RESEARCH ARTICLE

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2017, 8, 1235Pyrazolo[3,4-*d*]pyrimidines as sigma-1 receptor
ligands for the treatment of pain. Part 1:
4-acylamino derivatives†‡José Luis Díaz, , Jordi Corbera, Rosa Cuberes, Montserrat Contijoch,
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The synthesis of a new series of 4-acylaminopyrazolo[3,4-*d*]pyrimidines active on the sigma-1 receptor (σ_1R) is reported. Compounds were efficiently prepared using a two to three step process starting from commercially available 1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine. A SAR study shows that the σ_1R requires the presence of relatively highly lipophilic substituents at opposite sides of the central scaffold, while selectivity *versus* the σ_2R can be improved by shortening the distance of the basic nitrogen to it. Compound **9a** was among the most active and selective *in vitro* derivatives and exhibited potent antinociceptive properties in several pain models in mice, indicating its antagonistic behaviour.

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Introduction

The σ receptor (σR) was initially recognized as a new addition to the opioid receptor family, but later it has been shown to be a unique molecular chaperone, which modulates the function of different proteins when they become challenged by disease-related stress or mutations.¹ The σR presents at least two different subtypes, σ_1R and σ_2R , which show distinct functions and are related to different potential therapeutic indications. The σ_1R was cloned from guinea pig liver in the late nineties² and this discovery paved the way for the search for selective σ_1R ligands. Since the receptor is widely distributed in peripheral organs and in different areas of the central nervous system (CNS), which are involved in memory, emotion, sensory and motor function control, σ_1R ligands were mainly studied in the past for treating different CNS-related pathologies such as schizophrenia and depression.³ The σ_2R has remained more elusive and is often associated with proliferation, synaptogenesis and cell plasticity. Ligands acting on the σ_2R have been proposed as biomarkers for tumour cell proliferation and show pro-apoptotic properties, thus suggesting a potential role in cancer imaging and treatment.⁴

More recently, studies on the σ_1R knock-out (σ_1R -KO) mouse and several σ_1R ligands have been key to establishing the role of the σ_1R in opioid analgesia modulation and in

pain control, when central sensitization occurs, as is the case with several neuropathic pain states.⁵ A growing body of evidence supports the role of the σ_1R in pro-nociception, acting through the up-regulation of sodium and calcium channels as well as NMDA receptor function, all of them linked to pain facilitation and amplification of nociceptive messages. Another important factor is the localization of the σ_1R in key areas involved in pain control, such as the superficial layers of the dorsal horn, the periaqueductal grey matter, the locus coeruleus and the rostroventral medulla.⁶

The reduced levels of hyperalgesia observed in several models, both in σ_1R -KO mice and in rodents treated with σ_1R ligands, have helped to build up substantial evidence of the role of σ_1R antagonism in the treatment of pain.^{6,7} However, the determination of the functionality of σ_1R ligands is not obvious, since the protein is mainly located intracellularly and it exerts a modulatory action by regulating the action of different proteins. Probably, this is why some σ_1R ligands do not show the classical linear dose-response curves and the assay conditions and readouts used markedly

Fig. 1 General structure of pyrazolo[3,4-*d*]pyrimidines **I** and reference compound **1**.

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influence the outcome, and some compounds identified as agonists using one readout act as antagonists using a different one. For these reasons, *in vivo* tests are frequently used to determine the functional nature of σ_1 R ligands.⁸

Good antinociception in rodents has been shown by several types of σ_1 R antagonists, such as those developed by the group of A. Marrazzo around compound (+)-MR200 (ref. 9) and several series of spirocyclic derivatives studied by the group of B. Wünsch.¹⁰ Our group has been involved in the search for σ_1 R antagonists for several years and developed the highly selective σ_1 R antagonist E-52862 (**1**, Fig. 1 and Table 1)¹¹ up to phase II clinical trials for the treatment of different pain conditions.^{12,13}

As a back-up program in the development of **1**, we undertook several approaches in order to identify novel scaffolds binding to the σ_1 R and showing acceptable drug-like properties. A high throughput screening (HTS) campaign over our internal library led to the identification of an interesting new framework,¹⁴ while a scaffold hopping strategy toward **1** provided a series of potent triazole derivatives.¹⁵ As a different approach, we report here the synthesis and structure–activity relationship (SAR) studies of a

new series of pyrazolo[3,4-*d*]pyrimidines¹⁶ (**I**, Fig. 1), which were designed on the basis of Glennon's σ_1 R pharmacophore¹⁷ and taking into account the information generated in previous SAR studies.

Results and discussion

Chemistry

The synthesis of the final compounds was quite straightforward, since it involved a two to three step process starting from commercially available 1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine **2**. As indicated in Scheme 1, the reaction of **2** with an alkylating agent **3** in the presence of a strong base provided the *N*¹-substituted pyrazoles **4**.¹⁸ Alternatively, compounds **4** were obtained in two steps by reaction of **2** with a dihalo derivative **5** to afford **6**, followed by substitution with an amine **7** under standard conditions.

Both routes provided the *N*¹-substituted pyrazoles **4** as major isomers. *N*²-regioisomers were observed in the crude reaction mixture (1–30% by MS), but were isolated in very low yields or not isolated at all. This result could be attributed to lower solubility. The characterization of regioisomers was

Table 1 Amide derivatives **8**–**10**

Comp	R	K_i σ_1^a (h, nM)	HLM ^c Cl _{int} (μ l min ⁻¹ mg ⁻¹ prot.)	clog <i>P</i> ^d
1		17	—	—
8a	Cyclopropyl	32 731	—	1.0
8b	Cyclobutyl	716	—	1.3
8c	Cyclopentyl	116	—	1.9
8d	Cyclohexyl	98	13.9	2.4
9a	<i>tert</i> -Butyl	51	2.4	2.2
9b	Adamantyl	25	5.0	3.6
9c	Noradamantyl	55	6.2	3.0
9d	2,4-Dichlorophenyl	292	—	3.0
10a	<i>tert</i> -Butyl	9	3.2	2.7
10b	Adamantyl	9	11.7	4.2
10c	Noradamantyl	15	18.0	3.6
10d	Cyclopentyl	16	7.9	3.0
10e	Cyclohexyl	18	51.7	3.5
10f	4,4-Difluorocyclohexyl	55 ^b	12.2	3.9
10g	Tetrahydropyran-4-yl	514	—	1.1
10h	2,2,3,3-Tetramethylcyclopropan-1-yl	11 ^b	—	4.7
10i	2,4-Dichlorophenyl	58 ^b	—	3.6
10j	4-Fluorophenyl	156 ^b	—	3.1

^a Binding affinity (K_i) to human σ_1 R in transfected HEK-293 membranes using [³H]-(+)-pentazocine as radioligand. Each value is the mean of two determinations. ^b The compound also shows binding affinity to the σ_2 R in guinea pig brain membranes using [³H]-di-*o*-tolylguanidine as radioligand. Each value is the mean of two determinations. The % inhibition at 1 μ M is 81% (**10f**), 79% (**10h**), 75% (**10i**), and 57% (**10j**).

^c Intrinsic clearance in human liver microsomes as a measure of metabolic stability. ^d clog *P* calculated using ChemDraw Ultra 10.0.3.

Scheme 1 Reagents and conditions: (a) NaH, DMF, rt, 16 h; (b) K₂CO₃, NaI, DMF, rt, 16 h; (c) R₃COCl, pyridine, TEA, DMAP, 130 °C, MW; (d) R₃NCX, acetonitrile or toluene, 120–150 °C, MW.

effected over the pyrrolidinyethyl derivatives shown in Fig. 2. The minor isomer, **16**, which was isolated in 1% yield, showed a NOE effect between the pyrazole proton at position 8 and the methylene protons linked to N9, while the major isomer **4a**, isolated in 40% yield, did not show any NOE effect. The identity of **16** was confirmed by the presence of an NH₂ signal in the ¹H NMR spectrum in CDCl₃, thus discarding **17**. The amino substituted isomer analogues to **17** were not detected in any case.

The last step of the synthesis involved the derivatization of **4** with suitable reagents. Thus, amides **8-12** were obtained with acyl chlorides in pyridine under microwave heating, while reaction with alkyl isocyanates or alkyl isothiocyanates gave ureas **13** and **14** and thioureas **15**, respectively.

The compounds were isolated as hydrochloride, maleate or citrate salts, prepared by standard methods from the corresponding bases, which were usually obtained as oils.

Fig. 2 Potential regioisomeric composition in the alkylation of **2**.

SAR

A new series of pyrazolo[3,4-d]pyrimidines **1**¹⁶ was designed taking into account Glennon's σ₁R pharmacophore,¹⁷ which consists of a positive ionisable group (*i.e.*, a basic amino group) and two opposite hydrophobic regions at a distance of 2.5–3.9 Å and 6–10 Å (Fig. 4A). This elegant and simple model is nowadays still a good reference for designing new σ₁R ligands. Only very recently the σ₁R structure has been solved by X-ray diffraction,¹⁹ and therefore the rational design of σ₁R modulators has up to now mostly relied on ligand-based approaches, as recently summarized.²⁰

It was hypothesized that the pyrazolo[3,4-d]pyrimidine scaffold could place the primary hydrophobic group and the nitrogen atom in an adequate spatial disposition. Additionally, previous reports on bicyclic frameworks, such as **18** (ref. 21) and **19** (ref. 22) (Fig. 3), suggested that this disposition could be tolerated and that the ideal distance between the nitrogen atom and the central bicycle could be of two to three methylene groups. As shown in Fig. 4B, compound **18** fitted well

Fig. 3 Compounds **18** (ref. 21) and **19** (ref. 22).

All the compounds synthesized were evaluated in a primary σ_1 R binding assay using [3 H](+)-pentazocine²⁴ as radioligand (Tables 1–3). Because the two σ receptors are pharmacologically differentiated and selectivity among them is desired, the binding affinities to the σ_2 R were also measured, using [3 H]-di-*o*-tolylguanidine²⁵ as radioligand. Since, in general, the compounds showed low affinity for the σ_2 R, only the values for the σ_1 R appear in Tables 1–3, and σ_2 R

Taking into account the SAR study of compound **1**,¹¹ the amines piperidine, azepane and pyrrolidine were chosen as initial surrogates for the secondary hydrophobic group. As indicated in Table 1, several acylated derivatives (**8–10**) were prepared. The most active compounds exhibited nanomolar affinity for the σ_1 R. In the pyrrolidinyl series (**8**) it was shown that a certain degree of lipophilicity in the acyl substituent was needed, since the activity increased in the order cyclopropyl (**8a**), cyclobutyl (**8b**), cyclopentyl (**8c**), and cyclohexyl (**8d**). Regarding the amine counterpart, activity also improved when the lipophilicity increased, since the azepanyl

derivatives **10a–c** showed increased affinity in relation to the piperidinylamides **9a–c**, while **10d** and **10e** were more active than their pyrrolidinyl counterparts **8c** and **8d**. As expected, the ethoxy derivative **20** (Fig. 5) was completely devoid of activity, confirming that the amino group was a requirement for activity. The best activities were obtained with highly lipophilic and bulky acyl substituents, such as *tert*-butyl (**9a**, **10a**), adamantyl (**9b**, **10b**) and noradamantyl (**9c**, **10c**), but the *tert*-butyl derivatives were somewhat superior in terms of clearance.

The introduction of four methyl groups in the cyclopropyl ring substituent provided the highly active derivative (**10h**), with less selectivity for the σ_2R . The substituted aryl derivatives **9d** and **10i** showed diminished activity, as did the more polar tetrahydropyranyl derivative **10g**, while the fluoro derivative **10j**, with intermediate polarity, showed intermediate affinity. Compound **10f**, although improving the metabolic stability of **10e**, was less stable than **10a**.

Two of the best acyl substituents, *tert*-butyl and adamantyl, were chosen to study the variation of the amine group NR^1R^2 (Table 2). Introducing a *tert*-butyl group in position 4 of the piperidine ring (**11a**) improved the affinity for but impaired microsomal stability vs. **9a**, probably due to the lipophilicity increase. The acyclic di-isopropylamino derivatives showed reduced affinity both in the *tert*-butyl (**11b**) and the adamantyl (**12a**) series. Although **12a** was profiled further, it was discarded because of its low metabolic stability. The 4-morpholinyl (**11c** and **12c**) and homomorpholinyl (**11d**) derivatives were substantially less active than their piperidinyl counterparts (**9a**, **9b** and **10a**, respectively). The methylpiperazinyl derivative **11e** displayed submicromolar affinity, which was not sufficiently improved by the homopiperazinyl (**11f**) and isopropyl (**12b**) analogues. Overall, these results show that, as in the case of the acyl substituents, introducing heteroatoms in the carbocyclic rings in this part of the molecule is detrimental, probably due to the increase in polarity. In fact, all the compounds with *clogP* below 1.6 showed K_i

values above 1000 nM, indicating that a minimum overall lipophilicity is required by the σ_1R .

Elongation of the distance between the basic nitrogen and the pyrazolopyrimidine central scaffold to three (**11g**, **h**) or four (**11i**) methylene groups retained activity, while further elongation (**11j**) was detrimental. However, selectivity for the σ_1R vs. the σ_2R decreased in the case of **11i** and **11j**, a result consistent with that obtained in the SAR study of **1**.¹¹

The piperidinyl and azepanyl ethyl derivatives were selected to study the introduction of urea and thiourea groups to replace the acyl moiety (Table 3). The piperidinyl ureas **13** were less active than their azepanyl counterparts **14**, which showed interesting potencies but were either less selective (**14d**) or showed lower metabolic stabilities (**14b–h**). Regarding the thioureas **15a–d**, all of them were not selective for the σ_2R .

One of the best compounds, **9a**, was profiled further and confirmed to be fairly selective, since in addition to its low affinity for the σ_2R ($K_i > 10\,000$ nM), it failed to show any significant affinity (inhibition at $10\ \mu M < 50\%$) in a panel of 65 receptors.²⁷ Moreover it did not bind significantly to recombinant human cytochrome P450 (rhCYP) isoforms (1A2, 2C9, 2C19, 2D6, and 3A4).²⁸ Furthermore, it had good permeability in Caco-2 cells²⁹ ($P_{app} = 390\ \text{nm s}^{-1}$) and an efflux ratio of 0.9, indicating that it was not a P-gp substrate. Its metabolic stability in rodent liver microsomes was high (mouse $Cl_{int} = 6.6$; rat $Cl_{int} = 8.9\ \mu\text{L min}^{-1}\ \text{mg}^{-1}\ \text{prot.}$) like in humans ($Cl_{int} = 2.4\ \mu\text{L min}^{-1}\ \text{mg}^{-1}\ \text{prot.}$). This good *in vitro* ADME profile was complemented by a low blockage of the human ether-a-go-go-related gene (hERG) potassium channel,³⁰ an interesting result taking into account that it has been described that the pharmacophoric requirements of the σ_1R are reported to be similar to those of the hERG channel.³¹

The *in vivo* activity of **9a** was measured in three different pain models in mice (Table 4). In all of them **9a** displayed potent antinociceptive properties, indicating that it acts as an antagonist of the σ_1R .¹¹ Compound **9a** was active in both phases of the formalin test, where the intraplantar (i.pl.) injection of formalin elicits an early phase (phase I) and a delayed phase (phase II) of pain characterized by paw licking, biting and other behaviours. Phase I is predominantly caused by direct activation of C-fibers, whereas phase II is a result of the combination of an inflammatory reaction in the peripheral tissue and functional changes in the spinal cord, thus involving both peripheral and central sensitization.³² Compound **9a** exerted a dose-dependent analgesic effect on both phase I and phase II, with similar efficacy and potency in both phases.

It also showed a moderate, but significant, antiallodynic effect in the mouse model of mechanical hypersensitivity induced by i.pl. capsaicin, which produces increased sensitivity to pain, resulting from central sensitization.³³ This confirms previous studies, which indicate that the σ_1R is essential for the development of capsaicin-induced mechanical hypersensitivity.¹²

Fig. 5 Compounds **4b** and **20** versus **9a**.

Table 2 Amide derivatives 11–12

Comp	<i>n</i>	NR ₂	K _i σ ₁ ^a (h, nM)	HLM ^c Cl _{int} (μl min ⁻¹ mg ⁻¹ prot.)	clog P ^d
11a	1	4- <i>tert</i> -Butyl-piperidin-1-yl	12	8.6	3.9
11b	1	Diisopropylamino	399	—	2.8
11c	1	Morpholin-4-yl	>1000	—	1.0
11d	1	Homomorpholin-4-yl	184	—	0.9
11e	1	4-Methylpiperazin-1-yl	42 923	—	0.1
11f	1	4-Methylhomopiperazin-1-yl	813	—	0.6
11g	2	Piperidin-1-yl	45	1.1	2.5
11h	2	Homopiperidin-1-yl	33	0.2	3.1
11i	3	Homopiperidin-1-yl	62 ^b	—	3.2
11j	3	4- <i>tert</i> -Butyl-piperidin-1-yl	946 ^b	—	4.3
12a	1	Diisopropylamino	99	31.2	4.3
12b	1	4-Isopropylpyperazin-1-yl	573	—	2.3
12c	1	Morpholin-4-yl	774	—	2.4

^a Binding affinity (K_i) to human σ₁R in transfected HEK-293 membranes using [³H]-(+)-pentazocine as radioligand. Each value is the mean of two determinations. ^b The compound also shows binding affinity to the σ₂R in guinea pig brain membranes using [³H]-di-*o*-tolylguanidine as radioligand. Each value is the mean of two determinations. The % inhibition at 1 μM is 89% (11i) and 77% (11j). ^c Intrinsic clearance in human liver microsomes as a measure of metabolic stability. ^d clog P calculated using ChemDraw Ultra 10.0.3.

Compound 9a was also active in the partial sciatic nerve ligation (PSNL) model in mice, representative of neuropathic pain.³⁴ This model of peripheral nerve injury leads to a pain syndrome which is characterized by pain appearance in response to normally innocuous stimuli (allodynia) and exaggerated pain feeling in response to noxious stimuli (hyperalgesia). Hyperalgesia to noxious thermal stimuli and allodynia to cold and mechanical stimuli were used as outcome measures of neuropathic pain by using the plantar, cold plate and von Frey test, respectively. Compound 9a dose-dependently inhibited both mechanical and thermal hyperalgesia, achieving complete suppression at 80 mg kg⁻¹. In thermal allodynia (cold plate test), it exhibited a significant effect at three different doses. Post-treatment values on day 14 (1 day after treatments) were not significantly different from the pre-treatment post-surgery values on day 10, indicating that the effect of drug treatment was reversible (data not shown). Altogether, compound 9a exhibited significant antinociceptive effects, which were comparable in efficacy and potency with those displayed by the reference compound 1, demonstrating its antagonistic nature and reinforcing the role of σ₁R antagonists in the treatment of pain.

Evaluation of the physicochemical properties of compound 9a (Fig. 5) indicated that it is a highly soluble compound (thermodynamic solubility > 2 g mL⁻¹), with an experimental log P (2.6) in accordance with the calculated value (clog P 2.2), which together with its low molecular

weight makes it fully compliant with Lipinski's rule. However, compound 9a (and several additional analogues) showed an indication of suboptimal chemical stability at pH 2, since it slowly converted to the de-acetylated amine 4b, which was poorly active against the σ₁R (K_i 18 μM). Thus, after treatment with 0.01 M HCl, a decrease in area of the parent compound 9a over time was observed, which was accompanied by a corresponding increase in 4b (1.5% after 1 h, 3.4% after 2 h, 5.3% after 3 h). Although these results did not preclude a potential development, we decided to look for more stable alternatives, which are disclosed in the following paper.

The low binding affinity of primary amine 4b for the σ₁R was not surprising since, in accordance with the pharmacophoric features of the σ₁R ligands and the results shown in Table 1, a lipophilic substituent linked to the amino group was needed for activity. Hence, it can be concluded that the relatively high polarity of scaffold I is acceptable as long as lipophilic substituents are present in NR¹R² and R³. Indeed, a more lipophilic moiety in NR¹R² was needed in this case in order to achieve high affinity, compared to the series related to 1.¹¹ Whereas in the pyrazole series the morpholine group showed a similar potency to more lipophilic substituents, in this family it was detrimental. It seems that the increased polarity of the central scaffold has to be somehow compensated for by the increased lipophilicity in side chain moieties.

Table 3 Urea and thiourea derivatives **13–15**

Comp	R	$K_i \sigma_1^a$ (h, nM)	HLM ^c Cl_{int} ($\mu\text{l min}^{-1} \text{mg}^{-1} \text{prot.}$)	clog P^d
13a	Ethyl	371	—	1.6
13b	Propyl	85	5.7	2.3
13c	Cyclopentyl	192	—	2.6
13d	Cyclohexyl	818	—	3.1
13e	Adamantyl	181	—	3.7
13f	2,4-Dichlorophenyl	141	—	3.9
14a	Ethyl	79	2.8	4.2
14b	Propyl	24	22.2	3.1
14c	Isopropyl	23	31	2.5
14d	Butyl	29	60.3	3.2
14e	Cyclopropyl	100	—	2.2
14f	Cyclopentyl	21	112.5	3.1
14g	Cyclohexyl	119	—	3.7
14h	<i>tert</i> -Butyl	41	63.9	2.9
15a	Ethyl	9 ^b	—	2.0
15b	Propyl	7 ^b	—	2.5
15c	Butyl	13 ^b	—	3.0
15d	Cyclopropyl	22 ^b	—	2.0

^a Binding affinity (K_i) to human σ_1 R in transfected HEK-293 membranes using [³H]-(+)-pentazocine as radioligand. Each value is the mean of two determinations. ^b The compound also shows binding affinity to the σ_2 R in guinea pig brain membranes using [³H]-di-*o*-tolylguanidine as radioligand. Each value is the mean of two determinations. The % inhibition at 1 μM is: 61% (14d), 68% (15a), 82% (15b), 74% (15c), and 75% (15d). ^c Intrinsic clearance in human liver microsomes as a measure of metabolic stability. ^d clog P calculated using ChemDraw Ultra 10.0.3.

Table 4 *In vivo* activity of **9a** in comparison to **1**

	Formalin ^a				PSNL ^c										
	40 mg kg ⁻¹		80 mg kg ⁻¹		Capsaicin ^b		20 mg kg ⁻¹ (%)			40 mg kg ⁻¹ (%)			80 mg kg ⁻¹ (%)		
	Phase I	Phase II	Phase I	Phase II	40 mg	80 mg	VF	PT	CP	VF	PT	CP	VF	PT	CP
	(%)	(%) ^o	(%)	(%)	kg ⁻¹ (%)	kg ⁻¹ (%)									
1	50 ± 8	50 ± 13	79 ± 6	84 ± 8	52 ± 10 ^d	69 ± 9 ^e	23 ± 21 ^f	39 ± 15 ^f	—	72 ± 29 ^d	80 ± 40 ^d	—	98 ± 10 ^e	109 ± 38 ^e	—
9a	45 ± 12	45 ± 8	85 ± 6	81 ± 7	19 ± 3	49 ± 7	32 ± 7	65 ± 22	83 ± 9	57 ± 6	84 ± 26	85 ± 9	102 ± 15	122 ± 17	93 ± 6

^a Percentage of antinociception in the formalin test evaluated as the licking-biting time in drug-treated animals vs. vehicle. ^b Percentage reduction of mechanical hypersensitivity in the capsaicin test evaluated as the latency time to the paw withdrawal response to upward pressure by von Frey filament stimulation in drug-treated animals vs. vehicle, with 50 s cut-off. ^c Percentage reduction of mechanical (VF: von Frey test) and thermal (CP: cold plate test) hypersensitivity and thermal hyperalgesia (PT: plantar test) induced by partial sciatic nerve ligation, as described in the Experimental section. ^d Dose: 16 mg kg⁻¹. ^e Dose: 32 mg kg⁻¹. ^f Dose: 64 mg kg⁻¹.

Conclusions

The synthesis and pharmacological activity of a new series of pyrazolo[3,4-*d*]pyrimidines, designed according to known pharmacophoric features of the σ_1 R, is reported. The most active compounds exhibited nanomolar affinity for the σ_1 R and generally good selectivity for the σ_2 R. The σ_1 R required the presence of relatively highly lipophilic substituents at op-

posite sides of the central scaffold and selectivity was improved by shortening the distance of the basic nitrogen to it. Compound **9a** was among the most interesting derivatives and exhibited potent antinociceptive properties in several pain models in mice, indicative of its antagonistic behaviour over the σ_1 R. However, a suboptimal stability of **9a** at pH 2 led us to explore alternative substituents in the 4-position of the pyrazolopyrimidine scaffold that would overcome this

potential drawback. The results of this new approach are reported in the following paper.

Experimental

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Microwave assisted reactions were conducted using an Explorer Synthesizer from CEM. Flash chromatography was performed with a forced flow of the indicated solvent system on SDS silica gel Chromagel 60 ACC-(230-400 mesh) or on a CombiFlash Companion system with Redisep Rf disposable columns. ^1H NMR spectra were recorded on an Agilent UNITY 300 MHz spectrometer (fitted with a 5 mm H/F/X ATB probe) or an Agilent Mercury 400 MHz spectrometer (fitted with a 5 mm ID/PFG probe) with 2 H lock in deuterated solvents. Chemical shifts (δ) are in parts per million. Commercially available reagents and solvents (HPLC grade) were used without further purification for all the analytical tests. Analytical HPLC–MS analyses were performed on a Waters 2795-MS ZQ system using reverse phase XBridge C18 columns (4.6 $\times$ 50 mm, 2.5 μm), gradient 2–95% B (A = 10 mM ammonium bicarbonate, B = acetonitrile) over 4 min, injection volume: 5 μL , flow: 1.5 mL min^{-1} (method A) or gradient 2–95% B (A = 10 mM ammonium bicarbonate, B = acetonitrile) over 3.7 min, injection volume 5 μL , flow: 2.0 mL min^{-1} (method B). PDA spectra were recorded at 220–310 nm using a Waters 2996 PDA detector. Mass spectra were obtained over the range m/z 100–800 at a sampling rate of 0.3 scans per second using a Waters ZQ system. Data were integrated and reported using Water Masslynx software; all compounds displayed purity higher than 95% as determined by this method. Accurate mass measurements were carried out using an Agilent 6540 UHD Accurate-Mass QTOF system and obtained by electron spray ionization (ESI) in positive mode.

1-[2-(Pyrrolidinyl-1-yl)ethyl]-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (4a, $n = 1$, $\text{NR}^1\text{R}^2 = \text{pyrrolidine}$)

NaH (60% in mineral oil, 312 mg, 7.8 mol) was added under an argon atmosphere to a solution of 4-amino-1H-pyrazolo[3,4-*d*]pyrimidine (2, 1.0 g, 7.4 mmol) in anhydrous DMF (30 mL) at 0 $^\circ\text{C}$. The mixture was left to reach room temperature for 1 h, and then, after cooling to 0 $^\circ\text{C}$ again, a solution of 1-(2-chloroethyl)pyrrolidine (3, $n = 1$, $\text{NR}^1\text{R}^2 = \text{pyrrolidine}$) (0.989 g, 7.4 mmol) in anhydrous DMF (10 mL) was added dropwise. The mixture was stirred at room temperature for 18 h and then quenched with H_2O . The mixture was concentrated in vacuum and partitioned between EtOAc and H_2O . The combined organic layers were dried over anhydrous Na_2SO_4 , filtered and concentrated to dryness to yield a crude residue that was purified by flash chromatography (SiO_2 , EtOAc/MeOH gradient from 9:1 to 7:3) to give 4a as a solid (0.687 g, 40% yield) together with the N^2 -regioisomer 2-(2-(piperidin-1-yl)ethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine, 16 (13 mg, 1% yield). 4a: ^1H NMR (400 MHz, CDCl_3) δ 8.39 (1H, s, 9-H), 7.92 (1H, s, 2-H), 5.47 (2H,

br s, 10-H), 4.59 (2H, t, $J = 7.0$ Hz, 11-H), 3.07 (2H, t, $J = 7.0$ Hz, 12-H), 2.69–2.60 (4H, m, 14-H, 17-H), 1.82–1.71 (4H, m, 15-H, 16-H). HPLC (method B) Rt 1.58 min, 98% purity. MS (ESI) m/z : $[\text{M} + \text{H}]^+$ 233.2; 16: ^1H NMR (300 MHz, CDCl_3) δ 8.47 (1H, s, 8-H), 8.08 (1H, s, 2-H), 5.45 (2H, br s, 7-H), 4.53 (2H, t, $J = 6.3$ Hz, 11-H), 3.13 (2H, t, $J = 6.3$ Hz, 12-H), 2.70–2.51 (4H, m, 14-H, 17-H), 1.89–1.73 (4H, m, 15-H, 16-H). HPLC (method B) Rt 1.47 min, 93.6% purity, MS (ESI) m/z : 233.2.

1-[2-(Piperidin-1-yl)ethyl]-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (4b, $n = 1$, $\text{NR}^1\text{R}^2 = \text{piperidine}$)

Using a similar procedure to that previously described, 4b was obtained as a solid (1.63 g, 45% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.38 (1H, s, 8-H), 7.94 (1H, s, 2-H), 5.55 (2H, br s, 7-H), 4.62 (2H, t, $J = 7.1$ Hz, 11-H), 3.12–2.91 (2H, m, 12-H), 2.66–2.51 (4H, m, 14-H, 18-H), 1.67–1.61 (4H, m, 15-H, 17-H), 1.47–1.42 (2H, m, 16-H). HPLC (method A) Rt 2.29 min, 96% purity. MS (ESI) m/z : $[\text{M} + \text{H}]^+$ 247.2.

1-[2-(1,4-Oxazepan-4-yl)ethyl]-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (4c, $n = 1$, $\text{NR}^1\text{R}^2 = \text{homomorpholine}$)

Compound 2 (1.0 g, 7.4 mmol) was added in portions to a suspension of NaH (60% in mineral oil, 320 mg, 8.0 mmol) in anhydrous DMF (15 mL) under an argon atmosphere. After stirring for 2 h at room temperature, a solution of 1-bromo-2-chloroethane (5, $n = 1$, X = Br, Y = Cl) (0.68 mL, 8.14 mmol) in anhydrous DMF (2 mL) was added dropwise. The reaction was stirred at room temperature for 18 h, cooled to 0 $^\circ\text{C}$ and quenched with H_2O . The mixture was concentrated in vacuum, treated with EtOAc and filtered. The solution was concentrated and the crude product thus obtained purified by flash chromatography (SiO_2 , gradient from 100% EtOAc to EtOAc/MeOH 9:1) to yield 1-(2-chloroethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (6, $n = 1$, Y = Cl) as a white solid (0.82 g, 56% yield). A mixture of the previous derivative (0.250 g, 1.26 mmol), 1,4-oxazepane hydrochloride (0.313 g, 2.27 mmol), K_2CO_3 (0.524 g, 3.79 mmol) and a catalytic amount of NaI was dissolved in anhydrous DMF (10 mL) and the mixture stirred at 95 $^\circ\text{C}$ for 16 h under argon atmosphere. The mixture was concentrated under reduced pressure, treated with EtOAc and filtered. The solution was evaporated to dryness and the residue was treated with petroleum ether and decanted to give a crude product that was purified by flash chromatography (SiO_2 , EtOAc/MeOH 9:1) to yield 4c (0.08 g, 24% yield). ^1H NMR (300 MHz, CD_3OD) δ 8.19 (1H, s, 8-H), 8.09 (1H, s, 2-H), 4.47 (2H, t, $J = 6.6$ Hz, 11-H), 3.67 (2H, t, $J = 5.9$ Hz, 18-H), 3.64–3.56 (2H, m, 16-H), 3.05 (2H, t, $J = 6.6$ Hz, 12-H), 2.82–2.70 (4H, m, 14, 19-H), 1.79 (2H, p, $J = 5.9$ Hz, 15-H). HPLC (method B) Rt 1.51 min, 95.6% purity, MS (ESI) m/z : 263.1.

N-{1-[2-(Piperidin-1-yl)ethyl]-1H-pyrazolo[3,4-*d*]pyrimidin-4-yl}-pivalamide maleate (9a)

Compound 4b (0.50 g, 2.03 mmol), pivaloyl chloride (0.44 g, 3.66 mmol), triethylamine (1.13 mL, 8.12 mmol) and a catalytic amount of DMAP in anhydrous pyridine (5 mL) were

heated in a CEM microwave reactor at 130 °C for 20 min. The mixture was concentrated under reduced pressure and partitioned between EtOAc and 10% aqueous NaOH solution. The combined organic layers were washed with H₂O, dried and evaporated to dryness. The oily residue (0.5 g, 74% yield) was dissolved in ¹PrOH (3 mL) and cooled to 0 °C, and a solution of maleic acid (0.18 g in 2 mL of ¹PrOH) was added dropwise. After stirring for 30 min at room temperature, the solvent was removed under vacuum without heating and the residue was washed with Et₂O to give **9a** as a white solid (0.48 g, 71% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.77 (1H, s, 9-H), 8.72 (1H, s, 2-H), 8.47 (1H, s, 8-H), 6.03 (2H, s, 26-H, 27-H, maleate), 4.80 (2H, t, *J* = 6.3 Hz, 17-H), 3.58–3.53 (2H, m, 18-H), 3.45–3.20 (4H, m, 20-H, 24-H), 1.70–1.65 (4H, m, 21-H, 23-H), 1.54–1.49 (2H, m, 22-H), 1.31 (9H, s, 14-H, 15-H, 16-H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 177.76 (C-10), 167.10 (C-25, C-28, maleate), 154.63 (C-4), 154.54 (C-2), 152.83 (C-6), 136.88 (C-8), 135.67 (C-26, C-27, maleate), 103.99 (C-5), 54.36 (C-18), 52.49 (C-20, C-24), 41.41 (C-17), 39.77 (C-12), 26.55 (C-14, C-15, C-16), 22.68 (C-21, C-23), 21.32 (C-22). The assignment has been done according to the numbering in Fig. 5. HPLC (method B) Rt 2.80 min, 97% purity. HRMS (ESI) *m/z*: [M + H]⁺ calculated for C₁₇H₂₆N₆O 331.2241, found 331.2239.

1-Cyclopentyl-3-{1-[2-(piperidin-1-yl)ethyl]-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl}urea hydrochloride (**13c**)

A mixture of **4b** (0.050 g, 0.20 mmol) and cyclopentyl isocyanate (0.034 g, 0.31 mmol) in acetonitrile (2 mL) was heated in a CEM microwave reactor for 1 h at 120 °C. The mixture was concentrated under reduced pressure, treated with acetonitrile and filtered. The crude product was dissolved in anhydrous dichloromethane (1.5 mL) and 4 M dioxane–HCl solution (1 mL) was added at 0 °C. After stirring for 15 min the solid was filtered and washed with dioxane to give **13c** (55 mg, 70% yield). ¹H NMR (400 MHz, CD₃OD) δ 8.68 (1H, s, 16-H), 8.68 (1H, s, 2-H), 4.98 (2H, t, *J* = 6.0 Hz, 19-H), 4.20 (1H, p, *J* = 6.3 Hz, 11-H), 3.77 (2H, t, *J* = 6.0 Hz, 20-H), 3.72 (2H, d, *J* = 12.1 Hz, 22-H, 26-H), 3.07 (2H, td, *J* = 12.3, 3.1 Hz, 22-H, 26-H), 2.12–1.44 (14H, m, 12-H, 13-H, 14-H, 15-H, 23-H, 24-H, 25-H). ¹³C NMR (101 MHz, CD₃OD) δ 163.15 (C-8), 154.07 (C-2), 153.96 (C-4), 148.45 (C-6), 136.91 (C-16), 102.48 (C-5), 56.12 (C-20), 54.70 (C-22, C-26), 53.47 (C-19), 43.05 (C-11), 33.72 (C-12, C-15), 24.53 (C-13, C-14), 24.03 (C-23, C-25), 22.52 (C-24). HPLC (method A) Rt 3.56 min, 100% purity. HRMS (ESI) *m/z*: [M + H]⁺ calculated for C₁₈H₂₇N₇O: 358.235, found 358.2354.

1-{1-[2-(Azepan-1-yl)ethyl]-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl}-3-butylthiourea maleate (**15c**)

A mixture of 1-(2-(azepan-1-yl)ethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (0.10 g, 0.38 mmol) and butyl thioisocyanate (0.13 g, 1.15 mmol) in anhydrous toluene (4 mL) was heated in a CEM microwave reactor at 150 °C for 1 h. The mixture was concentrated under reduced pressure, purified by flash chromatography (SiO₂, EtOAc), treated with acetonitrile and filtered to afford the free base as a white solid

(22 mg, 15% yield). The previous solid was dissolved in MeOH (0.5 mL) and to this mixture a solution of maleic acid (7.5 mg, 0.06 mmol) in MeOH (0.5 mL) was added at 0 °C dropwise. After stirring for 15 min at room temperature, the solid was filtered, and the residue washed with Et₂O to give **15c** (26 mg, 90% yield). ¹H NMR (400 MHz, CD₃OD, 400 MHz) δ 8.62 (1H, s, 2-H), 8.52 (1H, s, 15-H), 6.22 (2H, s, 28-H, 29-H), 4.88 (2H, t, *J* = 5.9 Hz, 18-H), 3.79 (2H, t, *J* = 5.9 Hz, 19-H), 3.75 (2H, t, *J* = 7.1 Hz, 11-H), 3.49–3.44 (4H, m, 21-H, 26-H), 1.95–1.90 (4H, m, 22-H, 25-H), 1.80–1.68 (6H, m, 12-H, 23-H, 24-H), 1.56–1.42 (2H, m, 13-H), 1.01 (3H, t, *J* = 7.4 Hz, 14-H). ¹³C NMR (101 MHz, CD₃OD) δ 181.54 (C-8), 170.72 (C-27, C-30), 155.25 (C-2), 155.14 (C-4), 154.24 (C-6), 136.62 (C-15), 134.57 (C-28, C-29), 102.85 (C-5), 56.69 (C-19), 56.27 (C-21, C-26), 46.37 (C-18), 43.19 (C-11), 31.51 (C-12), 27.50 (C-22, C-25), 24.60 (C-23, C-24), 21.34 (C-13), 14.12 (C-14). HPLC (method B) Rt 3.70 min, 100% purity. HRMS (ESI) *m/z*: [M + H]⁺ calculated for C₁₈H₂₉N₇S: 376.2278, found 376.2276.

Determination of physicochemical properties

clog *P* was calculated using ChemDraw Ultra 10.0.3. Solubility was measured as thermodynamic solubility from the solid compound in phosphate buffer at pH 7.4 by HPLC. Log *P* was determined by using a pH metric technique³⁵ in a GpK_a Sirius Analytical instrument.

Stability determination

A solution of compound **9a** in DMSO (10 mM, 25 μL) was treated with 0.01 M HCl and the mixture was maintained at 37 °C for different time periods (1, 2 and 3 h), after which the mixture was left at room temperature for 5 min and the content monitored by HPLC. It was shown that the content of **9a** decreased with time (95.5% at 1 h, 93.6% at 2 h, 91.7% at 3 h), while the content of compound **4b** increased with time (1.5% at 1 h, 3.4% at 2 h, 5.3% at 3 h).

In vitro assays

Protocols for σ₁ and σ₂ receptor binding, human liver microsome stability and hERG inhibition tests are provided in the ESI.†

In vivo studies

Descriptions of the formalin, capsaicin and PSNL models are provided in the ESI.†

Abbreviations

ADMET	Absorption, distribution, metabolism, excretion and toxicity
Cl _{int}	Intrinsic clearance
clog <i>P</i>	Calculated logarithm of the octanol/water partition coefficient
CNS	Central nervous system
rhCYP	Recombinant human cytochrome P450

3D	Three-dimensional
hERG	Human ether-a-go-go-related gene
HLM	Human liver microsomes
HRMS	High resolution mass spectrometry
HTS	High throughput screening
NOE	Nuclear Overhauser effect
P_{app}	Apparent permeability coefficient
PSNL	Partial sciatic nerve ligation
Rt	Retention time
SAR	Structure–activity relationship
σ_R , σ_1R , σ_2R	Sigma, sigma-1 and sigma-2 receptor, respectively

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