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Pyrazolothiazolopyrimidine derivatives as a novel class of anti-inflammatory or antinociceptive agents: synthesis, structural characterization and pharmacological evaluation*

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Summary — As a part of a research program on anti-inflammatory-analgesic compounds, pyrazolothiazolopyrimidines **5a–f** and **5g-i** were prepared by cyclodehydration in 98% H_2SO_4 or PPA of the corresponding 6-thioketomethylene-substituted-4-hydroxy-pyrazolo[3,4-*d*]pyrimidines **2a–i** and **2g–i**. The results of the pharmacological *in vivo* screening indicate an interesting dissociation of the analgesic from the anti-inflammatory activity depending on aromatic or aliphatic substitution at the C4 of the thiazole ring. Analgesic activity was not associated with any narcotic effect; in addition, all the active compounds showed a remarkable systemic and gastric tolerance. This indicated a mode of action different from that of the classical nonsteroidal anti-inflammatory drugs, acting on prostaglandin biosynthesis. To clarify the mechanism or the mechanisms underlying the pharmacological activity of these and other closely related compounds, we initiated a 'file chemical approach' to various systems involved in the inflammatory process. At present, some of the more active *in vivo* compounds tested as substance P antagonists showed a moderate and possibly non-specific effect on NK_1 and NK_2 receptors.

pyrazolothiazolopyrimidine derivatives / anti-inflammatory–analgesic activity / substance P antagonists

Introduction

Since it became clear that the classical nonsteroidal anti-inflammatory drugs (NSAIDs) produced gastrointestinal side effects *via* cyclooxygenase (CO) inhibition, research in medicinal laboratories has been directed at finding compounds with different structural features able to act on other biological targets involved in the inflammatory response. In addition, recent studies have shown that prostaglandins are not always the carrier of the signals involved in the development of the process [1–3]. On the other hand, the complex and sequential network of the inflammation and the consequent heterogeneity of the inflammatory conditions require a different therapeutic approach aimed at blocking the underlying causes of the disorder or its specific evolutive state [1]. One avenue of research was to concentrate on substances able to act on the immune system [4, 5] or neuropeptides responsible for the neurogenic inflammation [6–10]. These are closely connected due to the ability of the latter to interact at different levels with the humoral or the cellular arms of the former [11, 12]. To date, very few chemicals are known to possess a selective mode of action on the named targets [6–12].

In this perspective, in our earlier studies on condensed heterocycles containing the pyrimidine ring as nonacidic anti-inflammatory-analgesic compounds, we noted the positive role of the triazolopyrimidine or thiazolopyrimidine moiety in joining with a large number of heterocyclic nuclei to obtain very effective compounds lacking in ulcerogenic or systemic effects [13]. On this basis, in recent years we decided to investigate the effect on the named moieties of the pyrazole nucleus in view of the importance and topicality that it holds in the field of the anti-inflammatory–analgesic drugs. The recently published promising results obtained by us with a series of pyrazolotriazolopyrimidines (Formula A) [13] encouraged us to synthesize the title compounds **5a–f** and

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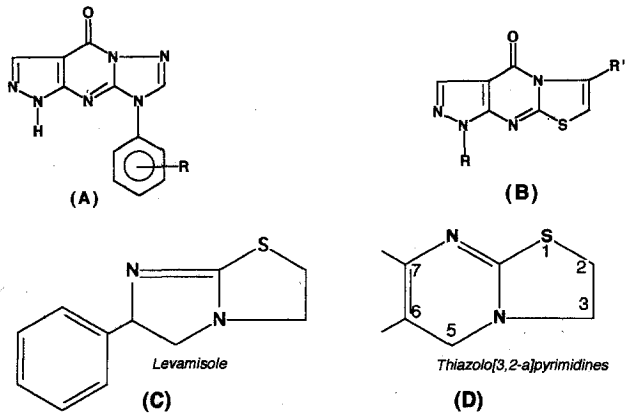
5g–i (Formula B). Recent papers also report on tricyclic compounds which contain the thiazole ring as anti-arthritic substances with a mode of action different from that of the classical NSAIDs [4] and the biological potential of the thiazolopyrimidine moiety as an anti-arthritic agent, also considering its structural analogy with the potent immunomodulator levamisole (C, D) [14].



At the outset, we chose the phenyl ring variously substituted as radical on the thiazole ring in view of the particular role that the extension of the aromatic–lipophilic parts of the molecule plays in the interaction with many biological systems involved in inflammation. To obtain more detailed information on structure–activity relationships, we subsequently evaluated the effect of an aliphatic substituent (methyl group) **5b** in the place of that aromatic on the thiazole ring and on N_1 of the pyrazole nucleus **5g–i** and compared the pharmacological properties of all the substituted compounds with the prototype **5a**. We also transformed some of the compounds **5b**, **c–f** into the corresponding 4-thione derivatives **6b**, **c–f**.

The aforementioned compounds were screened *in vivo* for their anti-inflammatory activity either *via* carrageenan paw oedema or acetic acid peritonitis, and for their analgesic activity by the phenylquinone writhing test.

To obtain a clearer idea of the mechanism or mechanisms of action of these and other related molecules, we also adopted a file chemical *in vitro* approach on the most active *in vivo* compounds, the first step of which was their evaluation as substance P antagonists since substance P has been suggested to be involved in mediating neurogenic inflammation and nociception [9, 11, 12, 15]. Besides, recently published papers report on tricyclic compounds as substance P antagonists [16–17]. Studies on compound **6c**, the most effective *in vivo* compound, are referred to below.

Results and discussion

Chemistry

*Syntheses of 1H-pyrazolo[3,4-d]thiazolo[3,2-a]4H-pyrimidin-4-one **5a–f** and their N_1-methyl analogs **5g–i***

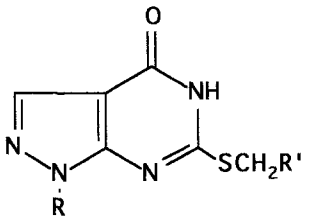
Pyrazolothiazolopyrimidine derivatives **5a–f** and **5g–i** (table II) were prepared by cyclodehydration in acidic media (98% H_2SO_4 or PPA) of the corresponding 6-thioketomethylene-substituted-4-hydroxy-pyrazolo-[3,4-*d*]pyrimidines **2a–f** or **2g–i** (table I). The latter were in their turn obtained by reacting 4-hydroxy-6-mercapto-pyrazolo[3,4-*d*]pyrimidines **1a**, **b** [18, 19] with aromatic or aliphatic commercially available α-haloketones/aldehydes. By hydrolysis of the intermediates **2c** and **2i** 4,6-dihydroxy-pyrazolo[3,4-*d*] pyrimidines **3c**, **i** were obtained. These compounds [20] were identical to those obtained by condensing commercially available 3-amino-4-carboethoxy-pyrazolo **4a**, or 5-amino-1 methylpyrazolo-4-carboxamide **4b** with urea in an oil bath at 180–200°C. This fact, in accordance with analytical instrumental data, IR (table I), ^{1}H-NMR (table IV) MS measurements (table VII) and UV spectra (table VIII), confirmed the presence of the ketomethylene group on the S6 atom of the pyrimidine ring. We assume the reaction to be common to the other compounds of the series **2a–f** and **2g–i** (table I).

Compounds **6b–f** (table III) were prepared by treating the corresponding 4-oxo compounds **5b–f** (table II) with Lawesson's reagent in anhydrous xylene. The above reactions are reported in scheme 1.

The elemental analyses, IR (table II), NMR (^{1}H, ^{13}C) (tables V, VI), MS measurements (table VII) and UV spectra (table VIII) of the tricyclic compounds **5a–f**, **5g–i** and **6b–f** were consistent with the assigned structures. Because of the possible double route in the final reaction we also ascertained the linear geometry of the latter (table V) *via* intramolecular NOE experiments. The ^{1}H-NMR of compound **5i** showed 4 well-resolved signals: a singlet at 3.952 ppm due to the methyl protons Me(1) bound to the nitrogen atom, a broad signal at 7.383 ppm due to the phenyl protons H(6) and 2 singlets at 6.523 ppm and 7.980 ppm. NOE experiments allowed resonance at 6.523 ppm to be assigned to the proton H(7) and hence the other signal at 7.980 ppm to H(3): the irradiation of the phenyl protons at 7.383 ppm produced relevant NOE at 6.523 ppm. This last experiment also allowed a linear geometry to be attributed to the compound **5i**; the saturation of the phenyl protons did not produce any NOE effects on Me(1), which could be expected in the case of an angular geometry.

Accordingly, the saturation of the methyl resonance at 3.952 ppm did not produce enhancement of the phenyl resonance. The unmethylated compound **5c**

Table I. Physico-chemical data of the compounds **2a–i**.



Compd	R	R'	m.p.(°C)	Yield%	Recr. solv.	I.R.(KBr, cm^{-1}) C=O	N-H	Formula
2a*	H	$CH(OC_2H_5)_2$	180-2	20	$EtOH/H_2O$	1700	3200	$C_{11}H_{16}N_4O_3S$
							3080	
2b*	H	$COCH_3$	220 dec.	35	AcOH	1670	3240	$C_8H_8N_4O_2S$
2c*	H	COC_6H_5	231-2	60	AcOH	1670	3120	$C_{13}H_{10}N_4O_2S$
2d*	H	$COC_6H_4Cl(p)$	286-7	70	DMF/H_2O	1680	3090	$C_{13}H_9ClN_4O_2S$
2e*	H	$COC_6H_4Br(p)$	273-5	80	DMF/H_2O	1675	3090	$C_{13}H_9BrN_4O_2S$
2f*	H	$COC_6H_4F(p)$	237-8 dec.	80	AcOH	1675	3090	$C_{13}H_9FN_4O_2S$
2g**	CH_3	$CH(OCH_3)_2$	> 300	25	EtOH	1730	-	$C_{10}H_{14}N_4O_3S$
						1670	-	
2h**	CH_3	$COCH_3$	197-9	35	EtOH	1730	-	$C_9H_{10}N_4O_2S$
						1670	-	
2i**	CH_3	COC_6H_5	225-6	25	$AcOH/H_2O$	1710	-	$C_{14}H_{12}N_4O_2S$
						1680		

TLC system: *ethyl acetate/methanol 9:1; **ethyl acetate.

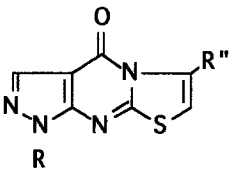
showed a very similar proton spectrum: a signal at 6.566 ppm due to H(7), a singlet at 7.980 ppm due to H(3) and a broad signal at 7.402 ppm due to the phenyl protons H(6). The close similarity of the **5i** and **5c** proton spectra strongly suggested that this latter compound also existed in a linear geometry. Mass spectra of the intermediates **2a–f** and **2g–i**, reported for the samples **2c** and **2i** (table VII) show in addition to a molecular peak two significant peaks resulting from a fragmentation in the α-position to the heterocyclic ring and to the carbonyl group. In the mass spectra of the tricyclic compounds **5a–f** and **5–i** (table VII), reported for the sample **5c**, an intense peak is present corresponding to the molecular ion; fragmentation produces only very low intensity peaks due to the high stability of the polycyclic cation.

Pharmacology

In vivo evaluation

Behavioural effects and acute toxicity in mice. At doses of 1000 mg/kg *po* and 500 mg/kg ip in mice, the test compounds did not show any significant gross behavioural and toxicological effects, except for the N_1-methyl substituted **5g** and **5h** (650 mg/kg *po* and 300 mg/kg ip). Higher doses of 1000 mg/kg *po* and 500 mg/kg ip produced dose-related sedation, motor

Table II. Physico-chemical data of the compounds **5a–i**.



Compd	R	R''	m.p.(°C)	Yield%	Recr. solv.	I.R.(KBr,cm^{-1}) C=O	N-H	Formula
5a**	H	H	273-5	25[a];8[b]	EtOH	1730	3220	$C_7H_4N_4OS$
5b**	H	CH_3	236-8	30[a];15[b]	EtOH	1720	3180	$C_8H_6N_4OS$
5c*	H	C_6H_5	248-9	90[a];45[b]	AcOH	1715	3200 3140	$C_{13}H_8N_4OS$
5d**	H	$C_6H_4Cl(p)$	>300	90[a];50[b]	DMF/H_2O	1730	3200	$C_{13}H_7ClN_4OS$
5e**	H	$C_6H_4Br(p)$	>300	98[a];60[b]	DMF/H_2O	1730	3205	$C_{13}H_7BrN_4OS$
5f**	H	$C_6H_4F(p)$	>300	65[a];30[b]	AcOH	1730	3215	$C_{13}H_7FN_4OS$
5g**	CH_3	H	208	30[a];10[b]	EtOH	1730	-	$C_8H_6N_4OS$
5h**	CH_3	CH_3	207-8	98[a];45[b]	EtOH	1730	-	$C_9H_8N_4OS$
5i*	CH_3	C_6H_5	210-2	98[a];50[b]	AcOH	1715	-	$C_{14}H_{10}N_4OS$

[a]H_2SO_4c; bPPA. TLC system: **ethyl acetate **5a**, **b**, **d–h**; *ethyl acetate/methanol 80:20 **5c**, **i**.

incoordination, hypotonia and bradypnoea. As tables IX and X show, the approximate LD_{50} values were ≈ 100 mg/kg *po* and 500 mg/kg ip, except for compounds **5g** and **5h** (650 mg/kg *po* and 300 mg/kg ip). At these doses, mortality generally occurred at 6–12 h postdrug in 20–60% of animals.

Phenylquinone writhing test. At 10 mg/kg *po* in the mouse phenylquinone-induced writhing test, compound **5c** showed dose-dependent analgesic activity that was approximately equal to phenylbutazone at the same dosage.

At 10 mg/kg *po*, thione derivatives showed remarkable analgesic activity, being as active as mephenamic acid **6d**, **f** and **6c**, **e** more active than the latter and the other 2 reference drugs, phenylbutazone and acetylsalicylic acid. At a dose of 1 mg/kg *po*, only compound **6c** was clearly more active than phenylbutazone at the same dosage, whereas the other compounds **6d–f** showed equal activity.

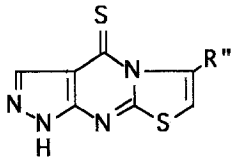
Anti-inflammatory activity. At a dose of 10 mg/kg *po* in the acetic acid peritonitis assay, only compound **5g** exhibited fairly satisfactory antiexudate activity by affording 40% protection. At the same dosage the reference drug phenylbutazone was inactive. In the rat paw oedema test, compounds **5b** and **5g** showed remarkable activity; their potency was comparable to PBZ.

Ulcerogenic activity. No compound showed any ulcerogenic effects or hyperaemia and mucus effusion in the gastric mucosa at the total dose of 400 mg/kg *po* administered twice (2-h interval) in fasted rats, whereas ASA (2 x 200 mg/kg), MFA (2 x 400 mg/kg) and PBZ (2 x 100 mg/kg) caused gastric ulcers in all animals.

In vitro evaluation

NK_1. It can be seen from figure 2 that **6c** (0.1 and 1 μM) had little effect on SPOMe-evoked responses,

Table III. Physico-chemical data of the compounds **6b–f**.



Compd	R"	m.p. (°C)	Yield%	Recr. solv.	I.R.(KBr, cm^{-1})		Formula
					C=S	N-H	
6b	CH_3	249-50	40	EtOH	1070	3180	$C_8H_6N_4S_2$
6c	C_6H_5	218-20	52	EtOH/H_2O	1080	3180	$C_{13}H_8N_4S_2x1H_2O$
6d	$C_6H_4Cl(p)$	227-9	80	EtOH/H_2O	1070	3180	$C_{13}H_7ClN_4S_2$
6e	$C_6H_4Br(p)$	228-9	50	EtOH/H_2O	1075	3170	$C_{13}H_7BrN_4S_2$
6f	$C_6H_4F(p)$	244-5	36	EtOH	1075	3200	$C_{13}H_7FN_4S_2$

TLC system: ethyl acetate.

although **6c** (10 μM) caused 51% inhibition. The compound (10 μM) caused a rightward shift in the position of the SPOMe CRC. However, the shift was not parallel and there was a marked depression of the maximum of the curve, *ie* the curve plateaued at 41% of maximum (fig 3). Therefore a K_a for **6c** could not be determined (see *Experimental protocols, Analysis of in vitro data*).

NK_2. **6c** (10 μM) produced a rightward shift of the eledoisin dose–response curve (following NK_1-receptor desensitization) with a K_a of 3.2 ± 0.6 μM (see fig 4).

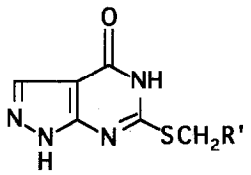
NK_3. From figure 5 it can be seen that there is no significant effect of **6c** (10 μM) on senktide-evoked responses in the superior cervical ganglia.

Conclusion

The results obtained (tables IX and X) indicate that these compounds represent a novel class of anti-inflammatory or antinociceptive agents devoid of any ulcerogenic effect with a different type of action from that of the common NSAID [29].

Based on the profiles discussed above, we have further concluded that the compounds **5b** and **5g** (table IX) were the best anti-inflammatory agents, while **5c** (table IX) and **6c** (table X) were the best non narcotic analgesic agents. On this basis, we formed the supposition that the aliphatic or aromatic substituent on the thiazole ring C_6 operate as carrier towards 2 different biological systems involved in the inflammation. This interesting hypothesis, leading to specific agents in the control of the complex network of the inflammatory process, was supported by the fact that the unsubstituted compound **5a** (table II) showed a mixed anti/inflammatory/analgesic action (fig 1). Also, an increased lipophilicity due to the presence of a sulphur atom in the place of the oxygen on the pyrimidine moiety produced an opposite effect on the activity of the aliphatic or aromatic compounds. In fact, analgesic activity was greatly enhanced whereas anti-inflammatory activity was clearly depressed. A negative effect on the analgesic activity was produced either from the introduction of halogens on the phenyl ring **5d–f** (table IX) and **6d–f** (table X) or from that of a methyl group on the pyrazole N_1 **5i** (table IX). In comparison with the recently reported analogs

Table IV. ^{1}H-NMR data of the compounds **2d–e**.



Compd	R'	^{1}H NMR (ppm)
2d	$COC_6H_4Cl(p)$	13.46,br.,s, (1H,NH);12.34 br.,s, (1H,NH); 8.10,s, (1H,H pyrazole);7.86,m, (J_{av}=8.4 Hz, J_{av}=34.8 Hz, 4H, phenyl); 4.88,s, (2H, methylene)
2e	$COC_6H_4Br(p)$	13.40,br.,s, (1H, NH);12.33,br.,s, (1H, NH);8.05,s,(1H, H, pyrazole);7.89m, (J_{av}=9.6 Hz, J_{av}=16.0 Hz 4H phenyl); 4.88,s, (2H, methylene)

containing a triazole ring [13] in the place of the thiazole ring, we noted an increase in analgesic activity in addition to a better systemic tolerance.

Further studies were carried out on the sample **6c** (figs 2–5), the most active *in vivo* of the tested compounds to clarify its mechanism of action. The compound was investigated as a tachykinin antagonist.

6c (10 μM) was shown to inhibit SPOMe-induced contractile responses in the guinea pig ileum LM/MP. **6c** (10 μM) produced a marked depression of SPOMe-responses and non-parallel displacement of the CRC. This effect of **6c** did not appear to be concentration-dependent, since **6c** (at concentrations of 0.1 and 1 μM) had little effect, whereas **6c** (at 10 μM) had a marked depressant effect. In contrast (±)CP 96,345 [30] caused a parallel shift in the SPOMe CRC, with little effect on the maximum of the curve (K_a for (±)CP 96,345 was 0.5 nM) (see figs 2, 3).

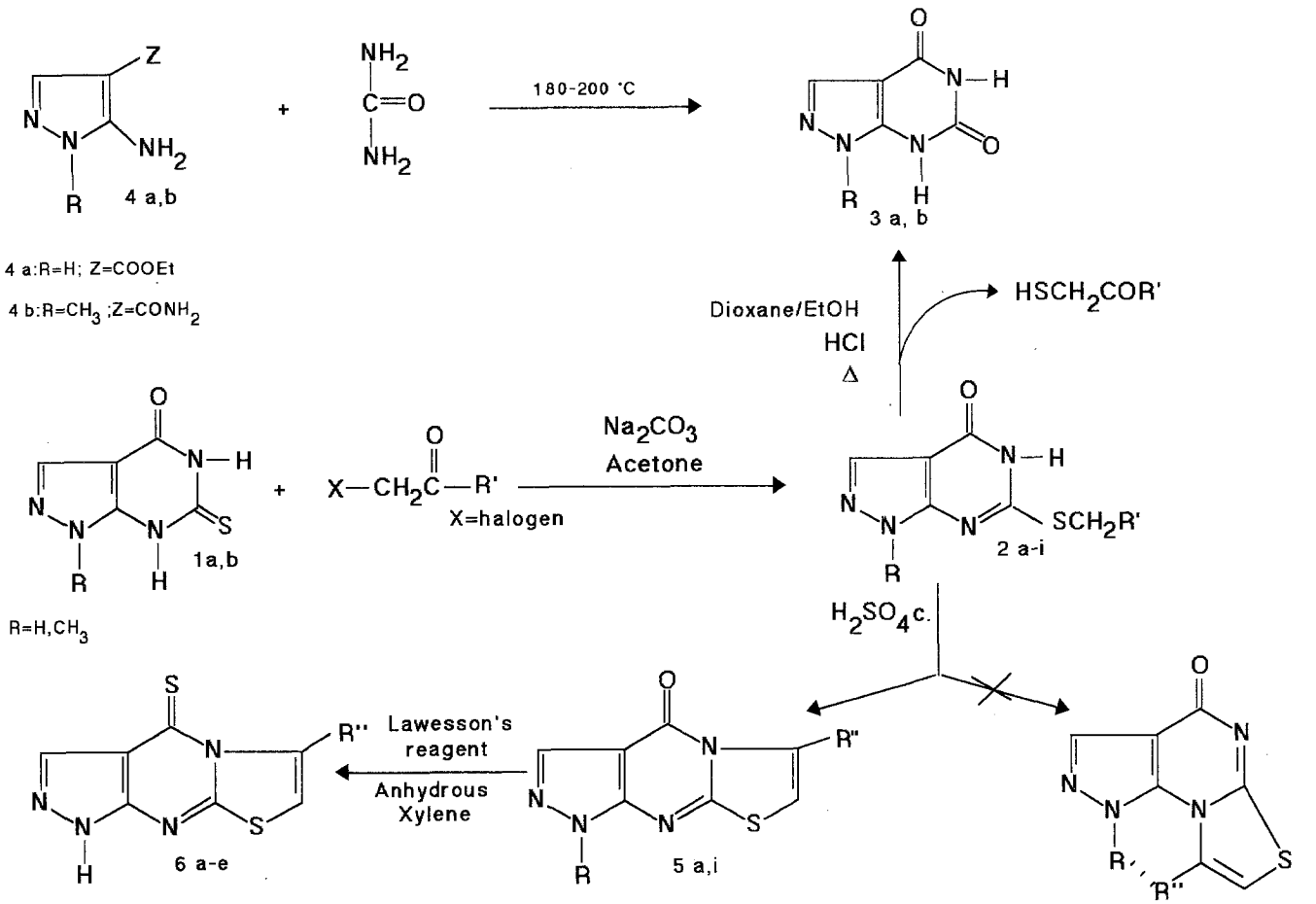
6c (10 μM) also inhibited NK_2-receptor mediated responses in the guinea-pig bladder preparation, suggesting that **6c** has weak NK_2-receptor blocking properties (fig 4). However, in the guinea-pig bladder **6c** also reduced the size of the contractile response evoked by 100 mM KCl (data not shown).

These observations suggest that **6c** may exert non-specific relaxant effects on smooth muscle tissues which may, at least in part, account for its apparent ability to block NK_1- and NK_2-receptor mediated contractile responses. This is supported by the observation that **6c** does not inhibit NK_3-mediated responses in neuronal tissues (fig 5).

Experimental protocols

Chemical methods

All melting points were taken in open capillaries using a Gallenkamp melting point apparatus with a digital thermometer MFB-595 and are uncorrected. The IR spectra were recorded with a Perkin–Elmer 281 spectrometer on KBr disks. Elemental analyses for C, H, N and S were obtained on a Carlo–Erba Mod EA 1108 Analyzer instrument. Analyses indi-



Scheme 1.

cated by the symbols of the elements or functions were within ± 0.4% of theoretical values.

The mp of the crude synthetic intermediates **2a–f** and **2g–i** (table I) were within ± 3°C in comparison with the pure product and they could be used without recrystallization, as could the final compounds **5a–f** and **5g–i** (table II). When the heating progression was very slow, the compounds **2a–f** and **2g–i** in part yielded the tricyclic derivatives **5a–f** and **5g–i**. The ^{1}H-NMR spectra (tables IV, V) were recorded in DMSO–d_6 on a Bruker WP 80 operating at a frequency of 80 MHz. The ^{13}C-NMR (table VI) were recorded on a Varian VXR-300 spectrometer operating at 75 MHz for ^{13}C in DMSO–d_6 as solvent at 25°C.

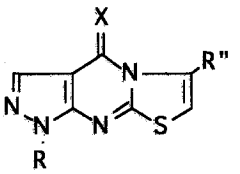
In some spectra the resonance relative to the pyrazole NH was undetectable due to the coupling with a quadrupolar ^{14}N nucleus and rapid exchange with the small amount of water present in the solvent.

NOE experiments (table V) were recorded on a Varian VXR-300 spectrometer operating at 300 MHz for ^{1}H in $CDCl_3$ as solvent at 25°C. The ^{1}H {^{1}H}-NOE experiments were performed on carefully degassed samples in the difference mode. The decoupler was placed at the required frequency to saturate the proton in question. The decoupler power used was the minimum required to saturate the spin in question. A period from 10–20s was used to allow the system to reach equilibrium. Each NOE experiment was repeated at least 4 times.

NMR data are reported in ppm with TMS as an internal reference and are given in δ; coupling constants are reported in Hz. Mass spectrometric measurements were performed on a VG 70-70E instrument in electron impact (EI) mode (70 eV, 100 μA). The samples were introduced by a direct inlet probe at the minimum temperature which gave an adequate vapour pressure: source temperature 150°C. UV maxima, reported for the samples **5c, d** and **6c, d** (table VIII), were measured on a Cary 219 spectrophotometer in methanol at 10^{-4} M concentration, and displayed the expected bathochromatic shifts of the 4-thione derivatives **6c, d** compared with the 4-one analogs **5c, d**.

The purity of the synthesized substances was assessed by thin-layer chromatography (TLC) on silica gel 60 F_{254} aluminium sheets (Merck); system: ethyl acetate/methanol (90:10, **2a–f**); ethyl acetate (**2g–i**; **5a**, **b**, **d–h**, and **6b**, **c–f**); ethyl acetate/ methanol 80:20 (**5c**, **5i**); detection: UV (λ = 254 and 366 nm). The solvents and reagents were purified in the usual manner.

Table V. ^{1}H-NMR data of the compounds **5c**, **d**, **e**, **i** and **6d**.



Compd	R	R''	X	^{1}H NMR (ppm)
5c*	H	C_6H_5	O	7.980,s (1H, H pyrazole); 7.402, br. s, (5H, phenyl); 6.566,s, (1H, H thiazole)
5d	H	$C_6H_4Cl(p)$	O	8.12,s, (1H, H pyrazole);7.46,s, (4H phenyl); 7.15,s,(1H,H thiazole)
5e	H	$C_6H_4Br(p)$	O	8.11,s, (1H, H pyrazole);7.49,m, (J_{av}=8.5 Hz, J_{av}=16.2 Hz,4H phenyl); 7.15,s, (1H,H, thiazole)
5i*	CH_3	C_6H_5	O	7.980,s, (1H, pyrazole); 7.363, br. s, (4H, phenyl); 6.523,s, (1H, thiazole); 3.952,s, (3H, methyl)
6d	H	$C_6H_4Cl(p)$	S	8.21,s, (1H, H pyrazole);7.46,s, (1H,H thiazole);7.39 m (4H phenyl)

*NOE experiments.

Syntheses of 6-thioketomethylene substituted 4-hydroxy-pyrazolo [3,4-d] pyrimidine derivatives 2a–f and 2g–i

General procedure: Method A. A saturated 4'-bromophenacylbromide solution (0.02 mol) was added slowly over a 2-h period to an already stirred (45 min) suspension of 4-hydroxy-6-mercapto-pyrazolo[3,4-*d*]pyrimidine **1a** or **1b** [18, 19] (0.02 mol) and an equimolar amount of Na_2CO_3 in 250 ml acetone. The mixture was then stirred for an additional 24 h and the solid was collected by filtration under reduced pressure, washed with water, dried and recrystallized from acetic acid. The compounds **2d–f** and **2g–i** were synthesized in the same manner. Yields, melting points, recrystallization solvents and IR data of the compounds have been reported in table I. ^{1}H-NMR and MS data respectively of the samples **2d**, **e** (table IV) and **2c** and **2i** (table VII) have been reported.

Method B. To a cooled suspension of 4-hydroxy-6-mercapto-pyrazolo[3,4-*d*]pyrimidine **1a** or **1b** sodium salt (0.01 mol) (obtained by adding equimolar NaOH in a small amount of H_2O to an ethanolic **1a** or **1b** [18, 19] suspension and heating for 30 min at 50–60°C), chloroacetone (0.01 mol) was slowly added. The reaction mixture was heated for 4.5 h and the resulting dark solution concentrated to dryness under reduced pressure. The residue, collected and washed with water, was recrystallized from acetic acid.

Compounds **2b** and **2h** were synthesized in this manner. Yields, melting points, recrystallization solvents and IR data for the compounds are reported in table I.

Method C. This method was like *Method B*, but using bromacetaldehyd-diethylacetal **2a** or dimethylacetal **2g**. Yields, melting points, recrystallization solvents and IR data of the compounds are reported in table I.

Hydrolysis of 4-hydroxy-6-phenacylthio-pyrazolo[3,4-d]pyrimidine 2c or its N_1-methyl analog 2i

A suspension of **2c** or **2i** (0.003 mol) in 60 ml of a mixture of dioxane/ethanol (80:20) was heated until dissolution and then a 6 N HCl solution (50 ml) added. The reaction mixture was refluxed under stirring for 10 h and cooled. The solid material was collected by filtration under reduced pressure, washed with water and dried. The crude product was purified by double precipitation with dilute acetic acid from a 10% Na_2CO_3 solution. The obtained products **3c** and **3i** were identical to the already noted 4,6-dihydroxy-pyrazolo [3,4-*d*]pyrimidines [20].

Syntheses of 1H-pyrazolo[3,4-d]thiazolo[3,2-a]4H-pyrimidin-4-one derivatives 5a–f and 5g–i

General procedures: Method A. A suspension of 4-hydroxy-6-phenacylthio-pyrazolo[3,4-*d*]pyrimidine **2c** (0.003 mol) in 5 ml concentrated sulphuric acid was stirred for 1 h and then

Table VI. ^{13}C-NMR data of the compounds **5d, b–c.**

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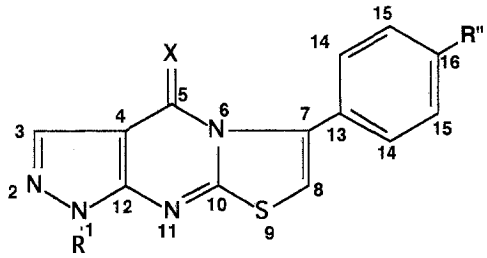


Compd	R"	X	^{13}C NMR (ppm)
5d	Cl(p)	O	109.46 (C8); 101.52 (C7); 127.13 (C13); 130.80 (C14) 133.01 (C13); 133.01 (C16);134.94 (C3); 162.86 (C4); 155.017, 153.373, 135.860 (quaternary carbon atoms C4, C5, C12, C10).
6c	H	S	112.15 (C8); 116.95 (C7);133.37 (C13); 127.02 (C14); 128.92 (C15); 128.03 (C16); 138.02 (C3); 176.87 (C5); 139.442,162.38, 143.21 (quaternary carbon atoms C4, C12, C10).

left at room temperature for 5 d, after which the reaction mixture was poured into 100 ml of ice-water under stirring. The formed precipitate was collected, washed with water, dried and recrystallized from acetic acid. The compounds **5a, b, d–f** and **5g–i** were synthesized in the same manner. Yields, melting points, recrystallization solvents and IR data for the compounds have been reported in table II. ^{1}H-NMR, ^{13}C-NMR, MS data and UV maxima respectively of the samples **5c–e, i** (table V), **5d** (table VI), **5c** (table VII) and **5c, d** (table VIII) have been reported.

Method B. A homogeneous mixture of 4-hydroxy-6-phenacylthio-pyrazolo[3,4-*d*]pyrimidine **2c** (0.003 mol) and an excess (6 g) of polyphosphoric acid (PPA) was heated at 140°C in an oil bath for 3 h. After cooling the melted yield was then neutralized with 10% NaOH and the precipitate formed was collected, dried and recrystallized from acetic acid. The compounds **5a, d–f** and **5g–i** were synthesized in the same manner. Yields, which were less favourable than those of *Method A*, melting points, recrystallization solvents and IR data of the compounds have been reported in table II. ^{1}H-NMR, ^{13}C-NMR, MS data and UV maxima respectively of the samples **5c–e, i** (table V), **5d** (table VI), **5c** (table VII) and **5c, d** (table VIII) have been reported.

*Syntheses of 1H-pyrazolo[3,4-d]thiazolo[3,2-a]4H-pyrimidin-4-thione derivatives **6b, c–f***

General procedure. A suspension of 1*H*-pyrazolo[3,4-*d*]thiazolo[3,2-*a*]4*H*-pyrimidin-4-one **5b** (table II) (0.004 mol) and Laweson's reagent (0.024 mol) in 120 ml anhydrous xylene was heated under stirring for 28 h until TLC (system: ethyl acetate) did not show the presence of the starting compound. The precipitate was collected, dried and recrystallized from a 50:50 mixture of EtOH/H_2O. A further small amount of product could be obtained by reducing the reaction solution to dryness under reduced pressure, treating the oil residue with ethanol and allowing the solution to stand for 2 d at room temperature. The compounds **6c–f**, were prepared in the same manner. Yields, melting points, recrystallization solvents and IR data of the compounds have been reported in table III. ^{1}H-NMR, ^{13}C-NMR and UV maxima respectively of the samples **6c** (tables V, VI, VIII) and **6c, d** (table VIII) have been reported.

Pharmacological in vivo and in vitro evaluation

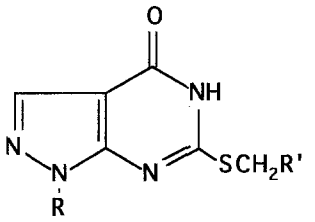
In vivo: experimental procedure

The compounds described in this paper were screened for their analgesic, anti-exudate and anti-inflammatory activities, as well as for their gross behavioural effects and acute toxicity. Acetylsalicylic acid (ASA), mephenamic acid (MFA) and phenylbutazone (PBZ) were used as reference drugs. Results for the tested compounds have been summarized in tables IX and X.

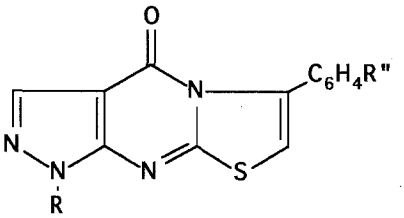
Pharmacology

Experiments were carried out on male albino Swiss mice (24– 26 g) and Sprague–Dawley rats (140–160 g). The test

Table VII. MS data of the compounds **2c**, **2i**, **5c**.



2c,i



5c

Compd	R	R'	R''	m/z (Relative abundance %)
2c	H	COC_6H_5	-	286(**M**$^{+\cdot}$, 12), 135(15), 105(100) 77(57), 51(15)
2i	CH_3	COC_6H_5	-	300 (**M**$^{+\cdot}$ 26),195 (4),149 (15),105 (100)
5c	H	-----	H	268 (**M**$^{+\cdot}$, 100), 269 (**M**+ 1,25), 270 (**M**+ 2,8), 267 (**M**- 1,51), 251(9), 134 (36), 102 (20)

compound were administered orally or ip in 0.5% methylcellulose suspension.

Statistically analysis was made using Students *t*-test *versus* controls. The significance level was set at $P < 0.05$.

Behavioural effects and acute toxicity in mice. The Irwin screening evaluative procedure [21] was used on groups of 5 animals. The compounds were administered orally at 3 dose levels (500, 700, and 1000 mg/kg). The animals were kept under observation for 6 h and the symptomatology was checked again 24 h later .The approximate LD_{50} was obtained from the mortality rate assessed 7 d later.

Phenylquinone writhing test. The test was performed following the technique of Berkowitz *et al* [22]. Groups of 5 mice were injected ip with 0.25 ml of a 0.02% solution of phenylquinone in 5% ethanol 60 min after oral administration of the test drugs. The writhing response frequency was counted in each animal for 5 min (between 5–10 min) after injection of the irritant. The analgesic effect was expressed as percentage of protection in comparison with controls.

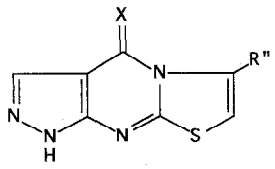
Anti-inflammatory activity

Anti-exudate activity. The acetic acid peritonitis method [23] was used. Groups of 5 rats were administered ip 10 ml of 0.5% CH_3COOH solution 1 h after oral administration of the test compounds. After 30 min the rats were killed with $(C_2H_5)_2O$, and peritoneal exudate collected and measured. The anti-exudate response was expressed as the percentage of the exudate volume reduction compared with controls.

Carrageenan-induced rat paw oedema. The test was performed following the technique of Winter *et al* [24] on groups of 5 rats. The test compounds were administered orally and 60 min later 0.1 ml of 0.1% solution was injected into the plantar aponeurosis of the rat hind paw. The volume of the paw was measured by a mercury plethysmometer prior to the injection of carrageenan and also 3 h later. Anti-inflammatory activity was given as percentage of inhibition of oedema in treated groups compared with controls.

Ulcerogenic activity. The experiment was performed on rats according to the procedure of Domenjoz [25]. The compounds were given orally to groups of 4 rats fasted for 24 h and after 2 h the treatment was again repeated. The animals were killed 6 h after the first dose with $(C_2H_5)_2O$ inhalation, their stomachs removed and examined with a dissecting microscope The severity of mucosal damage (ulcerogenic index) was graduated by means of scores ranging from 0 (= no lesion) to 4 (= exceptionally severe lesions). In order to take into account

Table VIII. UV data of the compounds **5c**, **d** and **6c**, **d**.



Compd	R''	X	UV λ	UV ε
5c	C_6H_5	O	228	15,000
			296	11,500
			314	7,250
5d	$C_6H_4Cl(p)$	O	228	14,000
			298	10,300
			316	6,580
6c	C_6H_5	S	228	15,300
			286	7,950
			356	8,290
			370	9,350
6d	$C_6H_4Cl(p)$	S	232	16,500
			286	11,000
			356	9,740
			369	10,200

the percentage of rats having ulcers, an index of ulceration was calculated on the basis of the following formula [26]:

$$\frac{\text{Mean degree of ulcers} \times \text{No of animals with ulcers}}{\text{No of animals}} \times 100$$

In vitro: experimental procedure

Compound **6c** was chosen for further studies and tested for its ability to inhibit tachykinin-mediated responses in isolated tissues. Its activity was assessed at the 3 tachykinin receptors, *ie* NK_1, NK_2 and NK_3. Tissues were obtained from male Dunkin–Hartley guinea pigs (300–500 g).

NK_1 assay: guinea-pig ileum. Compound **6c** was tested for its ability to inhibit NK_1-mediated contractile responses evoked by substance P-*O*-methylester (SPOMe), a NK_1 receptor selective agonist. Strips of guinea pig ileum myenteric plexus longitudinal muscle (MPLM) were prepared and mounted for isometric tension recording in 3-ml organ baths as previously described [27]. The baths were perfused with Krebs physiological salt solution (PSS); mM: NaCl 115, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.1 containing atropine sulphate (1 μM), indomethacin (1 μM) mepyramine (1 μM) and methylsergide (1 μM), pH 7.4, aerated with 95% O_2, 5% CO_2 at 37°C. Two assays were performed.

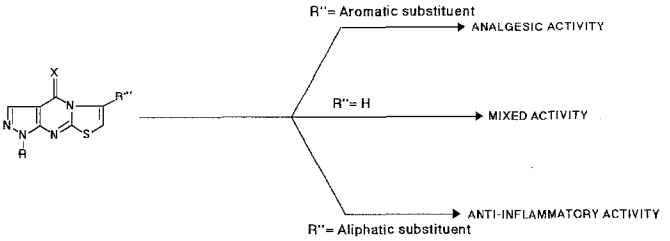


Fig 1. Summary of *in vivo* activity.

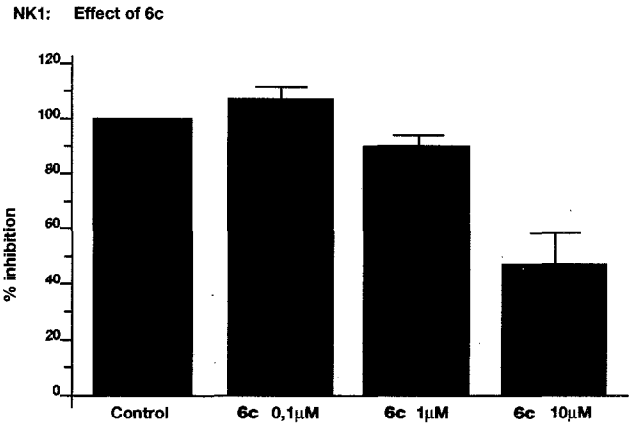


Fig 2. The effect of **6c** (0.1, 1 and 10 μM) on responses evoked by a single concentration (EC_{70}–EC_{80}) of SPOMe in guinea-pig ileum LM/MP. Histobars show mean values (n = 4) and vertical bars indicate ± SEM.

A. Effects of a single concentration of SPOMe. Following a 1-h equilibration period, a sequential concentration–response curve (CRC) to SPOMe was performed in order to ascertain the maximal effective concentration for each tissue. Three SPOMe (EC_{70-80}) concentrations were then added to the baths and the mean response calculated. The tissues were then exposed to **6c** (0.1 μM) for 45 min after which time the responses to SPOMe (EC_{70-80}) were reassessed. This sequence was repeated for 1 and 10 μM **6c**. The mean responses to SPOMe (EC_{70-80}) obtained in the presence of 0.1, 1 and 10 μM **6c** were then compared to those obtained in its absence.

B. Effect of SPOMe on CRC. The effects of **6c** on NK_1-mediated responses were further investigated. After a 1-h-equilibration period, a sequential SPOMe CRC was obtained in the absence (control) of **6c** and then the SPOMe CRC was repeated following the equilibration period (75 min) with **6c** (10 μM). For means of comparison, SPOMe CRCs were also obtained in

Table IX. Pharmacological data of the compounds **5a–g.**

Compd	LD_{50}		*Anti-inflammatory activity*		*Analgesic activity*	*Ulcerogenic index*[b] *(400 mg/kg x 2)*
	per os	*ip*	*Carrageenan paw oedema (100 mg/kg)*	*Acetic acid peritonitis (10 mg/kg)*	*Phenylquinone writhing test*[a] *(10 mg/kg)*	
5a	> 1000	> 500	45*	35*	22	0
5b	> 1000	>500	47*	26	11	0
5c	> 1000	> 500	0	0	26*	0
5d	> 1000	> 500	0	0	0	–
5e	> 1000	> 500	9	3	15	–
5f	> 1000	> 500	0	0	0	–
5g	650	300	56*	40*	25*	0
5h	650	300	9	4	10	–
5i	> 1000	> 500	22	17	8	–
PBZ	≈ 700	≈ 300	56*	7	27*	2.75[c]

Oral administration for all tests; substances partially solubilized in CMC 0.5%; [a]values are percent of controls; [b]more active compounds were tested for ulcerogenic activity; [c]PBZ 100 mg/kg x 2; *$P < 0.05$, Student *t*-test *vs* controls.

Table X. Pharmacological data of the compounds **6b–f.**

Compd	LD_{50}		*Anti-inflammatory activity*		*Analgesic activity*		*Ulcerogenic index*[b] *(400 mg/kg x 2)*
	per os	*ip*	*Carrageenan paw oedema*[a] *(100 mg/kg)*	*Acetic acid peritonitis (10 mg/kg)*	*Phenylquinone writhing test*[a] *(1 mg/kg)*	*(10 mg/kg)*	
6b	> 1000	> 500	10	15	17	19	0
6c	> 1000	> 500	25*	25*	40*	62*	
6d	> 1000	> 500	13	14	19	29	0
6e	> 1000	> 500	11	10	22	46*	0
6f	> 1000	> 500	28*	20	15	27*	0
ASA	≈ 1000	≈ 500	30*	0	0	10	2.50[c]
MFA	≈ 700	≈ 300	55*	0	7	25*	2.25
PBZ	≈ 2000	≈ 600	37*	6	19	30*	2.75[c]

Oral administration for all tests; substances partially solubilized in CMC 0.5%; [a]values are percent of controls; [b]more active compounds were tested for ulcerogenic activity; [c]PBZ 100 mg/kg x 2; ASA 200 mg/kg x 2; *$P < 0.05$, Student's *t*-test *vs* controls.

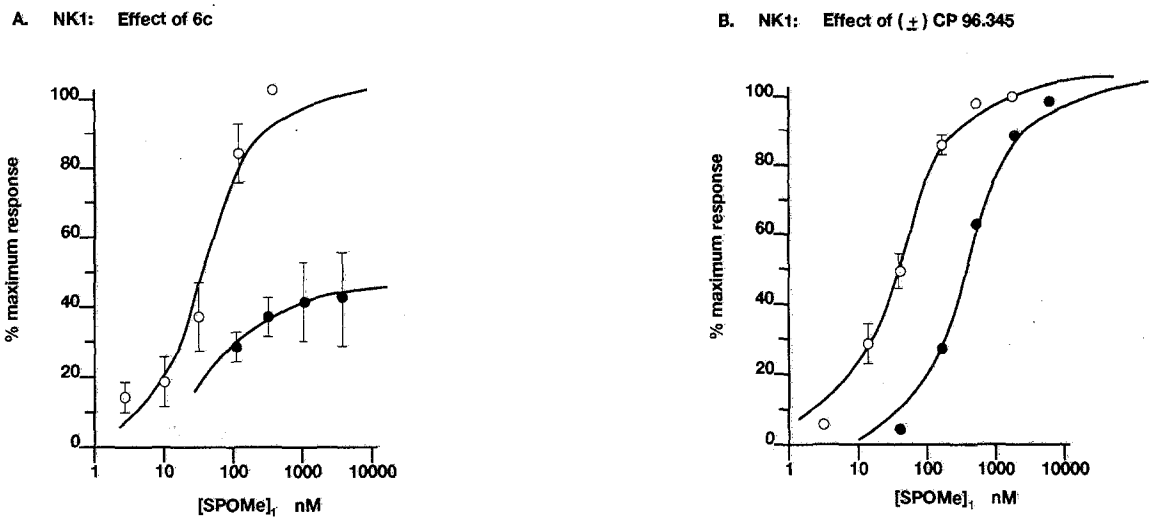


Fig 3. The effect of (**A**) **6c** (10 μM) and (**B**) (±) CP 96.345 (3 nM) on SPOMe-induced contractile responses in guinea-pig ileum LM/MP. Responses to SPOMe were measured in the absence (open circles) and presence (closed circles) of the antagonist. Points represent mean values (n = 4–6) and vertical bars indicate ± SEM. Curves were fitted by least squares analysis of variance (see text for details).

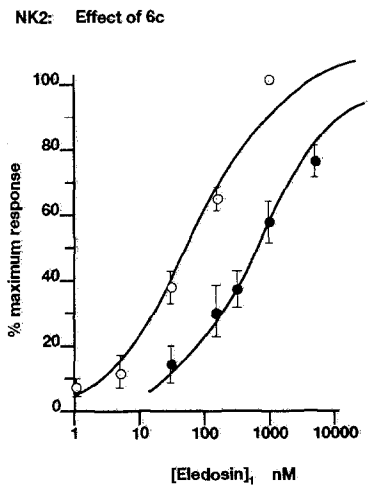


Fig 4. The effect of **6c** (10 μM) on NK_2-receptor mediated contractile responses in guinea-pig urinary bladder smooth muscle. Responses to eledoisin (following NK_1-receptor desensitization) were measured in the absence (open circles) of **6c** and presence (closed circles) of **6c** (10 μM). For details see legend to figure 3.

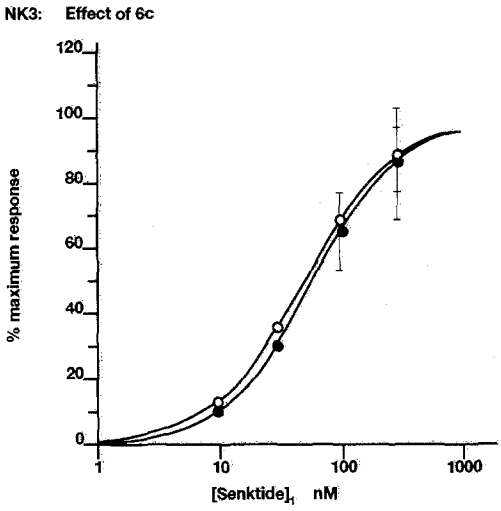


Fig 5. The effect of **6c** (10 μM) on NK_3-receptor mediated responses in guinea-pig superior cervical ganglia. Responses to senktide were measured in the absence (open circles) and presence (closed circles) of **6c** (10 μM). For details see legend to figure 3.

the presence and absence of ± CP 96,345 (a selective NK_1-receptor antagonist) [30]. The responses were expressed as percentage of maximum control response.

NK_2 assay: guinea-pig bladder. Transverse strips of guinea pig urinary bladder were mounted for isometric tension recording in 3-ml organ baths containing Krebs PSS, pH 7.4, aerated with 95% O_2: 5% CO_2 at 37°C. As the bladder has been shown to contain both NK_1 and NK_2 receptors, a desensitization procedure was employed in order to solely examine NK_2-mediated responses. Sar^9-Met $(O_2)^{11}$-substance P (100 nM; a selective NK_1 receptor agonist) was added to the tissue baths and left in contact for 8 min to desensitize NK_1 receptors [28]. NK_2-mediated responses were evoked by sequential addition of eledoisin (10 nM–1 μM).

The CRC was repeated following equilibriation (75 min) with **6c** (10 μM). Responses were measured by subtracting the integrated value of the area under the time/tension curve obtained over a 4-min period immediately prior to addition of each concentration of eledoisin from the integrated value obtained over an identical period immediately after addition of eledoisin.

NK_3 assay: guinea pig superior cervical ganglia. Desheathed superior cervical ganglia were placed in tissue baths that were divided into 3 chambers. The preganglionic terminal was positioned in the first chamber whilst the postganglionic terminal was placed in the third chamber with 1 sealed chamber separating them. The chambers were perfused with modified Krebs PSS: mM; NaCl 125, KCl 2, KH_2PO_4 1, $CaCl_2$ 2.5, $MgSO_4$ 1, $NaHCO_3$ 25, glucose 10 containing atropine (1 μM) and tetrodotoxin (0.1 μM). A sequential senktide CRC (10 nM–1 μM: a selective NK_3-receptor agonist) was performed both in the absence of **6c** and was repeated following equilibration (30 min) with **6c** (10 μM).

Analysis of in vitro data
Where appropriate the dose ratio (DR; *ie* the concentration of the agonist required to produce 50% maximal response in the presence and absence of **6c**) was obtained and used to calculate the disassociation constant (K_a value) for **6c**,using the following equation:

$$K_a = \frac{\text{Concentration of } \mathbf{6c} \text{ (10 M)}}{\text{(DR-1)}}$$

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