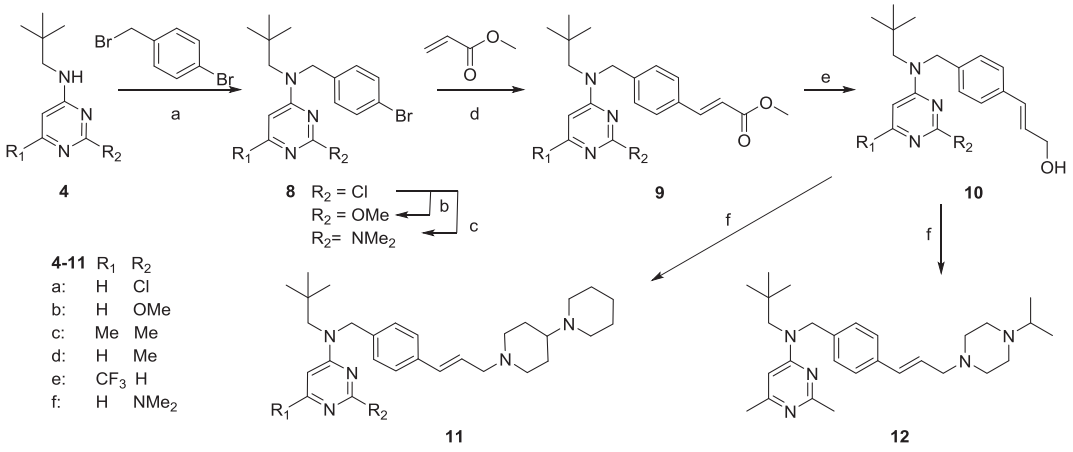
Table 2b
Allylamine analogues without cyano group.



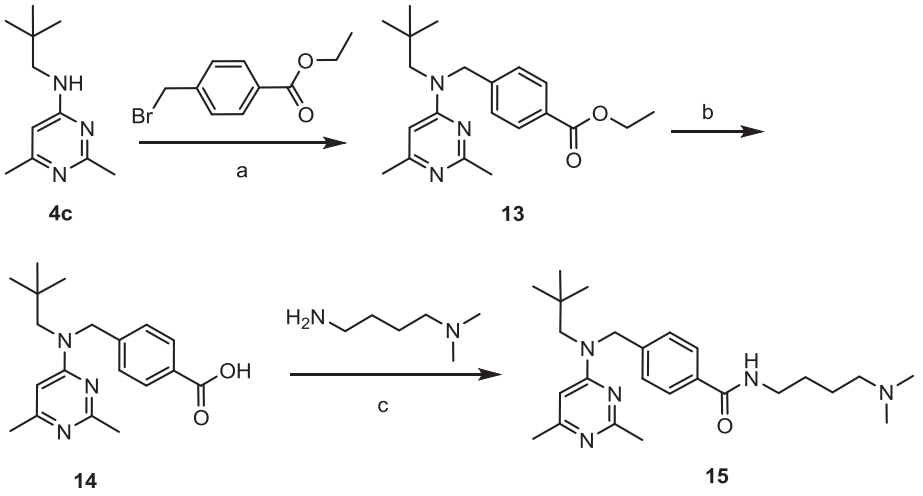
R_1	R_2	**11**	cAMP (HeLa) IC_{50} [μM]
H	Cl	**a**	0.36 ± 0.05 (n = 3)
H	OMe	**b**	0.70 ± 0.14 (n = 3)
H	Me	**c**	0.41 ± 0.05 (n = 3)
Me	Me	**d**	0.38 ± 0.06 (n = 4)
H	NMe_2	**e**	2.97 ± 0.70 (n = 3)
CF_3	H	**f**	4.80 ± 0.42 (n = 3)

Table 3
Linker variations.

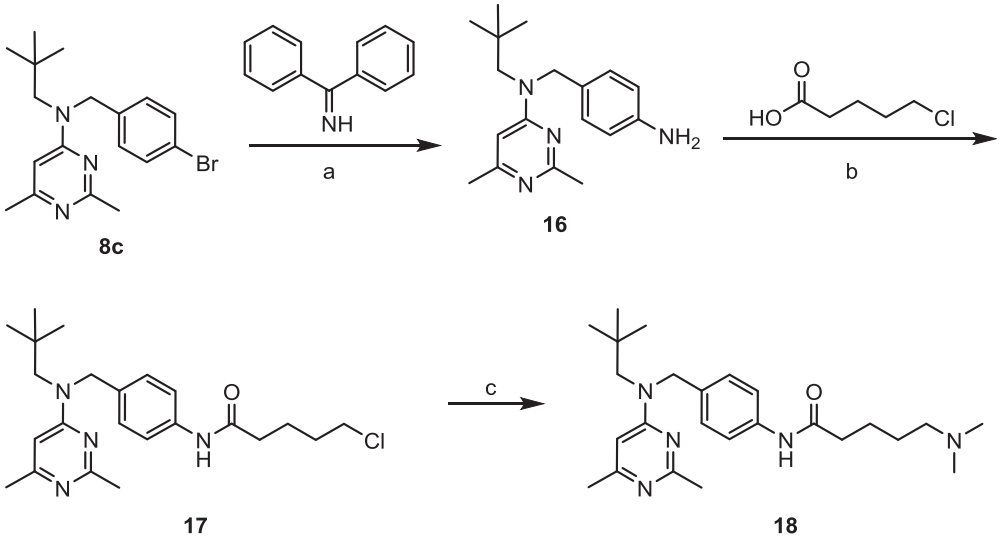
		cAMP IC_{50} [μM]		
		human cAMP (HeLa cells)	mouse cAMP (HEK cells)	rat cAMP (HEK cells)
12		0.33 ± 0.06 (n = 7)	1.63 ± 0.42 (n = 7)	1.59 ± 0.57(n = 5)
15		0.16 ± 0.03 (n = 11)	0.56 ± 0.07(n = 11)	1.26 ± 0.30 (n = 7)
18		0.20 ± 0.03 (n = 3)	0.76 ± 0.35 (n = 3)	1.99 ± 0.43 (n = 3)
21		0.05 ± 0.01 (n = 6)	0.18 ± 0.04 (n = 3)	0.48 ± 0.15 (n = 3)



Scheme 2. Preparation of the allyl series. Reagents and conditions: (a) NaH, DMF, 2 h, rt (26–69%); (b) NaOMe/MeOH, reflux, 16 h (100%); (c) Me_2NH/EtOH, 6 h, 60 °C (91%); (d) Pd(*t*-$Bu_3P)_2$, methyldicyclohexyl amine, dioxane, 130 °C, 5 min (52–99%); (e) DIBAH, CH_2Cl_2, −78 °C, 3 h (36–64%); (f) amine, cyanomethyl-trimethylphosphonium iodide, DIPEA, CH_3CH_2CN, 120 °C, 2 h (21–66%).

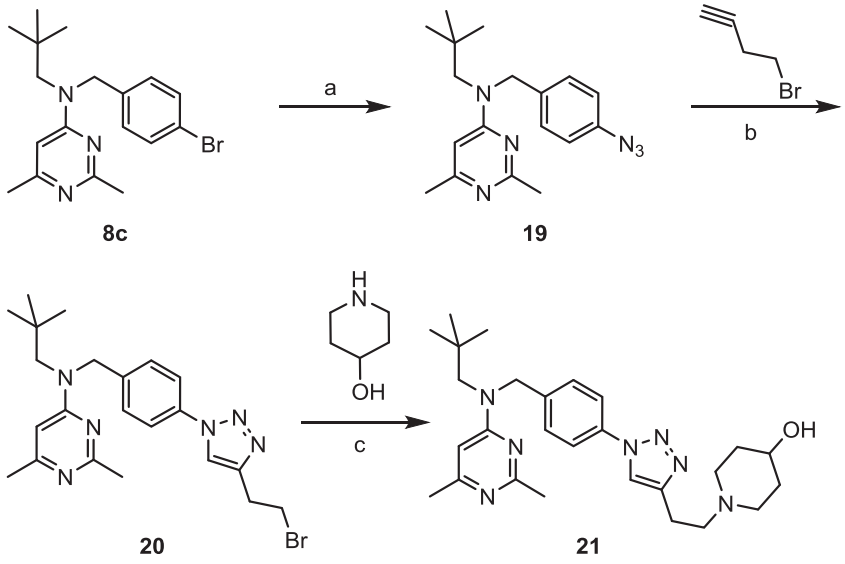


Scheme 3. Preparation of amide derivative **15**. Reagents and conditions: (a) NaH, DMF, 30 min, rt (58%); (b) 6 M HCl, 2 h, 100 °C (52%); (c) EDC, HOBT, DIPEA, CH_2Cl_2, 16 h, rt (54%).



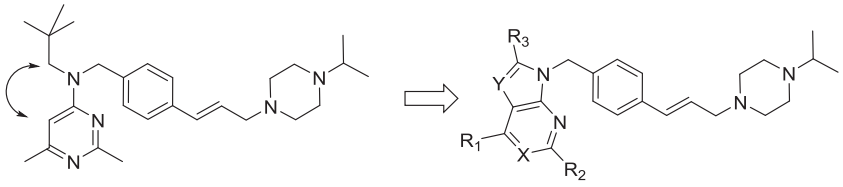
Scheme 4. Preparation of amide **18**. Reagents and conditions: (a) NaOMe, $Pd_2(dba)_3$, *rac*-BINAP, PhMe, 2 h, 100 °C (68%); (b) EDC, DIPEA, CH_2Cl_2, 1 h, rt (81%); (c) Me_2NH, THF, 4 d, 60 °C (98%).

Please cite this article in press as: Miltz W., et al. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.050



Scheme 5. Preparation of triazole **21**. Reagents and conditions: (a) NaN_3, CuI, *N,N′*-dimethylethylenediamine, sodium ascorbate, $EtOH/H_2O$, 1 h, 100 °C (75%); (b) CuI, CH_3CN, 18 h, rt (74%); (c) DMF, 30 min, 120 °C (44%).

Table 4
SAR of imidazopyridines.



Nr.	R1	R2	R3	X	Y	cAMP IC_{50} [μM]		
						Human (HeLa)	Rat (HEK)	Mouse (HEK)
29	Me	Me	Et	N	CH	11.6 ± 3.3 (n = 6)	nd	nd
39a	H	H	Et	CH	N	9.10 ± 3.97 (n = 3)	>20 (n = 4)	>20 (n = 4)
39b	Me	Me	Me	CH	N	0.41 ± 0.28 (n = 3)	6.10 ± 1.83 (n = 3)	1.84 ± 0.39 (n = 3)
39c	Me	Me	Et	CH	N	0.11 ± 0.01 (n = 427)	1.29 ± 0.17 (n = 108)	0.8 ± 0.12 (n = 110)
39d	Me	Me	Prop	CH	N	0.76 ± 0.47 (n = 2)	3.31 (n = 1)	5.22 (n = 1)
39e	Me	Me	iProp	CH	N	2.44 ± 1.28 (n = 7)	16.5 ± 3.48 (n = 3)	17.24 ± 2.76 (n = 3)
39f	Me	Me	iBu	CH	N	7.31 ± 1.99 (n = 2)	>20 (n = 2)	>20 (n = 2)

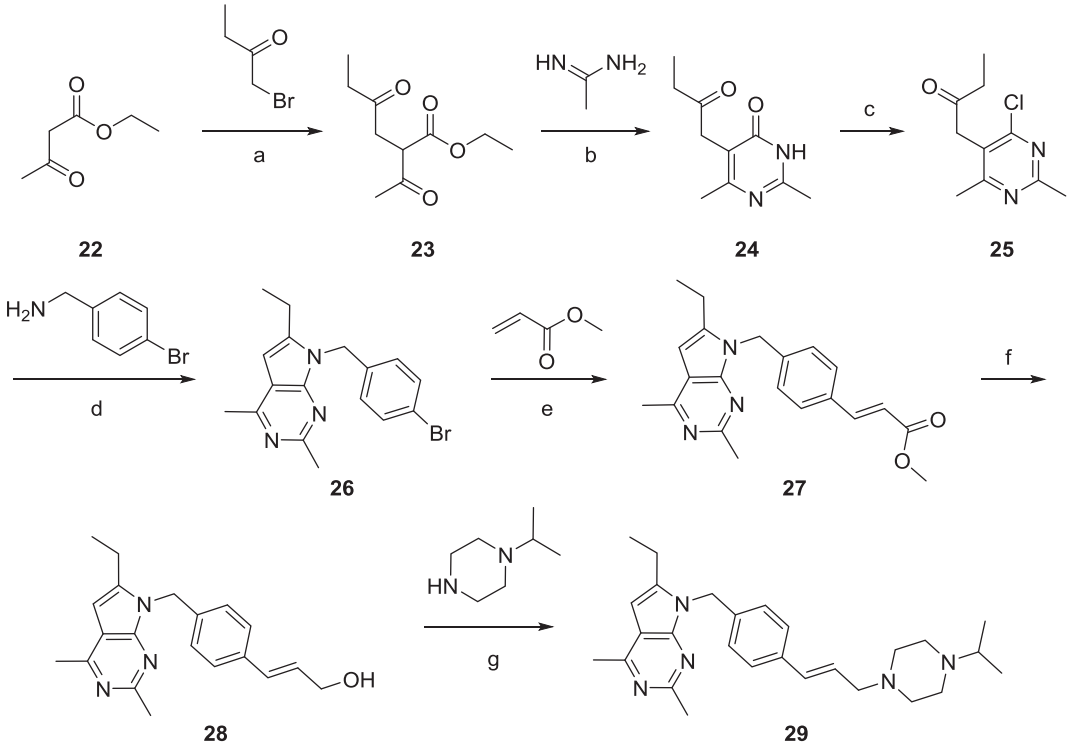
tried initially. Interestingly, compounds with other groups than cyano in this position (**7a–c** or **11a–f**; tables 2a,b) proved to have improved potency in inhibiting GPR4. While most of these derivatives showed good potency, analogs with larger substituents such as dimethylamino (**11e**) or trifluoromethyl (**11f**) were less tolerated by GPR4 receptor.

In a second step, the derivatization was focused on the replacement of the potentially labile propylene linker between the aminopyrimidine and the basic tail groups (**12**, Table 3). Interestingly, all derivatives with an increased polarity such as amides **15** and **18** or triazole **21** showed improved activity in both human and mouse cells.

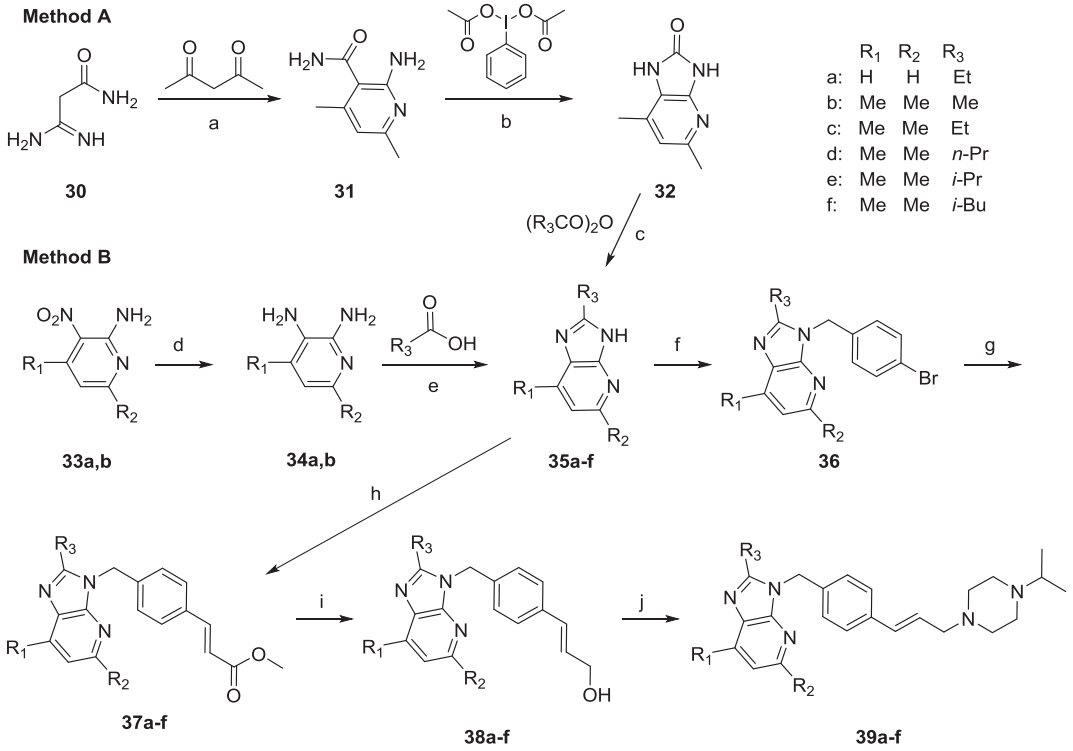
2.2.2. Imidazopyridines

The aminopyrimidine series was morphed into compounds with bicyclic scaffolds such as pyrrolopyrimidines or imidazopyridines. The nature of the bicyclic group seems to have high impact on activity. While pyrrolopyrimidine derivative **29** shows dramatic decrease in potency on GPR4 compared to its open version (**12**), the corresponding imidazopyridine derivative **39c** proved to be 100-fold more potent than **29** and also more potent than **12**. Furthermore, the two methyl groups in **39c** were found to contribute significantly to the binding into GPR4, since the des-methyl compound **39a** is 90-fold less potent than **39c**. The substituent in the 2-position of imidazole ring was found to be of great importance as well. The ethyl group in compound **39c** appeared to have the optimal size. While methyl (**39b**) is tolerated, but is binding with less efficiency to GPR4, larger substituents in this position such as *n*-Pr (**39d**) and especially the branched ones like *i*-Pr (**39e**) or *i*-Bu (**39e**) are much less accepted by the receptor.

The PK profiles of two pyrimidine compounds **15** and **21** were compared with compound **39c**, the most potent imidazopyridine analog (Table 5). Derivative **39c** shows the most promising PK properties (low clearance, good bioavailability and oral exposure)



Scheme 6. Preparation of pyrrolopyrimidine analog **29**. Reagents and conditions: (a) NaH, THF, 0 °C to rt, 16 h (87%); (b) Na/MeOH, 16 h, reflux (61%); (c) $POCl_3$, 15 min, reflux (70%); (d) $CHCl_3$, 12 h, 100 °C (100%); (e) Et_3N, $Pd(OAc)_2$, DMF, 15 min, 150 °C (56%); (f) DIBAL, CH_2Cl_2, 1 h, −78 °C (70%); (g) cyanomethyl trimethyl phosphonium iodide, DIPEA, CH_3CH_2CN, 1 h, 120 °C (59%).



Scheme 7. Preparation of the imidazopyridines. Reagents and conditions: (a) KOH, MeOH, 5 h, rt (100%); (b) KOH, MeOH, 16 h, rt (98%); (c) $MgCl_2$, 16 h, 100 °C (45–80%); (d) H_2, Pd/C, EtOH, 4 h, rt (99%); (e) PPA, 2 h, 190 °C (21–80%); (f) NaH, DMF, 4-bromobenzylbromide, 2 h, rt (20–70%); (g) $Pd(t\text{-}Bu_3P)_2$, dicyclohexylmethylamine, 5 min, 130 °C (59–92%); (h) NaH, methyl 4-bromomethylphenylacrylate, DMF, 2 h, rt (39%); (i) DIBAH, CH_2Cl_2, 3 h, −78 °C (65–97%); (j) amine, cyanomethyl-trimethylphosphonium iodide, DIPEA, CH_3CH_2CN, 120 °C, 2 h (59–86%).

Please cite this article in press as: Miltz W., et al. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.06.050

Table 5
Pharmacokinetics in female Sprague Dawley rats.

Rat PK (1 mg/kg iv, 3 mg/kg po)					
Nr.	CL [ml/ min.kg]	T ½ [h]	AUC po dn [ug.min/ ml], SD	Cmax dn [nM], SD	BAV [%], SD
15	48	34	424 ± 83	73 ± 25	17 ± 3
21	56	23	2051 ± 132	94 ± 54	96
39c	15 ± 2	5.6 ± 1.5	2196 ± 298	193 ± 39	86 ± 12

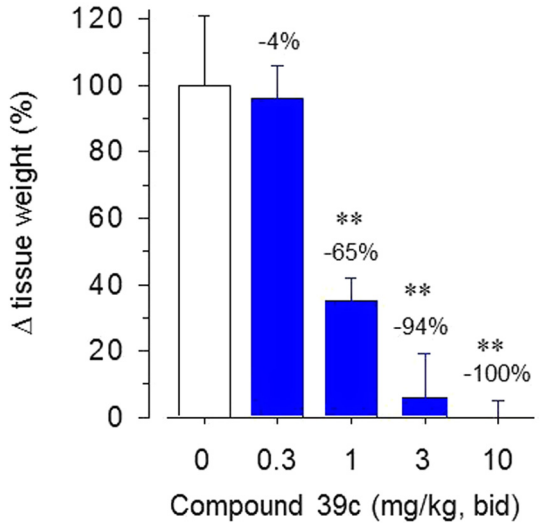


Fig. 2. Inhibition of VEGF-induced angiogenesis by compound **39c** in mouse chamber implant model. Statistics are One-Way ANOVA followed by Dunnett's test for multiple comparisons (** $p < 0.01$).

and was therefore chosen for further profiling. In order to assess the pharmacological effects described for GPR4 inhibition, compound **39c** was tested in animal models of angiogenesis, arthritis and pain.

2.3. In vivo pharmacology

2.3.1. Angiogenesis

Compound **39c** was first tested in a VEGF-induced angiogenesis model (Fig. 2). After implantation of a VEGF-containing agar chamber into mice, the compound was applied for three days at doses of 0.3, 1, 3 and 10 mg/kg po/bid. At day 4 the chamber was explanted and the weight of the tissue in the chamber was determined. Compound **39c** dose dependently inhibited the VEGF-induced angiogenesis showing a very strong effect already at 3 mg/kg po/bid dose.

2.3.2. Rat antigen-induced arthritis

For the evaluation of anti-inflammatory activity of GPR4 inhibitors, **39c** was tested in the rat antigen-induced arthritis model (Fig. 3). It was dosed for seven days after the day of intra-articular challenge with methylated BSA (mBSA) into the right knees and vehicle only into the left knees of Lewis rats being immunized with mBSA on day −21 and −14 (i.d.) on the back of the animal. Knee swelling was measured by digital calipers and the swelling ratio (R/L) was calculated. In addition, histology (inflammatory cell infiltration, joint damage and proteoglycan loss) was determined at day seven (Fig. 4).

Again, compound **39c** dose-dependently inhibited knee swelling in this model. Significant effect was observed already at 10 mg/kg po/bid dose even if 90 mg/kg po/bid dose was needed to reach the efficacy comparable to that of dexamethasone. In addition, inhibition of joint damage/erosions was also seen at 90 mg/kg with **39c**. Dexamethasone at 0.3 mg/kg represents the effective positive control compound and dose in this model (Fig. 4).

2.3.3. Rat hyperalgesia

Finally, the activity of the GPR4 inhibitor **39c** was tested for its antinociceptive effect using rat hyperalgesia model (Fig. 5). In this model, an injection of complete Freund's Adjuvant (CFA) into the hind paw on day 0 was followed by compound application (po) on day 3. Paw withdrawal measurements were performed at 1, 3 and 6 h. Compound **39c** showed a significant analgesic effect at

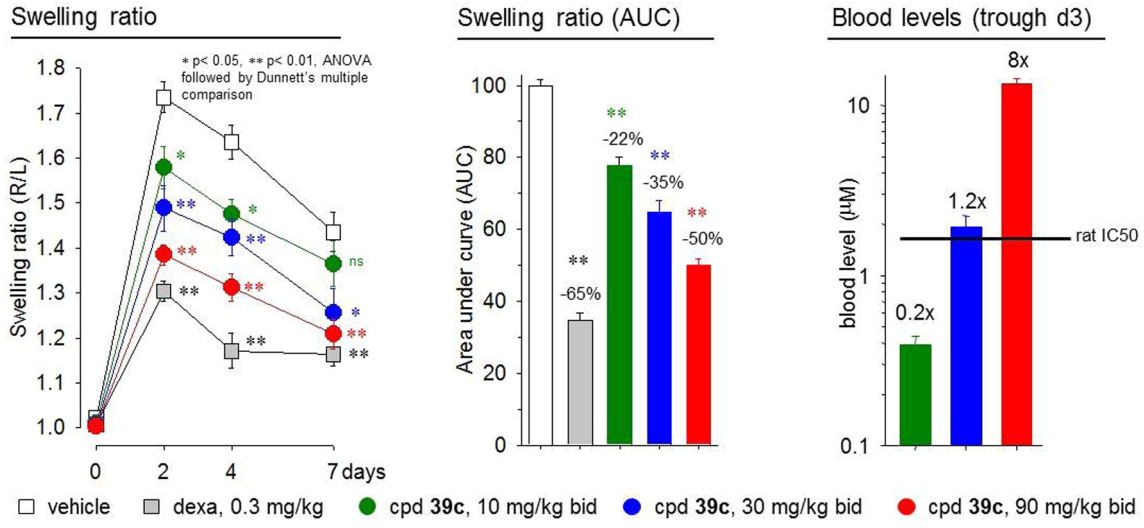


Fig. 3. Inhibition of swelling by compound **39c** in rat antigen-induced arthritis model and compound levels in blood at trough on day 3. Fold above or below the IC_{50} value of compound **39c** in the rat cAMP assay is indicated. Statistics are One-Way ANOVA followed by Dunnett's test for multiple comparisons ($^{*}p < 0.05$, $^{**}p < 0.01$). dexa, dexamethasone.

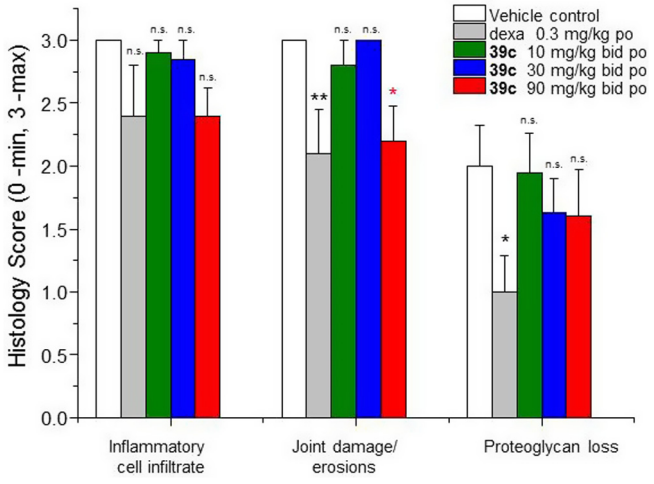


Fig. 4. Histological analysis of rat antigen-induced arthritis model. Inhibition of inflammatory cell infiltration, joint damage/erosions and proteoglycan loss was determined at day seven. Statistics are One-Way ANOVA followed by Dunnett's test for multiple comparisons ($^{*}p < 0.05$, $^{**}p < 0.01$). dexa, dexamethasone.

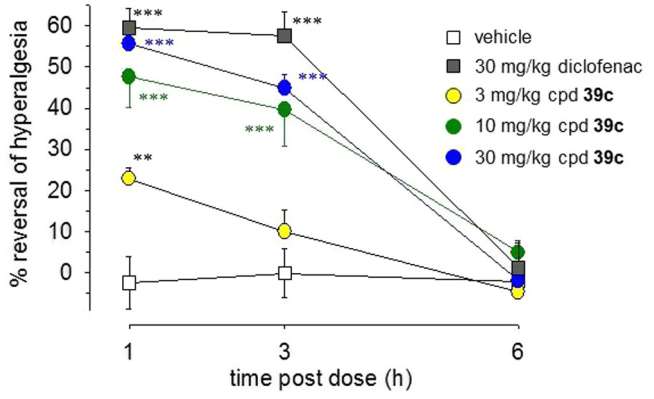


Fig. 5. Anti-nociceptive effect of compound **39c**. Statistics are One-Way ANOVA followed by Dunnetts test compared to matched vehicle group $^{**}p < 0.01$, $^{***}p < 0.001$.

10 and 30 mg/kg po dose. With the latter dose, an effect close to that of the NSAID diclofenac could be reached in this model.

3. Conclusion

In this paper we have described the discovery of a novel GPR4 antagonist **39c** starting from HTS hits **1** and **2**. Despite the high species specificity between human and rodent, which was reflected by lower activity in mouse and rat cellular assays, compound **39c** showed a strong efficacy in three different animal models: mouse VEGF-induced angiogenesis model, rat antigen-induced arthritis model and rat hyperalgesia model. *In vitro* this compound showed no cross-activity in a panel of kinase and proteases assays including Cathepsin D. It was also highly selective over other receptors as assessed by its screening in our internal receptor panel, except its activity on the histaminic 3 receptor (H_3-antagonist: IC_{50} of 0.93 ± 0.07 μM). Also, **39c** showed inhibition of the HERG channel (IC_{50} of 1.00 ± 0.32 μM was determined by binding assay using radiolabeled 3H-dofetilide binding to HEK293 cell membranes expressing human recombinant HERG K+ channels). While the compound was well tolerated during the course of animal models, the two mentioned liabilities prevented this compound to be further considered as a clinical candidate and required further optimization to find H_3 and hERG selective compounds with similar properties as **39c**.

Compound **39c**[16] is now commercially available as a tool GPR4 inhibitor[29] and has been used to demonstrate the suppression of acidosis stimulated expression of inflammatory genes in endothelial cells.[7] The imidazopyridine seems to be a privileged structure in medicinal chemistry field. It not only is an important motif for binding into GPR4 receptor as shown by other research groups (Fig. 6),[30–37] but was also used e.g. in the field of angiotensin 2 receptor antagonist reasearch.[38]

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers and used without further purification. All reactions were performed under inert conditions (argon) unless otherwise stated. ^{1}H NMR spectra were recorded on a Bruker 400 MHz or a Bruker 600 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) relative to an internal solvent reference. Significant peaks are tabulated in the order multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quintet; m, multiplet; br, broad), coupling constants, and number of protons. Final compounds were purified to ≥95% purity as assessed by analytical liquid chromatography with the following method: Waters Acquity UPLC–MS; column HSS T3 1.8 μm, 2.1 × 50 mm; A, water + 0.05% formic acid + 3.75 mM ammonium acetate; B, acetonitrile + 0.04% formic acid5–98% B in 1.4 min (short method) and 9.4 min (long method), flow 1.0 (short method) and 0.8 (long method) mL/min, flow 1.0 (short method) and 0.8 (long method) mL/min; column temperature 60 °C. The analyses were performed by using electrospray ionization in positive ion modus after separation by liquid chromatography (Nexera from Shimadzu). The elemental composition was derived from the mass spectra acquired at the high resolution of about 30'000 on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The high mass accuracy below 1 ppm was obtained by using a lock mass.

4.1.1. Synthesis of 2-chloro-N-(4-(3-(4-isopropylpiperazin-1-yl)prop-1-yn-1-yl)benzyl)-N-neopentyl pyrimidin-4-amine (**7a**)

(The synthesis of compounds **7b-c** is described in the supplementary data file).

4.1.1.1. Synthesis of 2-chloro-N-neopentylpyrimidin-4-amine (**4a**).

2,4-Dichloropyrimidine (5 g, 33.2 mmol) was dissolved in 10 ml THF and after addition of neopentylamine (3.48 g, 39.9 mmol) and potassium carbonate (6.96 g, 49.9 mmol) the mix-

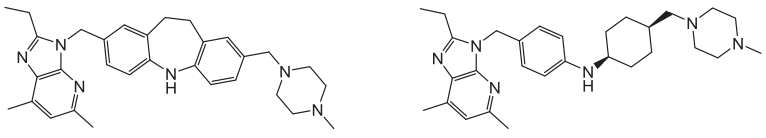


Fig. 6.

ture was stirred at 75 °C for 2 h min. The TLC showed 2 products (regioisomers). The mixture was diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4, filtrated and concentrated under reduced pressure. The crude product was purified by MPLC (silica gel, ethyl acetate/hexanes, 0–50%) to give 2.37 g (35.7%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (br, 1H), 6.28 (d, J = 6 Hz, 1H), 5.25 (br, 1H), 3.1 (br, 2H), 0.94 (s, 9H). MS (ESI): 200.2 $[M+H]^+$.

*4.1.1.2. Synthesis of 2-chloro-N-(4-iodobenzyl)-N-neopentylpyrimidin-4-amine (**5a**).* To a solution of 2-chloro-*N*-neopentylpyrimidin-4-amine (**4a**) (0.5 g, 2.48 mmol) and 4-iodobenzylbromide (810 mg, 2.73 mmol) in 10 ml of DMF was added sodium hydride (60% in mineral oil, 119 mg, 5 mmol) in portions. The mixture was stirred for 10 min at rt TLC indicated complete disappearance of **4a**. Then the mixture was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, ethyl acetate/hexanes, 0–50%) to give 1.01 g (83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.0 (d, J = 6 Hz, 1H), 7.68 (d, J = 8 Hz, 2H), 6.9 (d, J = 8 Hz, 2H), 6.32 (br, 1H), 4.8 (br s, 2H), 3.45 (br s, 2H), 0.96 (s, 9H); MS (ESI): 416.1 $[M+H]^+$.

*4.1.1.3. Synthesis of 3-(4-(((2-chloropyrimidin-4-yl)(neopentyl) amino)methyl)phenyl)prop-2-yn-1-ol (**6a**).* 640 mg (1.54 mmol) of 2-chloro-*N*-(4-iodobenzyl)-*N*-neopentylpyrimidin-4-amine (**5a**) was dissolved in 10 ml of DMF and after addition of propargyl alcohol (90 µl, 1.54 mmol), CuI (14.7 mg, 0.077 mmol), $PdCl_2(PPh_3)_2$ (54 mg, 0.077 mmol) and triethylamine (2.15 ml, 15.4 mmol) the mixture was heated for 1.5 h at 100 °C in a sealed tube. The mixture was partitioned between ethyl acetate and water. The organic layer was dried over Na_2SO_4 and concentrated to give 250 mg (47%) after purification by MPLC (silica gel, ethyl acetate/hexanes, 0–80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (br d, J = 6 Hz, 1H), 7.37 (d, J = 8 Hz, 2H), 7.07 (d, J = 8 Hz, 2H), 6.32 (br, 1H), 4.8 (br s, 2H), 4.48 (d, J = 6 Hz, 2H), 3.45 (br s, 2H), 2.77 (m, 1H), 1.0 (s, 9H); MS (ESI): 343.9, 345.8 $[M+H]^+$.

*4.1.1.4. Synthesis of 2-chloro-N-(4-(3-(4-isopropylpiperazin-1-yl) prop-1-yn-1-yl)benzyl)-N-neopentyl pyrimidin-4-amine (**7a**).* To a solution of 3-(4-(((2-chloropyrimidin-4-yl)(neopentyl)amino) methyl)phenyl) prop-2-yn-1-ol (**6a**) (125 mg, 0.364 mmol) in 90 ml of dichloromethane was added mesyl chloride (57 µl, 0.727 mmol) followed by triethylamine (150 µl, 1.09 mmol). After 5 min, 1-isopropylpiperazine (93 mg, 0.727 mmol) was added, followed by 10 ml of DMF and K_2CO_3 (150 mg, 1.09 mmol). The mixture was stirred for 15 min at 80 °C, then partitioned between ethyl acetate and water. The organic layer was dried over Na_2SO_4 and concentrated, which provided 97.6 mg (59%) after purification by MPLC (silica gel, ethyl acetate/hexanes, 0–80%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.9 (br, 1H), 7.37 (d, J = 8 Hz, 2H), 7.18 (d, J = 8 Hz, 2H), 6.55 (br, 1H), 4.87 (br s, 2H), 3.57 (br, 2H), 3.52 (s, 2H), 2.55–2.8 (m, 9H), 1.08 (d, J = 6 Hz, 6H), 1.0 (s, 9H); HRMS (ESI): calcd. For $C_{26}H_{36}ClN_5$ $[M+H]^+$ 454.27320, found 454.27313; HPLC (long method): t_R=4.68 min (91%).

*4.1.2. Synthesis of (E)-N-(4-(3-([1,4′-bipiperidin]-1′-yl)prop-1-en-1-yl)benzyl)-2-chloro-N-neopentyl pyrimidin-4-amine (**11a**)*

(The synthesis of compounds **11b–f** and **12** is described in the Supplementary data file).

*4.1.2.1. Synthesis of N-(4-bromobenzyl)-2-chloro-N-neopentylpyrimidin-4-amine (**8a**).* 2-Chloro-*N*-neopentylpyrimidin-4-amine (**4a**) (3.1 g, 15.5 mmol) was dissolved in 50 ml of DMF. After addition of sodium hydride (60% in mineral oil, 447 mg, 18.6 mmol) in portions, the mixture was stirred for 20 min at rt. 4-bromobenzylbromide (3.88 g, 15.5 mmol) was added slowly and the mixture was stirred for 2 h at rt. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, ethyl acetate/cyclohexane, 1:9) to yield 1.5 g (26%) of **8a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.02 (br, 1H), 7.52 (d, J = 8 Hz, 2H), 7.15 (d, J = 8 Hz, 2H), 6.94 and 6,53 (br, 1H), 4.81 (s, 2H), 3.61 and 3.43 (br s, 2H), 0.99 (s, 9H); MS (ESI):368.6, 370.6 $[M+H]^+$.

*4.1.2.2. Synthesis of (E)-methyl 3-(4-(((2-chloropyrimidin-4-yl) (neopentyl) amino)methyl)phenyl)acrylate (**9a**).* *N*-(4-Bromobenzyl)-2-chloro-*N*-neopentylpyrimidin-4-amine (**8a**) (1.5 g, 4.07 mmol) was dissolved in 10 ml of dioxane and after addition of methyl acrylate (420 mg, 4.9 mmol), dicyclohexylmethylamine (1.76 ml, 8.1 mmol) and $Pd(tBu_3P)_2$ (41 mg, 0.08 mmol) the mixture was heated for 5 min at 130 °C in a microwave. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated to give **9a** in 1.36 g (97%) yield after purification by MPLC (silica gel, ethyl acetate/cyclohexane, 1:9). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.02 (br, 1H), 7.68 (d, J = 8 Hz, 2H), 7.65 (d, J = 16 Hz, 1H), 7.21 (d, J = 8 Hz, 2H), 6.61 (d, J = 16 Hz, 1H), 6.5 (br, 1H), 4.85 (br s, 2H), 3.73 (s, 3H), 3.66 (br s, 2H), 0.98 (s, 9H); MS (ESI): 374.7 $[M+H]^+$.

*4.1.2.3. Synthesis of (E)-3-(4-(((2-chloropyrimidin-4-yl)(neopentyl) amino) methyl)phenyl)prop-2-en-1-ol (**10a**).* (*E*)-Methyl 3-(4-(((2-chloropyrimidin-4-yl)(neopentyl) amino)methyl)phenyl)acrylate (**9a**) (1.36 g, 3.64 mmol) was dissolved in 25 ml of dichloromethane and cooled to −78 °C. After slow addition of DIBAL (9.09 ml of a 1.2 M solution in toluene, 10.9 mmol) the mixture was stirred at −78 °C for 3 h. Then the mixture was quenched with water and concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, ethyl acetate/cyclohexane, 1:1) to give 600 mg (36%) of **10a** as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.99 (br, 1H), 7.38 (d, J = 8 Hz, 2H), 7.13 (d, J = 8 Hz, 2H), 6.97 (br, 1H), 6.52 (d, J = 16 Hz, 1H), 6.35 (dt, J = 16 and 6 Hz, 1H), 4.87 (t, J = 8 Hz, 1H), 4.81 (br s, 2H), 4.11 (m, 2H), 3.63 (br s, 2H), 0.98 (s, 9H); MS (ESI): 346.6 $[M+H]^+$.

*4.1.2.4. Synthesis of (E)-N-(4-(3-([1,4′-bipiperidin]-1′-yl)prop-1-en-1-yl)benzyl)-2-chloro-N-neopentyl pyrimidin-4-amine (**11a**).* (*E*)-3-(4-(((2-Chloropyrimidin-4-yl)(neopentyl)amino)methyl)phenyl) prop-2-en-1-ol (**10a**) (150 mg, 0.434 mmol) was dissolved in 5 ml of propionitrile and after addition of 4-piperidinyl piperidine (73 mg, 0.434 mmol), cyanomethyl-trimethyl-phosphonium iodide (freshly recrystallized from acetonitrile, 248 mg, 1.09 mmol) and diisopropylethylamine (354 µl, 2.17 mmol) the mixture was stirred for 2 h at 120 °C. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with 10% aqueous K_2CO_3 solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, methanol/ethyl acetate, 40:60) to give 102 mg (48%) of the product as a brown oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.00 (br, 1H), 7.39 (d, J = 8 Hz, 2H), 7.11 (d, J = 8 Hz, 2H), 6.5 (br, 1H), 6.47 (d, J = 16 Hz, 1H), 6.25 (dt, J = 16, 6 Hz, 1H), 4.80 (br s, 2H), 3.63 (br s, 2H), 3.03 (d, J = 6 Hz, 2H), 2.88 (m, 2H), 2.42 (m, 4H), 2.14 (m, 1H), 1.88 (m, 2H), 1.65 (m, 2H), 1.3–1.5 (m, 8H), 0.97 (s, 9H); HRMS (ESI): calcd. For $C_{29}H_{42}ClN_5$ $[M+H]^+$ 496.32015, found 496.32010.

*4.1.3. Synthesis of N-(4-(dimethylamino)butyl)-4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino) methyl) benzamide (**15**)*

*4.1.3.1. Synthesis of 2,6-dimethyl-N-neopentylpyrimidin-4-amine (**4c**).* 4-Chloro-2,6-dimethylpyrimidine (0.5 g, 3.47 mmol) was dissolved in 10 ml DMF and after addition of neopentylamine (363 mg, 4.16 mmol) and potassium carbonate (727 mg, 5.21 mmol) the mixture was stirred at 100 °C for 2 h. Then the mixture was concentrated under high vacuum. The residue was diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4, filtrated and concentrated under reduced pressure. The crude product was purified by MPLC (silica gel, MeOH/CH_2Cl_2, 0–10%) which provided 384 mg (57%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.96 (br t, J = 8 Hz, 1H), 6.21 (s, 1H), 3.15 (br, 2H), 2.27 (s, 3H), 2.15 (s, 3H), 0.90 (s, 9H); MS (ESI): 194 $[M+H]^+$.

*4.1.3.2. Synthesis of ethyl 4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl)benzoate (**13**).* Sodium hydride (902 mg, 35.7 mmol) was added portionwise to a solution of 2,6-dimethyl-*N*-neopentylpyrimidin-4-amine (**4c**) (5 g, 23.8 mmol) in 90 ml of DMF and the mixture was stirred for 15 min at rt. Then a solution of ethyl bromomethyl benzoate (6.63 g, 26.2 mmol) in 15 ml DMF was added dropwise over 30 min. The dark brown reaction mixture was quenched with 400 ml ice water and extracted six times with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated to give 12 g of a yellow oil, which was further purified by filtration over a silica pad (ethyl acetate/hexane 0–40%). Yield: 5.46 g (58%) of a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (d, J = 8 Hz, 2H), 7.15 (d, J = 8 Hz, 2H), 6.1 (br s, 1H), 4.92 (br s, 2H), 4.81 (br s, 2H), 4.35 (q, J = 8 Hz, 2H), 2.44 (s, 3H), 2.25 (s, 3H), 1.37 (t, J = 8 Hz, 3H), 0.96 (s, 9H); MS (ESI): 356.2 $[M+H]^+$.

*4.1.3.3. Synthesis of 4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino) methyl)benzoic acid (**14**).* Ethyl 4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl)benzoate (**13**) (3.32 g, 9.34 mmol) was dissolved in 10 ml of methanol. After addition of 4 N NaOH solution (23.3 ml, 93.4 mmol) the mixture was stirred for 1 h at 40 °C. The reaction mixture was concentrated, diluted with 10 ml ice water and then 6 N HCl solution was added until a pH of 6 was reached. The organic layer was separated, dissolved in acetone, dried over Na_2SO_4. Evaporation gave 2.1 g (62%) of a light yellow foam, which was used in the next step without further purification. MS (ESI): 328.1 $[M+H]^+$.

*4.1.3.4. Synthesis of N-(4-(dimethylamino)butyl)-4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl)benzamide (**15**).* A solution of 4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino) methyl)-benzoic acid (**14**) (1.95 g, 4.98 mmol) in 70 ml of CH_2Cl_2 was treated with EDC·HCl (1.91 g, 9.97 mmol), HOBT (1.91 g, 12.5 mmol) and diisopropylethylamine (4.35 ml, 24.9 mmol). Then 4-dimethylaminobutylamine (0.984 ml, 5.23 mmol) was added and the light yellow solution was stirred overnight at room temperature. The reaction mixture was filtrated, concentrated, diluted with 150 ml ethylacetate and washed with 100 ml of water and 50 ml of brine. The organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by HPLC (C18, acetonitrile/water gradient). Yield: 1.3 g (54%) of a white foam. ^{1}H NMR (400 MHz, CD_3OD): δ 7.74 (d, J = 8 Hz, 2H), 7.23 (d, J = 8 Hz, 2H), 6.32 (br s, 1H), 4.92 (br s, 2H), 3.6 (br s, 2H), 3.38 (t, J = 6 Hz, 2H), 2.41 (t, J = 6 Hz, 2H), 2.38 (s, 3H), 2.3 (s, 6H), 2.22 (s, 3H), 1.6 (m, 4H), 1.0 (s, 9H). %); HRMS (ESI): calcd. For $C_{25}H_{39}N_5O$ $[M+H]^+$ 426.32274, found 426.32266; HPLC (long method): tR = 2.39 min (100%).

*4.1.4. Synthesis of 5-(dimethylamino)-N-(4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl) phenyl)pentanamide (**18**)*

*4.1.4.1. Synthesis of N-(4-bromobenzyl)-2,6-dimethyl-N-neopentylpyrimidin-4-amine (**8c**).* 2,6-Dimethyl-*N*-neopentylpyrimidin-4-amine **(4c)** (1.53 g, 7.92 mmol) was dissolved in 50 ml of DMF. After addition of sodium hydride (60% in mineral oil, 228 mg, 9.5 mmol) in portions, the mixture was stirred for 20 min at rt. 4-Bromobenzylbromide (1.98 g, 7.92 mmol) was added slowly and the mixture was stirred for 2 h at rt. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated. Purification by MPLC (silica gel, ethyl acetate/cyclohexane, 1:1) provided 841 mg (29%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.5 (d, J = 8 Hz, 2H), 7.1 (d, J = 8 Hz, 2H), 6.4 (br s, 1H), 4.8 (br s, 2H), 3.44 (br s, 2H), 2.31 (s, 3H), 2.18 (s, 3H), 0.96 (s, 9H); MS (ESI): 362, 364 $[M+H]^+$.

*4.1.4.2. Synthesis of N-(4-aminobenzyl)-2,6-dimethyl-N-neopentylpyrimidin-4-amine (**16**).* *N*-(4-Bromobenzyl)-2,6-dimethyl-*N*-neopentylpyrimidin-4-amine (**8c**) (1.5 g, 4.14 mmol) was dissolved in 30 ml of toluene and after addition of benzophenone imine (831 µl, 4.97 mmol), sodium methylate (858 mg, 6.21 mmol), $Pd_2(dba)_3$ (94.8 mg, 0.103 mmol) and *rac*-BINAP (193 mg, 0.31 mmol) the mixture was stirred at 100 °C for 2 h. Then the mixture was cooled to rt and after addition with 30 ml of 2 M aqueous HCl the mixture was stirred at rt for 30 min. The mixture was filtrated over Celite and partitioned between ethyl acetate and 2 N HCl. The water layer was neutralized by slowly addition of Na_2CO_3 (pH >7), followed by extraction with ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated to give 840 mg (68%) of a colorless resin, which was used in the next step without further purification. MS (ESI): 299.2.

*4.1.4.3. Synthesis of 5-chloro-N-(4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl) phenyl)-pentanamide (**17**).* *N*-(4-Aminobenzyl)-2,6-dimethyl-*N*-neopentylpyrimidin-4-amine (**16**) (300 mg, 1.01 mmol), 5-chloro valeric acid (165 mg, 1.21 mmol), EDC·HCl (295 mg, 1.51 mmol) and diisopropylethylamine (527 µl, 3.02 mmol) were dissolved in 6 ml of CH_2Cl_2 and the mixture was stirred for 1 h at rt. The reaction mixture was directly purified by MPLC (silica gel, ethyl acetate/hexanes 0–50%). Yield: 340 mg (81%) as a colorless resin. MS (ESI): 417.2 $[M]^+$.

*4.1.4.4. Synthesis of 5-(dimethylamino)-N-(4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl) phenyl)pentanamide (**18**).* 5-Chloro-*N*-(4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)-methyl)-phenyl)pentanamide (**17**) (50 mg, 0.12 mmol) and a 2 M solution of dimethylamine in THF (3 ml, 6 mmol) were dissolved in THF in a closed vial and stirred for 4 d at 60 °C. The reaction mixture was concentrated and purified by HPLC (RP-18, acetone/water gradient). Yield: 50 mg (98%) as a colorless oil. ^{1}H NMR (400 MHz, CD_3OD): δ 7.58 and 7.54 (d, J = 8 Hz, 2H), 7.22 and 7.18 (d, J = 8 Hz, 2H), 6.99 and 6.62 (s, 1H), 5.14 and 4.95 (s, 2H), 3.9 and 3.53 (s, 2H), 3.2 (m, 2H), 2.9 (s, 6H), 2.6, 2.55, 2.45 and 2.35 (s, 6H), 2.45 (m, 2H), 1.75 (m, 4H), 1.1 and 1.05 (s, 9H); HRMS (ESI): calcd. For $C_{25}H_{39}N_5O$ $[M+H]^+$ 426.32274, found 426.32275; HPLC (short method): t_R = 0.72 (90%).

*4.1.5. Synthesis of 1-(2-(1-(4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl)phenyl)-2,3-dihydro-1H-1,2,3-triazol-4-yl)ethyl)piperidin-4-ol (**21**)*

*4.1.5.1. Synthesis of N-(4-azidobenzyl)-2,6-dimethyl-N-neopentylpyrimidin-4-amine (**19**).* A vial containing a solution of *N*-(4-bromobenzyl)-2,6-dimethyl-*N*-neopentylpyrimidin-4-amine (**8c**) (12.02 g, 31.5 mmol), sodium azide (4.1 g, 63 mmol), copper (I)iodide (600 mg, 3.15 mmol), *N,N′*-dimethylethylenediamine

(509 µl, 4.73 mmol) and sodium ascorbate (312 mg, 1.58 mmol) in ethanol (47 ml) and water (15.7 ml) was heated for 1 h at 100 °C in a microwave oven. The reaction mixture was diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$ solution, water and brine. The organic layer was dried over Na_2SO_4, concentrated and purified by MPLC (ethyl acetate/heptane 5–40%). Yield: 8.09 g (75%) as an yellow oil. ^{1}H NMR (400 MHz, CD_3OD): δ 7.68 (d, J = 8 Hz, 2H), 7.35 (d, J = 8 Hz, 2H), 6.4 (br s, 1H), 5.01 (s, 2H), 3.56 (s, 2H), 2.4 (s, 3H), 2.25 (s, 3H), 1.05 (s, 9H); MS (ESI): 309.5 $[M+H]^+$.

*4.1.5.2. Synthesis of N-(4-(4-(2-bromoethyl)-2,3-dihydro-1H-1,2,3-triazol-1-yl)benzyl)-2,6-dimethyl-N-neopentylpyrimidin-4-amine (**20**).* A solution of *N*-(4-azidobenzyl)-2,6-dimethyl-*N*-neopentylpyrimidin-4-amine (**19**) (530 mg, 1.63 mmol), 4-bromo-1-butyne (0.237 ml, 2.45 mmol) and copper (I) iodide (622 mg, 3.27 mmol) in 15 ml of acetonitrile was stirred at rt for 18 h. The reaction mixture was diluted with ethyl acetate and washed with saturated $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4, filtrated and concentrated under reduced pressure. The crude product was purified by MPLC (silica gel, ethyl acetate/CH_2Cl_2, 30–80%). Yield: 635 mg (74%) of a brown solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.9 (br, 1H), 7.7 (d, J = 8 Hz, 2H), 7.31 (d, J = 8 Hz, 2H), 6.21 (br s, 1H), 4.98 (br s, 2H), 3.75 (t, J = 8 Hz, 2H), 3.5 (br, 2H), 3.42 (t, J = 8 Hz, 2H), 2.56 (s, 3H), 2.4 (s, 3H), 1.05 (s, 9H); MS (ESI): 457, 459 $[M+H]^+$.

*4.1.5.3. Synthesis of 1-(2-(1-(4-(((2,6-dimethylpyrimidin-4-yl)(neopentyl)amino)methyl)phenyl)-2,3-dihydro-1H-1,2,3-triazol-4-yl)ethyl)piperidin-4-ol trifluoroacetyl salt (**21**).* A solution of *N*-(4-(4-(2-bromoethyl)-2,3-dihydro-1*H*-1,2,3-triazol-1-yl)benzyl)-2,6-dimethyl-*N*-neopentylpyrimidin-4-amine (**20**) (74 mg, 0.162 mmol) and 4-hydroxypiperidine (50 mg, 0.485 mmol) in 0.6 ml of DMF was heated for 30 min at 120 °C in a microwave oven. Then, the reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4, filtrated, concentrated. The crude product was purified by HPLC (RP-18, acetonitrile/water gradient). Yield: 50.8 mg (44%). ^{1}H NMR (400 MHz, CD_3OD, 2 rotamers): δ 8.48 and 8.47 (s, 1H), 7.88 and 7.83 (d, J = 8 Hz, 2H), 7.5 and 7.46 (d, J = 8 Hz, 2H), 7.06 and 6.65 (s, 1H), 5.25 and 5.08 (s, 2H), 4.18 and 4.0 (m, 1H), 3.9 (m, 1H), 3.78 (m, 1H), 3.65 (m, 1H), 3.58 (m, 2H), 3.53 (m, 4H), 3.4 (m, 1H), 3.18 (m, 1H), 2.65, 2.55, 2.5 and 2.4 (s, 6H), 2.22 (m, 1H), 2.03 (m, 2H), 1.8 (m, 1H), 1.12 and 1.1 (s, 9H); HRMS (ESI): calcd. For $C_{27}H_{39}N_7O$ $[M+H]^+$ 478.32888, found 478.32886; HPLC (6 min method): tR = 2.60 min (100%).

*4.1.6. Synthesis of (E)-6-ethyl-7-(4-(3-(4-isopropylpiperazin-1-yl)prop-1-en-1-yl)benzyl)-2,4-dimethyl-7H-pyrrolo[2,3-d]pyrimidine (**29**)*

*4.1.6.1. Synthesis of ethyl 2-acetyl-4-oxohexanoate (**23**).* A suspension of sodium hydride (60% in mineral oil, 690 mg, 17.2 mmol) in 80 ml THF was cooled to 0 °C and ethyl acetoacetate (**22**) (2 ml, 16 mmol) was added dropwise. The reaction mixture was stirred for 30 min at 0 °C. Then 1-bromo-2-butanone (1.85 ml, 17.2 mmol) was added. The mixture was stirred another 2 h at 0 °C and then allowed to warm up to rt. The mixture was quenched with 1 N HCl and extracted with diethyl ether. The organic layer was washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, filtrated and concentrated. Yield: 2.72 g (87%) of a light yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.12 (m, 2H), 4.0 (t, J = 8 Hz, 1H), 2.98 (m, 2H), 2.48 (q, J = 8 Hz, 2H), 2.25 (s, 3H), 1.19 (t, J = 8 Hz, 3H), 0.92 (t, J = 8 Hz, 3H).

*4.1.6.2. Synthesis of 2,6-dimethyl-5-(2-oxobutyl)pyrimidin-4(3H)-one (**24**).* Sodium (564 mg, 24.5 mmol) was added under argon to 25 ml of methanol, followed by acetamidine hydrochloride (1.19 g, 12.2 mmol) and ethyl 2-acetyl-4-oxohexanoate (**23**) (2.45 g, 12.25 mmol). The reaction mixture was refluxed for 16 h. The methanol was evaporated, 13 ml of water was added and the resulting solution was neutralized with 1 ml of acetic acid. The mixture was extracted with chloroform and the organic layer was concentrated. The crude product was recrystallized from toluene to give 1.45 g (61%) of the product as colorless needles. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.3 (s, 1H), 3.52 (s, 2H), 2.5 (m, 2H), 2.22 (s, 3H), 2.08 (s, 3H), 0.93 (t, J = 8 Hz, 3H); MS (ESI): 195.16 $[M+H]^+$.

*4.1.6.3. Synthesis of 1-(4-chloro-2,6-dimethylpyrimidin-5-yl)butan-2-one (**25**).* A mixture of 2,6-dimethyl-5-(2-oxobutyl)pyrimidin-4(3*H*)-one (**24**) (950 mg, 4.89 mmol) and 10 ml of $POCl_3$ was refluxed (110 °C) for 15 min. Then the mixture was concentrated. 20 ml of cold water was added, followed by 20 ml of $CHCl_3$. The mixture was the neutralized by addition of Na_2CO_3 solution. The organic layer was washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4, filtrated and concentrated to give the product in 724 mg (70%) yield as yellow needles. MS (ESI): 213.13 $[M+H]^+$.

*4.1.6.4. Synthesis of 7-(4-bromobenzyl)-6-ethyl-2,4-dimethyl-7H-pyrrolo[2,3-d]pyrimidine (**26**).* A solution of 1-(4-chloro-2,6-dimethylpyrimidin-5-yl)butan-2-one (**25**) (150 mg, 0.705 mmol) and 4-bromobenzylamine (273 mg, 1.41 mmol) in 5 ml of $CHCl_3$ was heated for 12 h at 100 °C in a microwave oven. Evaporation of solvent gave 242 mg (100%) of a yellow powder, which was used in the next step without further purification. MS (ESI): 345.8 $[M+H]^+$.

*4.1.6.5. Synthesis of (E)-methyl 3-(4-((6-ethyl-2,4-dimethyl-7H-pyrrolo[2,3-d]pyrimidin-7-yl)methyl)-phenyl) acrylate (**27**).* Methyl acrylate (39.6 µl, 0.435 mmol) and triethyl amine (122 µl, 0.871 mmol) were added under argon to a solution of 7-(4-bromobenzyl)-6-ethyl-2,4-dimethyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (**26**) (150 mg, 0.436 mmol) and $Pd(OAc)_2$ (20.8 mg, 0.044 mmol) in 1.6 ml of DMF. The mixture was heated for 15 min at 150 °C in a microwave oven. After cooling to rt, the reaction mixture was diluted with ethyl acetate and filtrated over Celite. The filtrate was washed with water, saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4, filtered and concentrated. The crude product was purified by MPLC (silica gel, ethyl acetate/heptane gradient). Yield: 85.4 mg (56%) as an yellow solid. MS (ESI): 350.9 $[M+H]^+$.

*4.1.6.6. Synthesis of (E)-3-(4-((6-ethyl-2,4-dimethyl-7H-pyrrolo[2,3-d]pyrimidin-7-yl)methyl)phenyl)prop-2-en-1-ol (**28**).* (*E*)-Methyl 3-(4-((6-ethyl-2,4-dimethyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)methyl)phenyl) acrylate (**27**) (85 mg, 0.244 mmol) was dissolved in 25 ml of dichloromethane and cooled to -78 °C. After slow addition of DIBAL (1 M in THF, 730 µl, 0.73 mmol) the mixture was stirred at -78 °C for 3 h. Then, the mixture was quenched with water and concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4 and concentrated to give 55 mg (70%) of the product as off-white crystals. MS (ESI): 322 $[M+H]^+$.

*4.1.6.7. Synthesis of (E)-6-ethyl-7-(4-(3-(4-isopropylpiperazin-1-yl)prop-1-en-1-yl)benzyl)-2,4-dimethyl-7H-pyrrolo[2,3-d]pyrimidine (**29**).* (*E*)-3-(4-((6-Ethyl-2,4-dimethyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)methyl)phenyl)-prop-2-en-1-ol (**28**) (28 mg, 0.087 mmol) was dissolved in 5 ml of propionitrile and after addition of 1-isopropylpiperazine (13 µl, 0.087 mmol), cyanomethyl-trimethyl-phosphonium iodide (50 mg, 0.217 mmol, freshly recrystallized from acetonitrile) and diisopropylethylamine (76 µl) the mixture was stirred for

2 h at 120 °C. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with 10% aqueous K_2CO_3 solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified HPLC (RP-18, acetonitrile/water gradient) to give 22 mg (59%) of a white powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.42 (d, J = 8 Hz, 2H), 7.07 (d, J = 8 Hz, 2H), 6.92 (s, 1H), 6.66 (d, J = 16 Hz, 1H), 6.26 (dt, J = 16 and 6 Hz, 1H), 5.57 (s, 2H), 2.9–4.0 (m, 11*H*), 2.87 (s, 3H), 2.78 (s, 3H), 2.7 (q, J = 7 Hz, 2H), 1.2–1.25 (m, 9H); HRMS (ESI): calcd. For $C_{27}H_{37}N_5$ $[M+H]^+$ 432.31217, found 432.31232.

*4.1.7. Synthesis of (E)-2-ethyl-3-(4-(3-(4-isopropylpiperazin-1-yl) prop-1-en-1-yl)benzyl)-5,7-dimethyl-3H-imidazo[4,5-b]pyridine (**39c**)*

(The synthesis of compounds **39a**,**b** and **d**–**f** is described in the supplementary data file).

*4.1.7.1. Synthesis of 2-amino-4,6-dimethylnicotinamide (**31b**).* Malonamidine hydrochloride (**30**) (20.4 g, 148.4 mmol) was dissolved in 1 L of methanol. After addition of acetylacetone (15.3 ml, 148 mmol) and KOH pellets (9.99 g, 178 mmol) the mixture was stirred for 24 h at rt. Evaporation gave 24.5 g (100%) of the product as a white solid. MS (ESI): 166.6 $[M+H]^+$.

*4.1.7.2. Synthesis of 5,7-dimethyl-1H-imidazo[4,5-b]pyridin-2(3H)-one (**32b**).* 2-Amino-4,6-dimethyl-nicotinamide (**31b**) (24.5 g, 148.3 mmol) was dissolved in 100 ml of methanol and after addition of a solution of 20.8 g (370.8 mmol) KOH pellets in 100 ml of methanol the mixture was stirred at rt for 30 min. Then, the mixture was cooled to -5 °C and iodobenzene diacetate (47.8 g, 148.3 mmol) was added within 30 min. Stirring was continued overnight at rt. Then, the product was filtered off, washed with methanol and dried under HV. Yield: 23.8 g (98%) of a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.34 (s, 1H), 5.0 (br, 2H), 2.28 (s, 3H), 2.17 (s, 3H); MS (ESI): 164.4 $[M+H]^+$.

*4.1.7.3. Synthesis of 2-ethyl-5,7-dimethyl-3H-imidazo[4,5-b]pyridine (**35c**).* A mixture of 5,7-dimethyl-1*H*-imidazo[4,5-*b*]pyridin-2(3*H*)-one (**32b**) (10 g, 61.3 mmol), propionic acid (87 ml, 1.18 mol), propionic anhydride (88 ml) and $MgCl_2$ (5.83 g, 61.3 mmol) was stirred overnight at 145 °C. Then the mixture was cooled to rt, 50 ml of methanol was added followed by 25% ammonia until a pH of 9 was reached. The mixture was extracted by ethyl acetate, the organic layers were washed with brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, methanol/CH_2Cl_2, 2–5%). Yield: 4.85 g (45%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.4 (br s, 1H), 6.85 (s, 1H), 2.82 (q, J = 8 Hz, 2H) 2.52 (s, 6H), 1.32 (t, J = 8 Hz, 3H); MS (ESI): 176.1 $[M+H]^+$.

*4.1.7.4. Synthesis of 3-(4-bromobenzyl)-2-ethyl-5,7-dimethyl-3H-imidazo[4,5-b]pyridine (**36c**).* 2-ethyl-5,7-dimethyl-3*H*-imidazo[4,5-*b*] pyridine (**35c**) (3 g, 17.1 mmol) was dissolved in 50 ml of DMF. After addition of sodium hydride (60% in mineral oil, 493 mg, 20.5 mmol) in portions, the mixture was stirred for 20 min at rt. 4-Bromobenzylbromide (4.28 g, 17.1 mmol) was added slowly and the mixture was stirred for 2 h at rt. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, ethyl acetate/cyclohexane 2:3) which provided 2.5 g (42%) of the product as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.5 (d, J = 8 Hz, 2H), 7.05 (d, J = 8 Hz, 2H), 6.93 (s, 1H), 5.41 (s, 2H), 2.76 (q, J = 8 Hz, 2H), 2.51 (s, 3H), 2.49 (s, 3H), 1.22 (t, J = 8 Hz, 3H); MS (ESI): 344, 346 $[M+H]^+$.

*4.1.7.5. Synthesis of (E)-methyl 3-(4-((2-ethyl-5,7-dimethyl-3H-imidazo[4,5-b]pyridin-3-yl)methyl) phenyl)acrylate (**37c**).* 3-(4-Bromobenzyl)-2-ethyl-5,7-dimethyl-3*H*-imidazo[4,5-*b*]pyridine (**36c**) (500 mg, 1.45 mmol) was dissolved in 15 ml of dioxane and after addition of methyl acrylate (654 mg, 7.26 mmol), dicyclohexylmethylamine (630 µl, 2.90 mmol) and $Pd(tBu_3P)_2$ (14.8 mg, 0.029 mmol) the mixture was heated for 5 min at 130 °C in a microwave. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4 and concentrated to give 300 mg (59%) of a colorless solid, which was used in the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.67 (d, J = 8 Hz, 2H), 7.62 (d, J = 16 Hz, 1H), 7.15 (d, J = 8 Hz, 2H), 6.96 (s, 1H), 6.6 (d, J = 16 Hz, 1H), 5.49 (s, 2H), 3.72 (s, 3H), 2.77 (q, J = 8 Hz, 2H), 2.52 (s, 3H), 2.50 (s, 3H), 1.23 (t, J = 8 Hz, 3H); MS (ESI): 350.8 $[M+H]^+$.

*4.1.7.6. Synthesis of (E)-3-(4-((2-ethyl-5,7-dimethyl-3H-imidazo[4,5-b]pyridin-3-yl)methyl)phenyl)prop-2-en-1-ol (**38c**).* (*E*)-Methyl 3-(4-((2-ethyl-5,7-dimethyl-3*H*-imidazo[4,5-*b*]pyridin-3-yl)methyl) phenyl)acrylate (**37c**) (500 mg, 1.43 mmol)) was dissolved in 25 ml of dichloromethane and cooled to −78 °C. After slow addition of DIBAL (3.05 ml of a 1.2 M solution in toluene, 4.29 mmol) the mixture was stirred at −78 °C for 3 h. Then the mixture was quenched with water and concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4 and concentrated to give **38c** in 300 mg (65%) yield as a colorless solid, which was used in the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.32 (d, J = 8 Hz, 2H), 7.03 (d, J = 8 Hz, 2H), 6.91 (s, 1H), 6.47 (d, J = 16 Hz, 1H), 6.33 (dt, J = 16, 6 Hz, 1H), 5.39 (s, 2H), 4.82 (t, J = 6 Hz, 1H), 4.06 (m, 2H), 2.73 (q, J = 8 Hz, 2H), 2.49 (s, 6H), 1.20 (t, J = 8 Hz, 3H); MS (ESI): 322 $[M+H]^+$.

*4.1.7.7. Synthesis of (E)-2-ethyl-3-(4-(3-(4-isopropylpiperazin-1-yl) prop-1-en-1-yl)benzyl)-5,7-dimethyl-3H-imidazo[4,5-b]pyridine (**39c**).* (*E*)-3-(4-((2-ethyl-5,7-dimethyl-3*H*-imidazo[4,5-*b*]pyridin-3-yl) methyl)phenyl)prop-2-en-1-ol (**38c**) (200 mg, 0.622 mmol) was dissolved in 6 ml of propionitrile and after addition of *N*-isopropyl-piperazine (89 µl, 0.622 mmol), cyanomethyl-trimethyl-phosphonium iodide (206 mg, 1.56 mmol) and diisopropylethylamine (534 µl, 3.1 mmol) the mixture was stirred for 2 h at 120 °C. Then the mixture was concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with 10% aqueous K_2CO_3 solution and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by MPLC (silica gel, methanol/ethyl acetate 1:9) which provided 244 mg (73%) of the product as a yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.37 (d, J = 8 Hz, 2H), 7.05 (d, J = 8 Hz, 2H), 6.94 (s, 1H), 6.46 (d, J = 16 Hz, 1H), 6.23 (dt, J = 16, 6 Hz, 1H), 5.43 (s, 2H), 3.03 (d, J = 6 Hz, 2H), 2.77 (q, J = 8 Hz, 2H), 2.59 (sept, J = 6 Hz, 1H), 2.25–2.5 (m, 8H), 1.23 (t, J = 8 Hz, 3H), 0.96 (d, J = 6 Hz, 6H); HRMS (ESI): calcd. For $C_{27}H_{37}N_5$ $[M+H]^+$ 432.31217, found 432.31219; HPLC (long method): t_R = 3.01 min (100**%**).

4.2. Biology

4.2.1. High throughput screen

The assay was performed with COS-7 cells transiently transfected with human GPR4 receptor cDNA with Lipofectamine. 900,000 compounds (Novartis owned library comprising diversity set and natural product compounds) were screened at a final compound concentration of 5 µM in 1536 well plates. pH-induced formation of cAMP was determined using the homogeneous time resolved fluorescence (HTRF) technology as provided by CisBio Inc. 1605 primary hits (threshold >40% inhibition) were tested in

a concentration-dependent manner of which 13% confirmed with an IC_{50} value below 23 μM. Compound specificity and selectivity were tested against TDAG8 and the endogenous beta2-adrenergic receptor in COS-7 cells.

The cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/HAM's tissue culture medium F12 (HAM's F12) medium supplemented with 10% fetal calf serum (FCS), 100 u/ml penicillin, 100 μg/ml streptomycin and 10 mM Hepes pH 8.2. Transiently transfected cells were harvested in Hepes buffer (pH = 8.2) (Hepes buffered saline (HBS), 20 mM Hepes). 100 nl compound was transferred prior to cell addition to the assay plate. 2 μl cells were seeded in 1536-well plates at a density of 2000 cells/well. 2 μl buffer (Hepes buffered saline (HBS), 8 mM Hepes/EPPS/MES, 2 mM 3-isobutyl-1-methylxanthin (IBMX)) at appropriate pH were added to the compound wells to reach the final pH of 6.5 for stimulation and pH 8.2 in control wells. Cells were incubated for 30 min at room temperature. 2 μl of cAMP-XL 665 and 2 μl anti cAMP-cryptate were dispensed and plates were read on a Viewlux (Perkin Elmer) reader after 90 min incubation at rt in the dark. Data were calculated from the 665 nm/620 nm ratio and% activity was normalized according to values at minimum and maximum of GPR4 activation.

4.2.2. pH-dependent cAMP release assay (human GPR4 in HeLa, rat and mouse GPR4 in HEK cells)

HeLa and HEK cells stably expressing human, rat and mouse GPR4, respectively, were established by transfecting the cells with a construct containing the respective GPR4 coding sequence. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/HAM's tissue culture medium F12 (HAM's F12) medium supplemented with 10% fetal calf serum (FCS), 100 u/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418 and 10 mM Hepes pH 8.0. pH-induced formation of cAMP was determined using the homogeneous time resolved fluorescence (HTRF) technology as provided by CisBio Inc. The cells were seeded in 384-well plates and cultured for 24 h at 37 °C, 5% CO_2 before performing the assay. Medium was removed and 10 μl buffer A (Hepes buffered saline (HBS), 10 mM Hepes, pH 8, 2 mM 3-isobutyl-1-methylxanthin (IBMX)) was added. For compound testing, buffer A with 2× concentrated compounds was used. Cells were incubated for 15 min at room temperature. 10 μl buffer B (HBS, 30 mM Hepes, specific pH) was added to reach the appropriate final pH for stimulation and incubation was continued for 15 min at room temperature. Finally, 10 μl of cAMP-XL 665 and 10 μl anti cAMP-cryptate were dispensed and plates were read on a Pherastar reader after 60 min incubation at rt. Data were calculated from the 665 nm/620 nm ratio and% activity was normalized according to values at minimum and maximum of GPR4 activation.

4.2.3. TDAG8 cAMP formation assay

Confluent TDAG8-transfected HeLa cells were grown in 24-well plates and labelled with [^{3}H]adenine (100 MBq/ml) for 4 h in serum-free DMEM medium. Cells were then incubated at 37 °C in a buffered salt solution containing 130 mM NaCl, 0.9 mM NaH_2PO_4, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM $CaCl_2$, 25 mM glucose, 20 mM Hepes. The pH of the solutions was adjusted to pH 7.9, pH 7.0 and pH 6.8 at room temperature. The phosphodiesterase inhibitor isobutylmethylxanthine (1 mM, IBMX) was added to allow accumulation of cAMP. Antagonist compounds were added 10 min prior to IBMX addition. Incubation was continued for 15 min. Cells were then extracted with ice-cold trichloroacetic acid and cAMP separated from free adenine and ATP using batch column chromatography.

4.2.4. hERG binding assay

HEK293 cells stably transfected with the HERG-1 gene (Swiss-Prot: Q12809) were obtained under license from the Wisconsin Alumni Research Foundation. Membranes prepared from these cells were used in the binding assay using the radioligand: [^{3}H] dofetilide with specific activity of 46.5 Ci/mmol. The assay contains in a 96-well Millipore GF/C filter plate 119 μl buffer, 1 μl compound, 40 μl 12.5 nM [^{3}H]dofetilide, 40 μl crude membrane suspension (15 μg protein). After for 90 min at room temperature incubations are terminated by rapid filtration, three washes with ice-cold buffer. The dried including 40 μl scintillant was read in a Wallac MicroBeta Trilux beta-counter.

4.2.5. Histamine H_3 receptor binding assay

Lyophilized PVT PEI-treated wheat germ agglutinin SPA beads, type A (RPNQ0003) were purchased from GE Healthcare (Buckinghamshire, UK). [3*H*]-*R*(−)-alpha-Methyl[imidazole-2.5(n)]histamine, an agonist radioligand, was purchased from GE Healthcare, TRK1017, specific activity 34 Ci/mmol, 1 mCi/mL). Membrane prepared from CHO-K1 cells stably expressing the human H_3 histamine receptor were purchased from Euroscreen (ES-392-M). The SPA assay was performed in a final volume of 50 μL in 384-well polystyrene plate (10 μL test compounds, total binding was determined by adding 10 μL water and non-specific binding was determined by the addition of 10 μL Clobenpropit, 20 μL 7.5 nM [^{3}H]-*R*-alpha-Methylhistamine in assay buffer [50 mM Tris-HCl, 5 mM EDTA, 1 mM EDTA, pH 7.4], 20 μL beads and membranes mixed suspension in assay buffer). The plates were shaken at room temperature, then allowed to stand for at least 1 h and then counted using a Perkin Elmer TopCount reader.

4.2.6. Mouse VEGF-induced angiogenesis model

The experiments were performed in accordance with the local VET authorities under license BS-1325. Porous tissue chambers made of perfluoro-alkoxy-Teflon (Teflon-PFA, 21 × 8 mm diameter, 550 μl volume) were filled with 0.8% agar and 20 U/ml heparin supplemented with or without 2 μg/ml recombinant human VEGF165. Female FVB mice of 8–10 weeks old were anesthetized using 3% Isoflurane inhalation. For subcutaneous implantation, a small skin incision was made on the flank and one chamber per animal was implanted under aseptic conditions onto the back of the animal. The skin incision was closed by wound clips. Animals were placed in groups of 6. On the 4th day after implantation, animals were sacrificed using CO_2. Chambers were excised and the vascularized fibrous tissue formed around each implant carefully removed and weighed. Body weight was used to monitor the general condition of the mice. Compound **39c** was dosed orally twice a day at 10, 3, 1 and 0.3 mg/kg for 4 days and was started 5 h prior to implantation of the chamber. The experiment was repeated twice for 30 mg/kg, 5 times for 10 mg/kg, 3 times for 3 mg/kg and was performed once for 1 and 0.3 mg/kg.

4.2.7. Rat antigen-induced arthritis

Female Lewis rats (120–150 g) were sensitized intradermally on the back at two sites with methylated bovine serum albumin (mBSA) homogenised 1:1 with complete Freund's adjuvant on days −21 and −14 (0.1 ml containing 1 mg/ml mBSA). On day 0, the right knee received 50 μl of 10 mg/ml mBSA in 5% glucose solution (antigen injected knee), while the left knee received 50 μl of 5% glucose solution alone (vehicle injected knee). The diameters of the left and right knees were then measured using calipers immediately after the intra-articular injections and again on days 2, 4 and 7. Treatments were administered daily as follows by oral gavage; vehicle (saline) at 5 ml/kg, compounds as indicated. Right

knee swelling was calculated as a ratio of left knee swelling, and the R/L knee swelling ratio plotted against time to give Area Under the Curve (AUC) graphs for control and treatment groups. Right and left knees were fixed, embedded and processed for histological analysis. Sections were stained for both nuclear morphology (Haematoxylin and Eosin) and proteoglycan content (Giemsa).

4.2.8. Mechanical rat hyperalgesia model

Naïve withdrawal thresholds of both hind paws were determined in male Wistar Han rats by using an increasing pressure stimulus placed onto the dorsal surface of each paw using an analgesymeter. Delayed inflammatory pain was then induced by intra-plantar injection of 25 µl of Complete Freund's Adjuvant (CFA) into one hindpaw with the contralateral paw acting as the control. After 3 days, compound **39c** (3, 10, and 30 mg/kg) or diclofenac (30 mg/kg) were administered by gavage as suspension in methylcellulose 5%. One hour later, paw withdrawal thresholds were re-measured on both the ipsilateral (CFA-injected) and contralateral (uninjected) paw (one measurement per paw and time point); measurements were repeated at 3 h and 6 h post dosing. The reversal of hyperalgesia was calculated using the following formula: Reversal (%) = 100 × (postdose ipsilateral threshold − predose ipsilateral threshold) (naïve ipsilateral threshold − predose ipsilateral threshold).

4.2.9. PK studies: animals and study design, bioanalytical evaluation and data processing

The PK studies were performed with adult female Sprague Dawley rats (weighing approx. 300 g).[39] For details see Supplementary data file.

Acknowledgements

The authors thank Dr. Ivan Cornella-Taracido, Dr. Timothy Hurley and Dr. Rene Lattmann for their valuable contributions to this work.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.06.050.

References

1. Seuwen K, Ludwig M-G, Wolf RM. *J Recept Signal Transduction*. 2006;26:599.
2. Ludwig M-G, Vanek M, Guerini D, et al. *Nature*. 2003;425:93.
3. Wyder L, Suply T, Ricoux B, et al. *Angiogenesis*. 2011;14:533.
4. Chen A, Dong L, Leffler NR, Asch AS, Witte ON, Yang LV. *PLoS ONE*. 2011;6:e275586.
5. Yang, L.; US20110081337.
6. Lardner A. *J Leukoc Biol*. 2001;69:522.
7. Dong L, Li Z, Leffler NR, Asch AS, Chi JT, Yang LV. *PLoS ONE*. 2013;8:e61991.
8. Giudici L, Velic A, Daryadel A, et al. *Cell Physiol Biochem*. 2013;32:1403.
9. Kumar NN, Velic A, Soliz J, et al. *Science*. 2015;348:1255.
10. Fukuda H, Ito S, Watari K, et al. *ACS Med. Chem. Lett.*. 2016;7:493.
11. Sanderlin EJ, Leffler NR, Lertpiriyapong K, et al. *Biochim. Biophys. Acta Mol. Basis Dis.*. 2017;1863:569.
12. Dong L, Krewson EA, Yang Li V. *Int J Mol Sci*. 2017;18:278.
13. Velcicky J, Miltz W, Oberhauser B, et al. *J Med Chem*. 2017;60:3672.
14. Brændvang M, Gundersen L-L. *Bioorg Med Chem*. 2007;15:7144.
15. Sonogashira K, Tohda Y, Hagihara N. *Tetrahedron Lett*. 1975;4467.
16. Ivan Cornella Taracido IC, Harrington EM, Hersperger R, Lattmann R, Miltz W, Weigand K, US 2009/0291942.
17. Zaragoza F, Stephensen H. *J Org Chem*. 2001;66:2518.
18. Carlsen PN, Jiang C, Herrick IR, Frontier AJ. *Tetrahedron*. 2015;71:5886.
19. Andersen J, Madsen U, Bjoerkling F, Liang X. *Synlett*. 2005;14:2209.
20. Kolb HC, Finn MG, Sharpless KB. *Angew Chem Int Ed*. 2004;2001:40.
21. Ichimoto TM. *Agric Biol Chem*. 1981;45:295.
22. Ellingboe JW, Antane M, Nguyen TT, Collini MD, Antane S. *J Med Chem*. 1994;37:542.
23. Chekmarev DS, Shorshnev SV, Stepanov AE, Kasatkin AN. *Tetrahedron*. 2006;62:9919.
24. Chang LCW, Von Frijtag Drabbe Kuenzel JK, Mulder-Krieger T, et al. *J Med Chem*. 2007;50:828.
25. Senanayake CH, Fredenburgh LE, Reamer RA, Larsen RD, Verhoeven TR, Reider PJ. *J Am Chem Soc*. 1994;116:7947.
26. Senanayake CH, Fredenburgh LE, Reamer RA, et al. *Heterocycles*. 1996;42:821.
27. Mantlo NB, Chakravarty PK, Ondeyka DL, et al. *J Med Chem*. 1991;34:2919.
28. Altmann E, Betschart C, Hayakawa K et al., WO 2004020441.
29. GPR4 receptor modulator 46, Dalton Pharma Services (Toronto Canada), MFCD18803646 (CAS 1197879-16-5).
30. Saki M, Nonaka H, Miyaji H, Takahashi C, Manabe H, Hiura N, WO2004/093912.
31. Saki M, Nonaka H, Miyaji H et al. EP 1 537 879, 2005.
32. Saki M, Nonaka H, Miyaji H, et al. EP 1 547 616, 2005.
33. Iida K, Otsubo N, Kuboyama T et al. WO 2005/082904, EP 1,724,271 2006
34. Saki M, Nonaka H, Miyaji H, et al. US 2006/0239999.
35. Saki M, Nonaka H, Miyaji Het al. US 2006/0252679.
36. Saki M, Miura K, Sasaki K. WO 2007/023880.
37. Tobo A, Tobo M, Nakakura T, et al. *PLoS ONE*. 2015;10. e0129334/1.
38. Dhanoa DS, Bagley SW, Chang RSL, et al. *J Med Chem*. 1993;36:4239.
39. Notari RE. *Biopharmaceutics and Clinical Pharmacokinetics*. 4th ed. New York: Marcel Dekker Inc.; 1987.