Full Paper

Synthesis and Receptor Binding of New Thieno[2,3-*d*]-pyrimidines as Selective Ligands of 5-HT$_3$ Receptors

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With the aim to develop new potent and selective ligands of 5-HT$_3$-type serotonin receptors and to acquire more information on their structure-affinity relationships, new thieno[2,3-*d*]pyrimidine derivatives **32**–**39** were synthesized and their binding to 5-HT$_3$ versus 5-HT$_4$ receptors was studied. Some of these new compounds exhibit good affinity for cortical 5-HT$_3$ receptors, but not for 5-HT$_4$ receptors. Among these derivatives, 6-ethyl-4-(4-methyl-1-piperazinyl)-2-(methylthio)-thieno[2,3-*d*]pyrimidine **32** is the most potent ligand (K_i = 67 nM); it behaves as a competitive antagonist of the 5-HT$_3$ receptor function in the guinea pig colon. Its binding interactions with 5-HT$_{3A}$ receptors were analysed by using receptor modelling and comparative docking.

Keywords: Antagonists / 5-HT$_3$ Receptors / Receptor docking / Thieno[2,3-*d*]pyrimidines

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Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic monoamine with a variety of functions in the peripheral and central nervous systems (CNS). It is involved in many physiological and pathophysiological processes, such as depression, anxiety, sleep, the circadian rhythm, aggression, feeding and sexual behaviour, schizophrenia, bulimia, anorexia and asthma [1].

The various biological effects of 5-HT are mediated through different 5-HT receptors and their signal transduction pathways. There are seven classes of serotonin receptors (5-HT$_1$R to 5-HT$_7$R) and some of them are further divided into several subtypes [2]. They are coupled to G proteins, except for the 5-HT type-3 receptor (5-HT$_3$R), which is a member of the cysteine (Cys)-loop family of ligand-gated ion channels as well as the nicotinic acetylcholine (nACh), A-type γ-aminobutyric acid (GABA$_A$) and glycine (Gly) receptors [2]. Their subunits form pentamers encircling an ion channel. Most 5-HT$_3$Rs are composed of subunits A and B [3]. Recently, 5-HT$_{3C}$, 5-HT$_{3D}$ and 5-HT$_{3E}$ subunits were cloned from human samples, but their functional roles are not yet known [4, 5]. 5-HT$_3$Rs can be presynaptic and postsynaptic, and are located both in the CNS and peripherally [2]. In the CNS, 5-HT$_3$Rs have been localized in the area postrema, nucleus tractus solitarii, nucleus caudatus, nucleus accumbens, amygdala, hippocampus, entorhinal, frontal and cingulate cortex, and the dorsal horn ganglia. In the periphery, they are found in autonomic neurons and in neurons of the sensory and enteric nervous systems, where they are involved in emesis, nociception and gut motility [6]. The 5-HT$_3$Rs modulate the release of neurotransmitters and neuropeptides such as dopamine, cholecystokinin, acetylcholine, GABA, substance P and 5-HT itself [6].

The 5-HT$_3$Rs have gained considerable attention because of the clinical use of 5-HT$_3$R antagonists (5-HT$_3$RAs) such as ondansetron, granisetron and tropisetron

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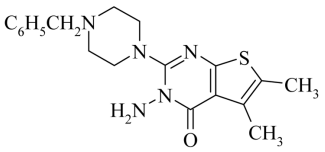
in the treatment of chemotherapy- and radiotherapy-induced nausea and vomiting and also in post-operative nausea and vomiting [7]. Moreover, a number of preclinical studies suggest that 5-HT_3RAs can be used in the treatment of various CNS disorders, such as anxiety, depression, schizophrenia, drug and alcohol abuse, chronic fatigue, withdrawal and age-associated memory impairments, pain (fibromyalgia and migraine) [6], and gastroenteric motility disorders such as diarrhoea accompanying the irritable bowel syndrome [8]. Recent data suggest that 5-HT_3RAs are also effective in the treatment of rheumatic diseases such as rheumatoid arthritis, tendinopathies, periarthropathies and myofascial pain [9].

The therapeutic potential of 5-HT_3 agonists is less well known; it was recently reported that they could be useful to treat or prevent neurodegenerative diseases such as ischaemic stroke, Alzheimer's disease, diabetic peripheral neuropathy, multiple sclerosis, amyotrophic lateral sclerosis, traumatic brain injury and spinal cord injury, Huntington's disease or Parkinson's disease [10].

In view of the wide-ranging involvement of 5-HT_3Rs in various physiological and pathological processes, a better understanding of the full therapeutic potential of their agonists and antagonists appears to be necessary.

For some years, we have been engaged in the development of subtype-selective ligands of 5-HTRs [11–15]. Considering the various therapeutic implications of 5-HT_3R ligands, we have recently focused our interest on developing novel 5-HT_3R ligands [16, 17]. As suggested by the literature [18], the three key pharmacophoric elements (an aromatic/heteroaromatic ring, a hydrogen-bond acceptor and a basic amino group) required for the interaction with 5-HT_3Rs, were taken into account for the synthesis of these compounds. They contain as "scaffold" a thienopyrimidine system selected for its broad range of biological activities [19], and differ particularly in the position of the piperazine ring. In one series of compounds, it is linked to the 2-position of the pyrimidine ring; among these derivatives, the most potent compound **1** (Fig. 1) behaves as an agonist in the Bezold–Jarisch reflex assay [16]. In a second series (compounds **2–6**), it is linked to the 4-position of the pyrimidine nucleus; among them, the most potent compound **2** (Fig. 1, R_1, R_2 = $-(CH_2)_3-$, R_3 = CH_3) behaves as a non-competitive antagonist in the isolated guinea-pig colon test [17]. The present work focuses on the acquisition of more information on the structure-affinity relationships of these latter derivatives. A new series of compounds **32–39** [20, 21] was synthesized (Fig. 1), which maintain the thieno[2,3-*d*]pyrimidine backbone and the piperazine ring linked to the 4-position of the pyrimidine nucleus, while the substituents at the 5- and 6-positions of the thieno[2,3-*d*]pyrimidine system were modified. In particular, alkyl moieties such as ethyl, *n*-propyl and *n*-butyl were attached to the 6-position, or a bulky tetrahydropyridine-substituted system was condensed at the 5- and 6-positions. This latter substitution has been done assuming a possible additional interaction between the lipophilic tetrahydropyridine-substituted system and the receptor counterpart which could be able to tolerate bulkier ligands.



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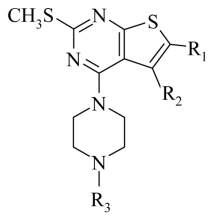


2-6, 32-39

Figure 1. Structures of published and new thieno[2,3-*d*]pyrimidines.

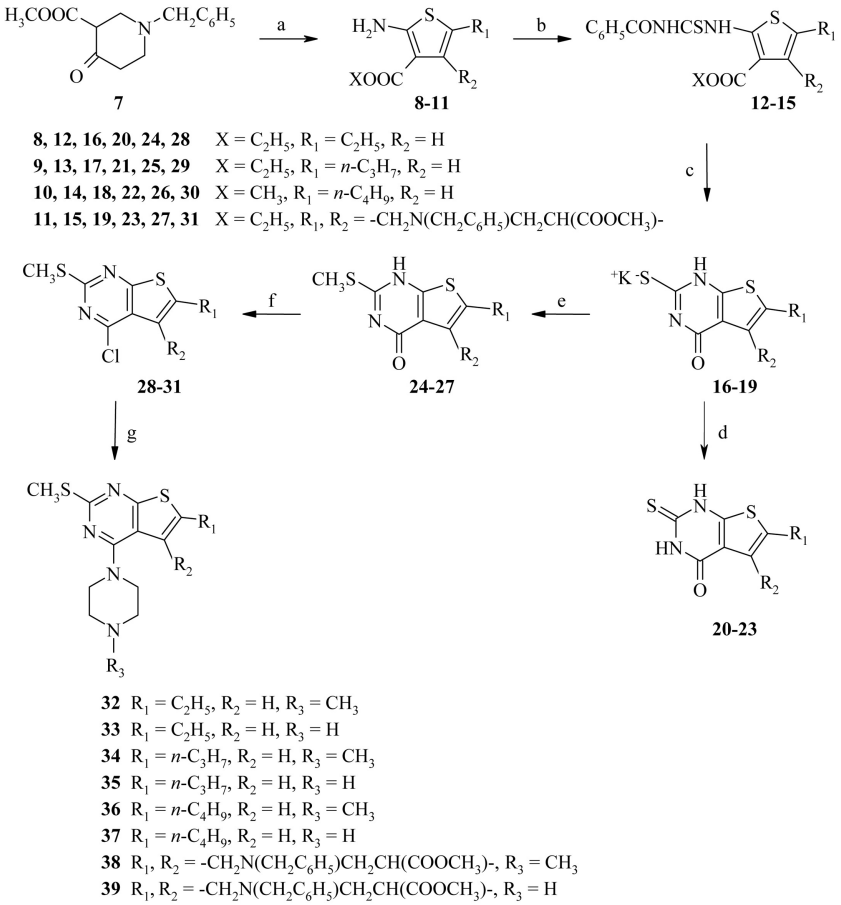
In some of the new compounds, a methyl group linked to N4 of the piperazine ring was inserted on the basis of well known literature data [16, 17, 22].

Moreover, the binding interactions of 5-HT_3R-selective ligands **2** and **32** were analysed via receptor modelling and comparative docking.

Chemistry

The synthetic procedure adopted for the preparation of thieno[2,3-*d*]pyrimidines **32–39** is depicted in Scheme 1. Aminoesters **8–10** were prepared as described [23–25], the unknown aminoester **11** was prepared from 4-oxo-1-(phenylmethyl)-3-piperidinecarboxylic acid methyl ester **7**, ethyl cyanoacetate, and sulphur following the procedure of Gewald *et al*. [23].

Derivatives **12–15** were obtained by refluxing the corresponding β-amino esters of 4,5-disubstituted-thiophenes **8–11** in acetone with benzoyl isothiocyanate, commercially available or prepared *in situ*. Successively, derivatives **12–15** were heated under reflux in ethanolic KOH solution to give the corresponding monopotassium salts of the 5,6-disubstituted-2-thioxothieno[2,3-*d*]pyrimi-



Reagents and conditions: (a) $CNCH_2COOC_2H_5$, sulphur, EtOH, 50°C, then diethylamine, 60°C, 5 h; (b) $SCNCOC_6H_5$, acetone, reflux, 2 h; (c) KOH, absolute EtOH, reflux, 3 h; (d) HCl, H_2O, rt, 30 min; (e) CH_3I, H_2O, rt, 1.5 h; (f) $POCl_3$, 150°C, 35 min or 2.5 h; (g) 1-methylpiperazine or piperazine, absolute EtOH, reflux, 1 or 3 h.

Scheme 1. Synthesis route of presented compounds **7**–**39**.

dinones **16**–**19**. Acidification with concentrated HCl of aqueous solutions of monopotassium salts **16**–**19** gave the corresponding thioxo compounds **20**–**23**, which were useful to confirm the structures of salts **16**–**19**. Reaction of the potassium salts **16**–**19** with CH_3I in water at room temperature furnished the 2-methylthio derivatives **24**–**27**, which were then converted into 4-chloro derivatives **28**–**31** by heating with an excess of $POCl_3$. 4-(1-Piperazinyl)thieno[2,3-*d*]pyrimidine derivatives **32**–**39** were finally synthesised by refluxing the 4-chloro derivatives **28**–**31** with piperazine or 1-methylpiperazine in ethanol. The proposed structures for compounds **8**–**39** were confirmed via elemental analyses and the IR and ^{1}H-NMR spectra (see the Experimental, Section 5).

Results and discussion

Pharmacology

Compounds **32**–**39** were tested in binding assays *in vitro* to determine their displacing potencies on [^{3}H]LY 278584 and [^{3}H]GR 113808 binding to 5-HT_3Rs and 5-HT_4 receptors (5-HT_4Rs), respectively, using the rat cortex for 5-HT_3Rs and the guinea pig striatum for 5-HT_4Rs. 5-HT and tropisetron were used as reference substances. The binding data reported in Table 1 are expressed as K_i values.

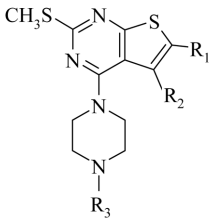
Compound **32**, which displayed the best affinity, was additionally tested in a functional assay *in vitro*, using the isolated guinea pig distal colon, in order to evaluate its putative agonistic or antagonistic properties (Fig. 2 and Table 2) [26].

Structure-affinity relationships of receptor binding

The results of the binding tests, presented in Table 1, demonstrate that compounds **32**–**39** (except for **38**) exhibit appreciable affinities for 5-HT_3Rs and high selectivity relative to the 5-HT_4Rs, since none of the new derivatives display significant affinity for the latter.

The new thienopyrimidines **32**–**39** were synthesized to explore how the affinity for 5-HT_3Rs is affected by alkyl substituents at the 6-position of the thienopyrimidine

Table 1. Chemical structures of new and published thieno[2,3-*d*]pyrimidines and their binding affinities to 5-HT_3Rs.



Compd.				K_i[a] (nM) (± SD)	
	R_1	R_2	R_3	5-HT_3	5-HT_4
2[b]	$-(CH_2)_3-$		CH_3	33 ± 5	na[c]
3[b]	$-(CH_2)_3-$		H	70 ± 7	na[c]
4[b]	$-(CH_2)_3-$		4-$OCH_3C_6H_4$	na[c]	na[c]
5[b]	$-CH_2CH_2CH_2CH(COOC_2H_5)-$		CH_3	1791 ± 187	na[c]
6[b]	$-CH_2CH_2CH_2CH(COOC_2H_5)-$		H	na[c]	na[c]
32	C_2H_5	H	CH_3	67 ± 7	na[c]
33	C_2H_5	H	H	124 ± 14	na[c]
34	*n*-C_3H_7	H	CH_3	298 ± 28	na[c]
35	*n*-C_3H_7	H	H	572 ± 86	na[c]
36	*n*-C_4H_9	H	CH_3	644 ± 44	na[c]
37	*n*-C_4H_9	H	H	1060 ± 104	na[c]
38	$-CH_2N(CH_2C_6H_5)CH_2CH(COOCH_3)-$		CH_3	na[c]	na[c]
39	$-CH_2N(CH_2C_6H_5)CH_2CH(COOCH_3)-$		H	3679 ± 327	na[c]
	5-HT			354 ± 97	79 ± 10
	tropisetron			6.58 ± 0.72	4357 ± 653

[a] Each value is the mean ± SD of the data from three separate experiments.
[b] These data are included for the sake of clarity [16, 17].
[c] Not active, percentage of inhibition of specific binding <50% at 10^{-5} M.

Table 2. Potencies (EC_{50}) and maximum effects (E_{max}) of 2-Me-5-HT in the presence of 10 nM tropisetron (trop) and compound **32** in increasing the contractions of guinea pig colon *in vitro*.

	EC_{50} (M) ± SEM	E_{max} (%) ± SEM[a]
2-Me-5-HT	$9.1 \times 10^{-6} \pm 0.9 \times 10^{-6}$	65.4 ± 13.6
2-Me-5-HT + trop	$60.8 \times 10^{-6} \pm 14.1 \times 10^{-6}$[b]	66.1 ± 32.6[c]
2-Me-5-HT + **32**	$22.1 \times 10^{-6} \pm 7.2 \times 10^{-6}$[d]	63.2 ± 11.4[c]

The level of significance is indicated next to the values as compared with 2-Me-5-HT.
[a] SEM, standard error of the mean.
[b] $p < 0.01$.
[c] Not significant.
[d] $p < 0.05$.

system and by a bulky tetrahydropyridine-substituted system condensed to the thiophene nucleus.

All the alkyl derivatives **32**–**37** possess noteworthy displacing potencies on 5-HT_3R binding, with K_i ranging from 67 nM to 1060 nM. Among them, 6-ethyl-4-(4-methyl-1-piperazinyl)-2-(methylthio)thieno[2,3-*d*]pyrimidine **32** demonstrated the highest affinity (K_i = 67 nM). As a general trend, the binding affinity of compounds **32**–**37** gradually decreases with the length of the alkyl substituent at the 6-position, from ethyl, **32** and **33**, to *n*-propyl, **34** and **35**, and to *n*-butyl derivatives **36** and **37** (K_i = 67, 124, 298, 572, 644 and 1060 nM, respectively). The ethyl moiety seems to fit better into the receptor binding pocket than the more bulky *n*-propyl and *n*-butyl groups. Condensation of a tetrahydropyridine-substituted ring at the 5- and 6-positions of the thieno[2,3-*d*]pyrimidine system leads to compounds exhibiting a complete loss, *e.g.* **38**, or a considerable decrease in affinity, **39** with K_i = 3679 nM. With regard to the substitution on the piperazine nucleus, the trend was that the displacing potencies of 4-methylpiperazine derivatives **32**, **34** and **36** were about twice as high (K_i = 67, 298 and 644 nM, respectively) as those of the corresponding unsubstituted piperazine derivatives **33**, **35** and **37** (K_i = 124, 572 and 1060 nM, respectively). The 4-methyl group might interact with a hydrophobic accessory site. Regarding the substitution on the piperazine ring, the new series of compounds display a general trend similar to that reported for derivatives **2** and **3**, where the 4-methyl derivative **2** is about twice as potent as the unsubstituted **3**.

Although tetrahydropyridine derivatives **38** and **39** are the least potent ligands in the series, the unsubstituted

piperazine derivative **39** still revealed a measurable affinity for 5-HT$_3$Rs in the micromolar range (K_i = 3679 nM), whereas the methylpiperazine derivative **38** had none. This finding is not in accordance with the trend shown by this series of derivatives (**32** – **37**) and with that of compounds **2** and **3**.

Moreover, the affinity displayed by compound **39** indicates that although it contains a bulky phenylmethyl substituent linked at the 7-position of the tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidine system, it can still interact with the receptor binding site. Comparison of the binding properties of compounds **32** and **2** show that **32** has a slightly lower affinity than that of **2** (Table 1). Thus, a 5-membered unsubstituted ring fused to the thieno[2,3-*d*]pyrimidine system, or an ethyl moiety linked to the 6-position of the same nucleus, appears to be optimal for strong interactions with the 5-HT$_3$R binding site.

Functional properties

Compound **32**, the new derivative with the highest affinity for 5-HT$_3$Rs, was also tested to evaluate its functional properties *in vitro* concerning the increase of the contractions of the isolated guinea pig colon. Tropisetron, a well-known 5-HT$_3$R antagonist, was used as a reference compound. In the isolated guinea pig colon, 10 nM tropisetron shifted the dose-response curve of the selective 5-HT$_3$R agonist 2-methyl-5-hydroxytryptamine (2-Me-5-HT) to the right, suggesting competitive antagonism (Fig. 2, Table 2). Compound **32** (10 nM) affected the 2-Me-5-HT-induced contractions similarly to tropisetron, but the right shift of the curve turned out to be smaller (Fig. 2, Table 2). Nevertheless, both compounds shifted the curve of 2-Me-5-HT to the right, but because of the high maximum concentration of 2-Me-5-HT alone (10^{-4} M), the maximum values in the presence of the compounds were impossible to measure in the isolated organ bath studies. For this reason, the maximum and the EC_{50} values were predicted via computer program. These findings can be reconciled with compound **32** being a competitive antagonist of 5-HT$_3$Rs, but a less potent one than tropisetron.

5-HT$_{3A}$R modelling

Receptor modelling was used to localize the binding sites of selective ligands **2** and **32** on 5-HT$_3$Rs. This was based on the building of a homology model of 3A – type 5-HT receptors (5-HT$_{3A}$Rs), obtained from the electron microscopic structure of the nACh receptor with agonist-free binding cavities at 4 Å resolution [27]. This model, used for the comparative docking of potent versus inactive ligands, could elucidate possible binding interactions of compound **2** with 5-HT$_{3A}$Rs.

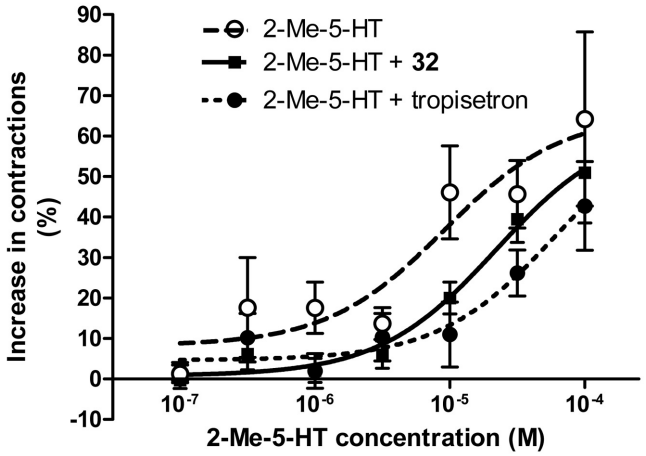


Figure 2. The effects of 10 nM tropisetron (●) and compound **32** (■) in increasing the contractions elicited by a selective 5-HT$_3$R agonist, 2-Me-5-HT (○), in the isolated guinea pig colon *in vitro*. The effects of 2-Me-5-HT were expressed as the percentage increases in contractions as compared with the basal colon activity (n = 6).

The homology model of 5-HT$_{3A}$Rs based on the structure of nACh receptors with an agonist-free (empty) binding cavity [27] is an improvement compared to previous models involving an acetylcholine-binding protein [28] where, according to an emerging consensus, bound agonists "pull in" loop C to close the binding cavity. In contrast, the open binding cavity can accommodate 5-HT$_3$RAs in more docking positions [27]. Consequently, the docking of **2** to 5-HT$_{3A}$Rs resulted in three predominant positions in the interface of the subunits under loop C (Fig. 3) in the binding cavity where 5-HT$_3$RAs [28] and agonists [29] can be docked and bound. How can we decide which docking position is relevant in binding? Comparative docking of active, **2** and **32**, versus less active and inactive, **4**, **5**, **6** and **38** analogues in displacement of the [^{3}H]LY 278584 and [^{3}H]zacopride bindings to the 5-HT$_3$Rs of the rat cortex were used to distinguish probable binding positions from docking artefacts. Overlapping docking positions of active and inactive derivatives were considered artefacts, while the similar dockings of the active ones support similar binding modes associated with high affinity. The light grey structure of compound **2** (next to 1. in Fig. 3) with best energy was in juxtaposition with the best docking of compound **32** (not shown for clarity), and it represents the most probable binding mode with its aromatic rings close to tyrosines Y143 and Y153 and the piperazine ring close to asparagines N128 and Y234. The two other dockings (medium grey (2.) and dark grey (3.) in Fig. 3) were in juxtaposition with the identical parts of the best docking positions of the less active and inactive compounds **4**, **5**, **6** and **38**, and

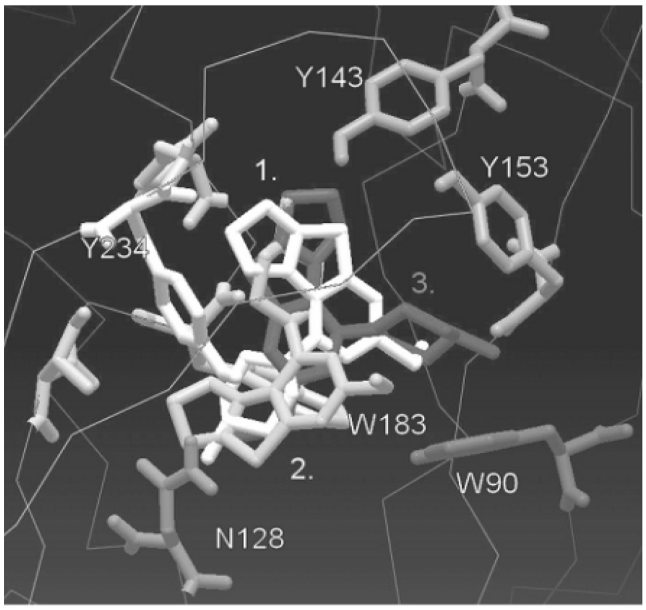


Figure 3. Three major docking positions of compound **2** with best energies and highest docking frequencies in the interface of two subunits of agonist-free 5-HT_{3A}Rs under loop C. The numbers (1. light grey, 2. medium grey, 3. dark grey) show the rank order of docking energies and frequencies. Amino acids which are crucial in the binding of granisetron to 5-HT_{3A}Rs are indicated [27].

they were therefore considered artefacts. The docking vicinity of the distal piperazinyl tertiary amino group of compounds **2** and **32** to N128 is in agreement with the docking vicinity of the analogous granatane amino group of granisetron, a 5-HT_3R antagonist (K_i = 0.20 nM) [30], to N128 [27]. Moreover, mutations of the homologous asparagine (N102 in a_1 Gly receptors) support the vicinity of N102 to the tropanic amino group of tropisetron in Gly receptor antagonism [31]. This asparagine residue is conserved in (Cys)-loop receptors and it can be associated with the requirement of the basic tertiary amino groups in the structure-affinity relationships for antagonist binding to 5-HT_3Rs.

On the basis of the best docking position of **2** in Fig. 3, the structure-affinity relationships observed for 5-HT_3R binding of compounds **2**–**6**, **32**–**39** could be explained. The R_3 groups being larger than methyl do not fit well in the binding cavity because of possible steric clashes around N128 and Y234. Small R_1 alkyl groups or a propyl chain bridging 5- and 6-positions are also tolerated, while large groups in these positions are probably sterically hindered by the wall of the cavity (Y234 in Fig. 3).

Further, docking studies also suggest that amino acid residues W90, N128, Y143, Y153, W183 and Y234 conserved in 5-HT_{3A}Rs play key roles in binding compound **2** (Fig. 3). Point mutations of these residues deteriorated the binding affinity of [^{3}H]granisetron for recombinant 5-HT_{3A}Rs [27]. This supports the view that compounds **2**, **32** and granisetron bind in the same binding cavity of 5-HT_{3A}Rs.

Receptor modelling and binding studies were performed with homomeric 5-HT_{3A}Rs and in the brain, respectively, while the functional test was carried out in the guinea pig colon probably containing peripheral-type heteromeric 5-HT_{3AB}Rs too. In fact, recent studies suggest that mainly homomeric 5-HT_{3A}Rs are present in the CNS, whereas both heteromeric 5-HT_{3AB}Rs and homomeric 5-HT_{3A}Rs exist in the periphery [32].

Consequently, receptor modelling can be correlated with binding data to 5-HT_3Rs in brain rather than with the peripheral functional assay.

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The authors have declared no conflict of interest.

Conclusion

In conclusion, a new series of thieno[2,3-*d*]pyrimidine derivatives has been prepared as ligands for 5-HT_3Rs. Some of the new compounds display good affinity for 5-HT_3Rs but not for 5-HT_4Rs. Among these derivatives, 6-ethyl-4-(4-methyl-1-piperazinyl)-2-(methylthio)thieno[2,3-*d*]pyrimidine **32** possesses the highest affinity and in an *in vitro* functional test behaves as a competitive antagonist, like tropisetron. Receptor modelling and comparative docking support the conclusion that compounds **2** and **32** and the reference antagonist granisetron bind to 5-HT_{3A}Rs in the same binding cavity. The modelling results rationalise the structure-affinity data of receptor binding with the surrounding amino acid residues.

Experimental

General methods

Melting points were determined in glass capillary tubes on a Gallenkamp apparatus with an MFB-595 digital thermometer (Weiss-Gallenkamp, London, UK) and are uncorrected. Elemental analyses for C, H, N and S were performed on a Fisons-Carlo Erba EA1108 Elemental Analyzer (Carlo Erba, Milan, Italy) and were within ± 0.4% of the theoretical values. Infrared spectra were recorded in KBr disks on a Perkin Elmer FT-IR 1600 spectrometer (Perkin-Elmer, Norwalk, CT, USA). ^{1}H-NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ solution at 200 MHz on a Varian Inova-Unity

200 spectrometer (Varian Inc., Palo Alto, CA, USA); chemical shifts are given in δ values (ppm), relative to tetramethylsilane as the internal standard; coupling constants (*J*) are given in Hz. Signal multiplicities are characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br s (broad singlet). The purities of all the synthesized compounds were checked by TLC on an aluminium sheet coated with silica gel 60 F_{254} (Merck, Germany) and visualized by UV (λ = 254 and 366 nm). All commercial chemicals and solvents were of reagent grade and were purchased from commercial vendors.

Chemistry

*2-Amino-6-(phenylmethyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3,4-dicarboxylic acid 3-ethyl 4-methyl diester **11***

To a mixture of 4-oxo-1-(phenylmethyl)-3-piperidinecarboxylic acid methyl ester **7** (16.80 g, 68.00 mmol), ethyl cyanoacetate (7.70 g, 68.00 mmol) and sulphur (2.20 g, 68.75 mmol) in ethanol (14 mL), stirred on an oil bath at 50°C, diethylamine (7.00 mL) was added slowly and the mixture was stirred at 60°C for 5 h. After the mixture had been cooled, the precipitate was filtered off, washed with cold ethanol, dried and recrystallised from ethanol to give **11** as a pure solid. Yield: 14.76 g (58%); mp. 193 – 194°C; IR (KBr, selected lines) cm^{-1} 3386, 3289, 1723, 1665, 1580, 1495, 1361, 1278, 1203, 1169. ^{1}H-NMR ($CDCl_3$) δ 1.25 (t, *J* = 7.0 Hz, 3H, CH_2CH_3), 2.75 (dd, ^2J = 11.8 Hz, ^3J = 4.9 Hz, 1H, $CH_AH_BCHCOOCH_3$), 3.15 (dd, ^2J = 11.8 Hz, ^3J = 3.6 Hz, 1H, $CH_AH_BCHCOOCH_3$), 3.30 (d, *J* = 14.6 Hz, 1H, $PhCH_2NCH_CH_D$), 3.60 (d, *J* = 13.1 Hz, 1H, $PhCH_AH_BNCH_2$), 3.64 – 3.68 (m, 1H + 3H, $PhCH_2NCH_CH_D$, $COOCH_3$), 3.73 (d, *J* = 13.1 Hz, 1H, $PhCH_AH_BNCH_2$), 3.90 – 3.95 (m, 1H, $CHCOOCH_3$), 4.19 (q, *J* = 7.0 Hz, 2H, CH_2CH_3), 5.95 (s, 2H, NH_2), 7.24 – 7.36 (m, 5H, aromatic). Anal. ($C_{19}H_{22}N_2O_4S$) C, H, N, S.

*2-[[(Benzoylamino)thioxomethyl]amino]-5-ethyl-3-thiophenecarboxylic acid ethyl ester **12***

Benzoyl chloride (4.08 mL, 35.13 mmol) was added under stirring to a solution of NH_4NCS (3.11 g, 40.86 mmol) in anhydrous acetone (28 mL). The mixture was heated at reflux under stirring for 5 min. A solution of the ethyl ester **8** (7.00 g, 35.13 mmol) in anhydrous acetone (70 mL) was then added and the mixture was stirred under reflux for 2 h. After the mixture had been cooled, a small amount of solvent was evaporated off under reduced pressure, and the solid obtained was collected, washed with water, dried and recrystallised from ethanol to give **12** as a pure solid. Yield: 11.40 g (89%); mp. 148 – 151°C; IR (KBr, selected lines) cm^{-1} 3280, 2970, 1691, 1563, 1527, 1464, 1232, 1193, 1152, 675. ^{1}H-NMR (DMSO-d_6) δ 1.24 (t, *J* = 7.6 Hz, 3H, CH_2CH_3), 1.33 (t, *J* = 7.0 Hz, 3H, CH_2CH_3), 2.75 (q, *J* = 7.6 Hz, 2H, CH_2CH_3), 4.35 (q, *J* = 7.0 Hz, 2H, CH_2CH_3), 7.05 (s, 1H, aromatic), 7.50 – 8.08 (m, 5H, aromatic), 11.92 (s, 1H, NH), 14.74 (s, 1H, NH). Anal. ($C_{17}H_{18}N_2O_3S_2$) C, H, N, S.

*2-[[(Benzoylamino)thioxomethyl]amino]-5-propyl-3-thiophenecarboxylic acid ethyl ester **13***

This compound was prepared from amino ester **9** by the same procedure as described for **12**, and was recrystallised from ethanol. Yield: 8.73 g (66%); mp. 148 – 150°C; IR (KBr, selected lines) cm^{-1} 3304, 2956, 1680, 1552, 1525, 1449, 1316, 1223, 1163, 1082. ^{1}H-NMR (DMSO-d_6) δ 0.93 (t, *J* = 7.2 Hz, 3H, $CH_2CH_2CH_3$), 1.33 (t, *J* = 7.0 Hz, 3H, CH_2CH_3), 1.57 – 1.70 (m, 2H, $CH_2CH_2CH_3$), 2.71 (t, *J* = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 4.36 (q, *J* = 7.2 Hz, 2H, CH_2CH_3), 7.05 (s, 1H, aromatic), 7.48-8.08 (m, 5H, aromatic), 11.93 (s, 1H, NH), 14.75 (s 1H, NH). Anal. ($C_{18}H_{20}N_2O_3S_2$) C, H, N, S.

*2-[[(Benzoylamino)thioxomethyl]amino]-5-butyl-3-thiophenecarboxylic acid methyl ester **14***

This compound was prepared from amino ester **10** by the same procedure as described for **12**, and was recrystallised from ethanol. Yield: 10.97 g (80%); mp. 150 – 152°C; IR (KBr, selected lines) cm^{-1} 3334, 2919, 1694, 1666, 1555, 1523, 1490, 1458, 1225, 1161. ^{1}H-NMR (DMSO-d_6) δ 0.91 (t, *J* = 7.2 Hz, 3H, $CH_2CH_2CH_2CH_3$), 1.22 – 1.42 (m, 2H, $CH_2CH_2CH_2CH_3$), 1.52 – 1.68 (m, 2H, $CH_2CH_2CH_2CH_3$), 2.73 (t, *J* = 7.2 Hz, 2H, $CH_2CH_2CH_2CH_3$), 3.88 (s, 3H, $COOCH_3$), 7.05 (s, 1H, aromatic), 7.50 – 8.10 (m, 5H, aromatic), 11.94 (s, 1H, NH), 14.75 (s, 1H, NH). Anal. ($C_{18}H_{20}N_2O_3S_2$) C, H, N, S.

*2-[[(Benzoylamino)thioxomethyl]amino]-6-(phenylmethyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3,4-dicarboxylic acid 3-ethyl 4-methyl diester **15***

This compound was prepared from amino ester **11** by the same procedure as described for **12**, with slight variations: the refluxing time was 3 h and, after cooling, the solvent was removed under reduced pressure and the resulting solid was collected, washed with water, dried and recrystallised from ethyl acetate. Yield: 15.49 g (82%); mp. 172 – 174°C; IR (KBr, selected lines) cm^{-1} 3251, 1718, 1709, 1673, 1534, 1423, 1323, 1246, 1181, 707. ^{1}H-NMR (DMSO-d_6) δ 1.22 (t, *J* = 7.0 Hz, 3H, CH_2CH_3), 2.60-2.70 (m, 1H, $CH_AH_BCHCOOCH_3$), 3.00-3.10 (m, 1H, $CH_AH_BCHCOOCH_3$), 3.46 (d, *J* = 15.1 Hz, 1H, $PhCH_2NCH_CH_D$), 3.54 – 3.56 (m, 1H + 3H, $PhCH_2NCH_CH_D$, $COOCH_3$), 3.72-3.90 (m, 2H, $PhCH_2N$), 3.97 – 4.07 (m, 1H, $CHCOOCH_3$), 4.28 (q, *J* = 7.0 Hz, 2H, CH_2CH_3), 7.23 – 8.11 (m, 10H, aromatic), 11.90 (s, 1H, NH), 14.77 (s, 1H, NH). Anal. ($C_{27}H_{27}N_3O_5S_2$) C, H, N, S.

*6-Ethyl-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one **20***

Benzoyl derivative **12** (10.28 g, 28.36 mmol) was added to a solution of KOH (3.18 g, 56.68 mmol) in absolute ethanol (62 mL) and the mixture was refluxed under stirring for 3 h. After the mixture had been cooled, a small amount of solvent was evaporated under reduced pressure, and the solid obtained was collected, washed with absolute ethanol and dried to give salt **16** (2.03 g, 28%). A suspension in water (10 mL) of potassium salt **16** (0.40 g, 1.60 mmol) was acidified with concentrated HCl and stirred for 30 min at room temperature. The solid obtained was collected, washed with water, dried and recrystallised from ethanol to give **20** as a pure solid. Yield: 0.12 g (35%); mp. 280 – 282°C; IR (KBr, selected lines) cm^{-1} 3046, 2920, 1668, 1566, 1535, 1466, 1264, 1188, 1127, 842. ^{1}H-NMR (DMSO-d_6) δ 1.21 (t, *J* = 7.6 Hz, 3H, CH_3), 2.76 (q, *J* = 7.6 Hz, 2H, CH_2), 6.93 (s, 1H, aromatic), 12.41 (s, 1H, NH), 13.78 (s, 1H, NH). Anal. ($C_8H_8N_2OS_2$) C, H, N, S.

*6-Propyl-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one **21***

Benzoyl derivative **13** (4.00 g, 10.62 mmol) was added to a solution of KOH (1.19 g, 21.20 mmol) in absolute ethanol (43 mL) and the mixture was refluxed under stirring for 4 h. After the mixture had been cooled, the solid was filtered off, washed with absolute ethanol and dried to obtain salt **17** (2.60 g, 92%). Potassium salt **17** (1.00 g, 3.78 mmol) was suspended in water (50 mL) and the suspension was then acidified with concentrated HCl

and stirred for 30 min at room temperature. The solid obtained was collected, washed with water, dried and recrystallised from ethanol to give **21** as a pure solid. Yield: 0.38 g (44%); mp. 248–250°C; IR (KBr, selected lines) cm^{-1} 2827, 1677, 1580, 1548, 1265, 1241, 1185, 1128, 886, 752. ^{1}H-NMR (DMSO-d_6) δ 0.90 (t, J = 7.4 Hz, 3H, CH_3), 1.52-1.70 (m, 2H, $CH_2CH_2CH_3$), 2.71 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_3$), 6.93 (s, 1H, aromatic), 12.40 (s, 1H, NH), 13.38 (s, 1H, NH). Anal. ($C_9H_{10}N_2OS_2$) C, H, N, S.

6-Butyl-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one ***22***

This compound was prepared from benzoyl derivative **14** by the same procedure as described for **17**. Potassium salt **18**: yield: 6.79 g (86%). After washing, compound **22** was recrystallised from ethanol. Yield: 0.22 g (58%); mp. 198–200°C; IR (KBr, selected lines) cm^{-1} 3049, 2865, 1678, 1580, 1547, 1268, 1188, 1124, 894, 750. ^{1}H-NMR (DMSO-d_6) δ 0.88 (t, J = 7.2 Hz, 3H, CH_3), 1.21–1.42 (m, 2H, $CH_2CH_2CH_2CH_3$), 1.42-1.65 (m, 2H, $CH_2CH_2CH_2CH_3$), 2.71 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_2CH_3$), 6.93 (s, 1H, aromatic), 12.40 (s, 1H, NH), 13.39 (s, 1H, NH). Anal. ($C_{10}H_{12}N_2OS_2$) C, H, N, S.

7-(Phenylmethyl)-4-oxo-2-thioxo-1,2,3,4,5,6,7,8-octahydropyrido[4′,3′:4,5]thieno[2,3-d]pyrimidine-5-carboxylic acid methyl ester ***23***

Benzoyl derivative **15** (3.50 g, 6.51 mmol) was added to a solution of KOH (0.73 g, 13.03 mmol) in absolute ethanol (30 mL) and the mixture was refluxed under stirring for 3 h. After the mixture had been cooled, the solvent was removed under reduced pressure and the solid obtained was collected, washed with a small amount of absolute ethanol and dried to give salt **19** (2.60 g, 93%). Potassium salt **19** (0.08 g, 0.18 mmol) was poured into water (10 mL) and the solution was acidified with concentrated HCl and stirred for 10 min at room temperature. The mixture was then extracted with ethyl acetate. The organic layer was collected, dried with anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude solid obtained was collected, washed with diethyl ether, dried and recrystallised from ethanol to give **23** as a pure solid. Yield: 0.02 g (26%); mp. 174–178°C; IR (KBr, selected lines) cm^{-1} 3431, 1693, 1546, 1455, 1368, 1200, 1144, 1027, 752. ^{1}H-NMR (DMSO-d_6) δ 2.60–2.71 (m, 1H, $CH_AH_BCHCOOCH_3$), 3.10–3.30 (m, 1H, $CH_AH_BCHCOOCH_3$), 3.46 (d, J = 15.1 Hz, 1H, $PhCH_2NCH_CH_D$), 3.54–3.56 (m, 1H + 3H, $PhCH_2NCH_CH_D$, $COOCH_3$), 3.82–4.35 (m, 1H + 2H, $CHCOOCH_3$, $PhCH_2N$), 7.20–7.57 (m, 5H, aromatic), 12.45 (s, 1H, NH), 13.43 (s, 1H, NH). Anal. ($C_{18}H_{17}N_3O_3S_2$) C, H, N, S.

6-Ethyl-2-(methylthio)thieno[2,3-d]pyrimidin-4(1H)-one ***24***

CH_3I (1.52 mL, 24.41 mmol) was added to a suspension of monopotassium salt **16** (2.03 g, 8.11 mmol) in water (95 mL) and the mixture was stirred at room temperature for 1.5 h. The solid was then filtered off, washed with water, dried and recrystallised from ethanol to give **24** as a pure solid. Yield: 1.50 g (82%); mp. 205–207°C; IR (KBr, selected lines) cm^{-1} 3056, 2961, 1661, 1563, 1476, 1287, 1188, 1115, 879, 764. ^{1}H-NMR (DMSO-d_6) δ 1.26 (t, J = 7.4 Hz, 3H, CH_3), 2.53 (s, 3H, SCH_3), 2.82 (q, J = 7.4 Hz, 2H, CH_2), 7.01 (s, 1H, aromatic), 12.70 (s, 1H, NH). Anal. ($C_9H_{10}N_2OS_2$) C, H, N, S.

2-(Methylthio)-6-propylthieno[2,3-d]pyrimidin-4(1H)-one ***25***

This compound was prepared from salt **17** by the same procedure as described for **24**, and was recrystallised from ethanol. Yield: 1.73 g (89%); mp. 174–176°C; IR (KBr, selected lines) cm^{-1} 2958, 1652, 1550, 1481, 1426, 1287, 1190, 1147, 1110, 888. ^{1}H-NMR (DMSO-d_6) δ 0.93 (t, J = 7.2 Hz, 3H, CH_3), 1.57-1.76 (m, 2H, $CH_2CH_2CH_3$), 2.52 (s, 3H, SCH_3), 2.77 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 7.01 (s, 1H, aromatic), 12.71 (s, 1H, NH). Anal. ($C_{10}H_{12}N_2OS_2$) C, H, N, S.

6-Butyl-2-(methylthio)thieno[2,3-d]pyrimidin-4(1H)-one ***26***

This compound was prepared from salt **18** by the same procedure as described for **24**, and was recrystallised from ethanol. Yield: 1.05 g (51%); mp. 188–190°C; IR (KBr, selected lines) cm^{-1} 2927, 1652, 1537, 1283, 1185, 1139, 1080, 1012, 882, 760. ^{1}H-NMR (DMSO-d_6) δ 0.90 (t, J = 7.2 Hz, 3H, CH_3), 1.22-1.42 (m, 2H, $CH_2CH_2CH_2CH_3$), 1.50–1.68 (m, 2H, $CH_2CH_2CH_2CH_3$), 2.53 (s, 3H, SCH_3), 2.80 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_2CH_3$), 7.00 (s, 1H, aromatic), 12.70 (s, 1H, NH). Anal. ($C_{11}H_{14}N_2OS_2$) C, H, N, S.

2-(Methylthio)-7-(phenylmethyl)-4-oxo-1,4,5,6,7,8-hexahydropyrido[4′,3′:4,5]thieno[2,3-d]pyrimidine-5-carboxylic acid methyl ester ***27***

This compound was prepared from salt **19**, by the same procedure as described for **24**, and was recrystallised from ethyl acetate. Yield: 2.15 g (66%); mp. 216–218°C dec; IR (KBr, selected lines) cm^{-1} 3392, 1741, 1658, 1537, 1495, 1452, 1396, 1305, 1264, 1196. ^{1}H-NMR (DMSO-d_6) δ 2.45 (s, 3H, SCH_3), 2.74 (dd, ^2J = 11.6 Hz, ^3J = 5.0 Hz, 1H, $CH_AH_BCHCOOCH_3$), 2.99 (dd, ^2J = 11.6 Hz, ^3J = 3.5 Hz, 1H, $CH_AH_BCHCOOCH_3$), 3.47-3.51 (m, 1H + 3H, $PhCH_2NCH_CH_D$, $COOCH_3$) 3.60 (d, J = 13.6 Hz, 1H, $PhCH_2NCH_CH_D$), 3.75-4.02 (m, 1H + 2H, $CHCOOCH_3$, $PhCH_2N$), 7.23–7.44 (m, 5H, aromatic), 12.59 (br s, 1H, NH). Anal. ($C_{19}H_{19}N_3O_3S_2$) C, H, N, S.

4-Chloro-6-ethyl-2-(methylthio)thieno[2,3-d]pyrimidine ***28***

A mixture of 2-methylthio derivative **24** (1.20 g, 5.30 mmol) and $POCl_3$ (6 mL) was heated at 150°C and stirred for 35 min. After the mixture had been cooled, the solution was poured into cold water and neutralised with a 10% solution of NaOH. The solution was then extracted with chloroform. The organic layers were collected, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting crude oil (1.15 g, 96%) was used without further purification for the synthesis of compounds **32** and **33**.

4-Chloro-2-(methylthio)-6-propylthieno[2,3-d]pyrimidine ***29***

This compound was prepared from 2-methylthio derivative **25** by the same procedure as described for **28**, with slight variations: the solid, obtained after neutralization with a 10% solution of NaOH, was filtered, washed with water, dried and recrystallised from ethanol/cyclohexane to give **29** as a pure solid. Yield: 0.48 g (65%); mp. 82–83°C; IR (KBr, selected lines) cm^{-1} 2952, 1639, 1554, 1478, 1361, 1295, 1233, 1128, 875, 833. ^{1}H-NMR (DMSO-d_6) δ 0.95 (t, J = 7.2 Hz, 3H, CH_3), 1.60–1.80 (m, 2H, $CH_2CH_2CH_3$), 2.57 (s, 3H, SCH_3), 2.91 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 7.19 (s, 1H, aromatic). Anal. ($C_{10}H_{11}ClN_2S_2$) C, H, N, S.

*6-Butyl-4-chloro-2-(methylthio)thieno[2,3-d]pyrimidine **30***

This compound was prepared from 2-methylthio derivative **26** by the same procedure as described for **28**. The resulting crude oil (1.23 g, 85%) was used without further purification for the synthesis of compounds **36** and **37**.

*4-Chloro-2-(methylthio)-7-(phenylmethyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-d]pyrimidine-5-carboxylic acid methyl ester **31***

This compound was prepared from 2-methylthio derivative **27** by the same procedure as described for **28**, with slight variations: the refluxing time was 2.5 h and, the resulting solid was collected, washed with water, dried and recrystallised from ethyl acetate to obtain **31** as a pure solid. Yield: 0.42 g (81%); mp. 141 – 144°C; IR (KBr, selected lines) cm^{-1} 1738, 1545, 1476, 1411, 1337, 1271, 1153, 839, 747, 699. ^{1}H-NMR (DMSO-d_6) δ 2.60 (s, 3H, SCH_3), 2.72 (dd, ^2J = 12.1 Hz, ^3J = 4.5 Hz, 1H, $CH_AH_BCHCOOCH_3$), 3.25 – 3.40 (m, 1H, $CH_AH_BCHCOOCH_3$), 3.43 – 3.49 (m, 1H + 3H, $PhCH_2NCH_CH_D$, $COOCH_3$), 3.56 (d, J = 13.6 Hz, 1H, $PhCH_2NCH_CH_D$), 3.63 – 4.25 (m, 1H + 2H, $CHCOOCH_3$, $PhCH_2N$), 7.30-7.36 (m, 5H, aromatic). Anal. ($C_{19}H_{18}ClN_3O_2S_2$) C, H, N, S.

*6-Ethyl-4-(4-methyl-1-piperazinyl)-2-(methylthio)thieno[2,3-d]pyrimidine **32***

A mixture of 1-methylpiperazine (0.65 mL, 5.86 mmol) and 4-chloro derivative **28** (0.80 g, 3.27 mmol) was heated under reflux and stirred for 2.5 h in ethanol (10 mL). After the mixture had been cooled, the solid was eliminated and the solution was concentrated under reduced pressure. The resulting crude oil was purified by flash column chromatography with methanol as eluent. The homogeneous fractions were evaporated *in vacuo* to furnish **32** as pure oil. Yield: 0.85 g (84%); IR (KBr, selected lines) cm^{-1} 2929, 2792, 1549, 1511, 1444, 1350, 1298, 1142, 996, 881. ^{1}H-NMR (DMSO-d_6) δ 1.26 (t, J = 7.6 Hz, 3H, CH_2CH_3), 2.21 (s, 3H, NCH_3), 2.38 – 2.50 (m, 4H + 3H, piperazine, SCH_3), 2.85 (q, J = 7.6 Hz, 2H, CH_2CH_3), 3.75 – 3.85 (m, 4H, piperazine), 7.22 (s, 1H, aromatic). Anal. ($C_{14}H_{20}N_4S_2 \cdot H_2O$) C, H, N, S.

*4-(4-Methyl-1-piperazinyl)-2-(methylthio)-6-propylthieno[2,3-d]pyrimidine **34***

This compound was prepared from 4-chloro derivative **29** by the same procedure as described for **32**. The resulting crude oil was purified by flash column chromatography with methanol/ethyl acetate (3 : 7, v/v) as eluent. Yield: 0.63 g (60%); IR (KBr, selected lines) cm^{-1} 2927, 1552, 1512, 1440, 1348, 1279, 1142, 995, 880, 770. ^{1}H-NMR (DMSO-d_6) δ 0.94 (t, J = 7.2 Hz, 3H, $CH_2CH_2CH_3$), 1.55-1.76 (m, 2H, $CH_2CH_2CH_3$), 2.22 (s, 3H, NCH_3), 2.37 – 2.49 (m, 4H + 3H, piperazine, SCH_3), 2.81 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_3$), 3.77-3.83 (m, 4H, piperazine), 7.24 (s, 1H, aromatic). Anal. ($C_{15}H_{22}N_4S_2$) C, H, N, S.

*6-Butyl-4-(4-methyl-1-piperazinyl)-2-(methylthio)thieno[2,3-d]pyrimidine **36***

This compound was prepared from 4-chloro derivative **30** by the same procedure as described for **32**. The crude oil obtained was purified by flash column chromatography with methanol/ethyl acetate (3 : 7, v/v) as eluent. Yield: 0.66 g (60%); IR (KBr, selected lines) cm^{-1} 2929, 2852, 2792, 1512, 1443, 1349, 1298, 1142, 997, 885. ^{1}H-NMR (DMSO-d_6) δ 0.90 (t, J = 7.2 Hz, 3H, $CH_2CH_2CH_2CH_3$), 1.22 – 1.43 (m, 2H, $CH_2CH_2CH_2CH_3$), 1.51-1.70 (m, 2H, $CH_2CH_2CH_2CH_3$), 2.21 (s, 3H, NCH_3), 2.38 – 2.50 (m, 4H + 3H, piperazine, SCH_3), 2.81 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_2CH_3$), 3.75 – 3.87 (m, 4H, piperazine), 7.22 (s, 1H, aromatic). Anal. ($C_{16}H_{24}N_4S_2$) C, H, N, S.

*4-(4-Methyl-1-piperazinyl)-2-(methylthio)-7-(phenylmethyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-d]pyrimidine-5-carboxylic acid methyl ester **38***

A mixture of 4-chloro derivative **31** (0.21 g, 0.48 mmol) and 1-methylpiperazine (0.10 g, 0.99 mmol) in absolute ethanol (10 mL) was refluxed and stirred for 1 h. After the mixture had been cooled, the solvent was removed under reduced pressure. The resulting oil was suspended in water and extracted with ethyl acetate. The organic layers were collected, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The sticky residue obtained was triturated with diethyl ether, collected, dried and recrystallised from *n*-hexane. Yield: 0.060 g (25%); mp. 123 – 125°C; IR (KBr, selected lines) cm^{-1} 2926, 2795, 1721, 1529, 1501, 1449, 1403, 1365, 1249, 1151. ^{1}H-NMR ($CDCl_3$) δ 2.18 (s, 3H, NCH_3), 2.20 – 2.39 (m, 2H, piperazine), 2.52 (s, 3H, SCH_3), 2.70 – 2.90 (m, 1H, $CH_AH_BCHCOOCH_3$), 2.98 – 3.18 (m, 2H + 1H, piperazine, $CH_AH_BCHCOOCH_3$), 3.25 – 3.45 (m, 4H, piperazine), 3.53 (s, 3H, $COOCH_3$), 3.62-3.83 (m, 2H + 2H, $PhCH_2NCH_2$), 3.95 – 4.10 (m, 1H, $CHCOOCH_3$), 7.20 – 7.39 (m, 5H, aromatic). Anal. ($C_{24}H_{29}N_5O_2S_2$) C, H, N, S.

*6-Ethyl-2-(methylthio)-4-(1-piperazinyl)thieno[2,3-d]-pyrimidine **33***

4-Chloro derivative **28** (0.44 g, 1.79 mmol) was added to a solution of piperazine (0.31 g, 3.59 mmol) in ethanol (15 mL). The mixture was heated under reflux, stirred for 3 h and then cooled. The resulting solid was eliminated by filtration and the solution was concentrated under reduced pressure. The solid obtained was collected with diethyl ether, dried and purified by flash column chromatography with methanol as eluent. The homogeneous fractions were evaporated *in vacuo* to afford **33** as a pure solid. Yield: 0.22 g (42%); mp. 119 – 121°C; IR (KBr, selected lines) cm^{-1} 3278, 2836, 1551, 1514, 1441, 1339, 1268, 1148, 998, 828. ^{1}H-NMR (DMSO-d_6) δ 1.27 (t, J = 7.4 Hz, 3H, CH_2CH_3), 2.47 (s, 3H, SCH_3), 2.73 – 2.95 (m, 2H + 4H, CH_2CH_3, piperazine), 3.70 – 3.80 (m, 4H, piperazine), 7.20 (s, 1H, aromatic). Anal. ($C_{13}H_{18}N_4S_2 \cdot H_2O$) C, H, N, S.

*2-(Methylthio)-4-(1-piperazinyl)-6-propylthieno[2,3-d]-pyrimidine **35***

This compound was prepared from 4-chloro derivative **29** by the same procedure as described for **33**. The crude solid obtained was purified by flash column chromatography with methanol/ethyl acetate (3 : 7, v/v) as eluent. Yield: 0.41 g (71%); mp. 93 – 95°C; IR (KBr, selected lines) cm^{-1} 2931, 2793, 1550, 1512, 1445, 1348, 1294, 1142, 997, 881. ^{1}H-NMR (DMSO-d_6) δ 0.94 (t, J = 7.2 Hz, 3H, $CH_2CH_2CH_3$), 1.53 – 1.76 (m, 2H, $CH_2CH_2CH_3$), 2.47 (s, 3H, SCH_3), 2.70-2.90 (m, 4H + 2H, piperazine, $CH_2CH_2CH_3$), 3.70-3.78 (m, 4H, piperazine), 7.21 (s, 1H, aromatic). Anal. ($C_{14}H_{20}N_4S_2 \cdot H_2O$) C, H, N, S.

*6-Butyl-2-(methylthio)-4-(1-piperazinyl)thieno[2,3-d]-pyrimidine **37***

This compound was prepared from 4-chloro derivative **30** by the same procedure as described for **33**. The sticky product obtained

was purified by flash column chromatography with methanol/ethyl acetate (3 : 7, v/v) as eluent. Yield: 0.11 g (20%); mp. 70–72°C; IR (KBr, selected lines) cm^{-1} 3268, 2922, 1553, 1509, 1437, 1336, 1143, 992, 878, 805. ^{1}H-NMR (DMSO-d_6) δ 0.93 (t, J = 7.2 Hz, 3H, $CH_2CH_2CH_2CH_3$), 1.26–1.48 (m, 2H, $CH_2CH_2CH_2CH_3$), 1.55–1.73 (m, 2H, $CH_2CH_2CH_2CH_3$), 2.49 (s, 3H, SCH_3), 2.72–2.95 (m, 2H + 4H, piperazine, $CH_2CH_2CH_2CH_3$), 3.68–3.95 (m, 4H, piperazine), 7.24 (s, 1H, aromatic). Anal. ($C_{15}H_{22}N_4S_2$) C, H, N, S.

2-(Methylthio)-7-(phenylmethyl)-4-(1-piperazinyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-d]pyrimidine-5-carboxylic acid methyl ester ***39***

A mixture of 4-chloro derivative **31** (0.16 g, 0.37 mmol) and piperazine (0.16 g, 1.90 mmol) was refluxed and stirred for 1 h in absolute ethanol (5 mL). After the mixture had been cooled, the solvent was removed under reduced pressure to furnish an oil, which was suspended in water and extracted with diethyl ether. The organic layers were collected, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure to afford **39** as pure oil. Yield: 0.080 g (46%); IR (KBr, selected lines) cm^{-1} 2921, 2808, 1735, 1650, 1500, 1449, 1364, 1260, 1156, 986. ^{1}H-NMR ($CDCl_3$) δ 2.59 (s, 3H, SCH_3), 2.78–2.95 (m, 4H, piperazine), 3.10–3.30 (m, 4H, piperazine), 3.38–3.50 (m, 2H, $CH_2CHCOOCH_3$), 3.61 (s, 3H, $COOCH_3$), 3.64–3.68 (m, 2H + 2H, $ArCH_2NCH_2$), 4.03–4.15 (m, 1H, $CHCOOCH_3$), 7.24–7.39 (m, 5H, aromatic). Anal. ($C_{23}H_{27}N_5O_2S_2$) C, H, N, S.

Binding assays

Male CRL:CD(SD)BR-COBS rats (about 150 g, Charles River Laboratories, Italy) and male CRL:(HA) BR albino guinea pigs (about 300 g, Charles River Laboratories, Italy) were killed by decapitation; their brains were rapidly dissected into the various areas (the rat cortex for 5-HT_3Rs and the guinea pig striatum for 5-HT_4Rs) and stored at −80°C until the day of assay.

Tissues were homogenized in 50 volumes of 50 mM ice-cold Tris HCl, pH 7.4 containing 0.50 mM EDTA and 10 mM $MgSO_4$ for 5-HT_3Rs or 50 mM Hepes HCl, pH 7.4, for 5-HT_4Rs, using an Ultra Turrax TP-1810 homogenizer (2 × 20 s) (Janke &. Kunkel, Staufen, Germany), and the homogenates were centrifuged at 50 000 *g* for 10 min (Beckman Avanti J-25 refrigerated centrifuge; Beckman, USA). Each pellet was resuspended in the same volume of fresh buffer, incubated at 37°C for 10 min and centrifuged again at 50 000 *g* for 10 min. The pellet was then washed once by resuspension in fresh buffer and centrifuged as before.

The pellet obtained was finally resuspended in the appropriate incubation buffer: 50 mM Hepes HCl, pH 7.4, containing 10 μM pargyline for 5-HT_4Rs, and 50 mM Tris HCl, pH 7.4, containing 10 μM pargyline, 0.50 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbic acid and 140 mM NaCl for 5-HT_3Rs.

The [^{3}H]LY 278584 [33] (84.0 Ci/mmol Amersham (USA), for 5-HT_3) binding was assayed in a final incubation volume of 1 mL, consisting of 0.50 mL of tissue (16 mg/sample), 0.50 mL of the [^{3}H]ligand (4 nM) and 0.02 mL of displacing agent or solvent; non-specific binding was measured in the presence of 1 μM quipazine. The [^{3}H]GR 113808 [34] (84.0 Ci/mmol, Amersham, for 5-HT_4) binding was assayed in a final incubation volume of 1.0 mL, consisting of 0.50 mL of tissue (20 mg/sample), 0.50 mL of the [^{3}H]ligand (0.1 nM) and 0.02 mL of displacing agent or solvent; non-specific binding was measured in the presence of 10 μM 5-HT. Incubations (30 min at 25°C for 5-HT_3Rs or 30 min at 37°C for 5-HT_4Rs) were stopped by rapid filtration under vacuum through GF/B filters which were then washed with 12 mL (4 × 3 times) of ice-cold 50 mM Tris HCl, pH 7.4, or 50 mM Hepes HCl, pH 7.4, using a Brandel M-48R cell harvester (Gaithersburg, MD, USA). Dried filters were immersed in vials containing 4 mL of Ultima Gold MV (Packard Instruments, USA) and counted in a Wallac 1409 liquid scintillation spectrometer (Wallac-PerkinElmer, USA) with a counting efficiency of about 50%. Drugs were tested in triplicate at different concentrations (from 10^{-5} to 10^{-10} M) and dose-inhibition curves were analysed by the Allfit program [35] to obtain the concentration of unlabelled drug that caused 50% inhibition of ligand binding. K_i values were derived from the IC_{50} values [36].

In vitro functional studies on isolated guinea pig colon

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1813-1/2002). Crl(HA)BR male guinea pigs (Charles-River Laboratories, Hungary) were kept at 22 ± 3°C; the relative humidity was 30–70% and the light/dark cycle was 12/12 h. They were maintained on a standard guinea pig pellet diet (Charles-River Laboratories, Hungary) with tap water available *ad libitum*. The animals were sacrificed by CO_2 inhalation. The distal portion of the colon was removed from a Hartley guinea pig (400–500 g) starved 24 h before experiments. The colon was cleaned in Krebs bicarbonate buffer (in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7; pH 7.4) at room temperature and cut into 2 cm segments. The segments were suspended longitudinally in an organ bath containing Krebs bicarbonate buffer warmed to 37°C and bubbled through with 95% O_2/5% CO_2. An amount of 1 g of loading tension was applied; the tissues were left to be incubated for 1 h. Isometric contractions were detected with the ISOSYS Data Acquisition System (Experimetria Ltd., Hungary). The contractile action of the selective 5-HT_3R agonist 2-Me-5-HT (Sigma, Hungary) was investigated in a cumulative way. Results were expressed as percentage contraction increases compared with the basal colon activity. Compound **32** and the specific 5-HT_3RA tropisetron (Sigma), 10 nM each were added to the bath 10 min before the application of 2-Me-5-HT. Dose–response curves were fitted to the contraction-increasing effects. The EC_{50} maximum inhibitions were calculated and statistics were determined by means of Prism 4.0 software (GraphPad Software, USA), using the ANOVA Newman-Keuls test.

5-HT_{3A}R modelling

The extracellular region of murine 5-HT_{3A}Rs was built up on the basis of the recently published three-dimensional structure of the nACh receptor [37] (protein data bank entry code 2BG9), as described [27]. Briefly, multiple sequence alignments were performed by "ClustalW" software. Homology model building of the extracellular region of murine 5-HT_{3A}Rs based on the a and d subunits of the nACh receptor and its refinement were carried out using the Jackal protein structure modelling package on a Silicon Graphics Octane workstation under Irix 6.5 operation system. The assembled homopentamer was energy-minimized. The quality of the model was verified using Procheck. Docking calculations were carried out at the interface of the α-subunit

based ("lid-open") and δ-subunit based ("lid-shut") dimeric part of the pentamer. AutoDock 3.0 [38] was applied for docking calculations, using the Lamarckian genetic algorithm (LGA) and the pseudo-Solis and Wets (pSW) methods. Gasteiger – Huckel partial charges were applied both for ligands and proteins. Solvation parameters were added to the protein coordinate file and the ligand torsions were defined using the "Addsol" and "Autotors" utilities, respectively. Random starting positions, orientations and torsions (for flexible bonds) were used for the ligands. Each docking run consisted of 100 cycles. Final structures with rmsd < 2 Å were considered to belong in the same docking cluster.

References

[1] G. B. Yang, C. L. Qiu, H. Zhao, Q. Liu, Y. Shao, *J. Neuroimmunol*. **2006**, *178*, 24 – 29.

[2] P. Chameau, J. A. Hooft, *Cell Tissue Res.* **2006**, *326*, 573 – 581.

[3] D. Hoyer, J. P. Hannon, G. R. Martin, *Pharmacol. Biochem. Behav*. **2002**, *71*, 533 – 554.

[4] A. M. Karnovsky, L. F. Gotow, D. D. McKinley, J. L. Piechan, *et al.*, *Gene* **2003**, *319*, 137 – 148.

[5] B. Niesler, B. Frank, J. Kapeller, G. A. Rappold, *Gene* **2003**, *310*, 101 – 111.

[6] L. Farber, U. Haus, M. Spath, S. Drechsler, *Scand. J. Rheumatol.* **2004**, *119*, 2 – 8.

[7] B. J. Jones, T. P. Blackburn, *Pharmacol. Biochem. Behav.* **2002**, *71*, 555 – 568.

[8] M. Camilleri, A. R. Northcutt, S. Kong, G. E. Dukes, *et al.*, *Lancet* **2000**, *355*, 1035 – 1040.

[9] T. Stratz, W. Müller, *Curr. Rheum. Rev.* **2005**, *1*, 295 – 298.

[10] D. Oksenberg, R. Urfer, U.S. Patent 2004191312, 2004 [Chem. Abstr. **2004**, 141, P289075].

[11] M. Modica, M. Santagati, F. Russo, L. Parotti, *et al.*, *J. Med. Chem.* **1997**, *40*, 574 – 585.

[12] M. Santagati, H. Chen, A. Santagati, M. Modica, *et al.*, *Proceedings of the 12th European Symposium on QSARs: molecular modelling and prediction of bioactivity, Copenhagen, Denmark, August 23 – 28*, **1998**, pp. 433 – 439.

[13] M. Modica, M. Santagati, A. Santagati, F. Russo, *et al.*, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1089 – 1092.

[14] M. Modica, M. Santagati, F. Russo, C. Selvaggini, *et al.*, *Eur. J. Med. Chem.* **2000**, *35*, 677 – 689.

[15] L. Salerno, M. Siracusa, F. Guerrera, G. Romeo, *et al.*, *Arkivoc* **2004**, *5*, 312 – 324.

[16] M. Modica, M. Santagati, S. Guccione, A. Santagati, *et al.*, *Eur. J. Med. Chem.* **2001**, *36*, 287 – 301.

[17] M. Modica, G. Romeo, L. Materia, F. Russo, *et al.*, *Bioorg. Med. Chem.* **2004**, *12*, 3891 – 3901.

[18] M. C. Menziani, F. De Rienzo, A. Cappelli, M. Anzini, P. G. De Benedetti, *Theor. Chem. Acc.* **2001**, *106*, 98 – 104.

[19] V. P. Litvinov, *Adv. Heterocycl. Chem.* **2006**, *92*, 83 – 143, and references therein.

[20] M. Modica, M. Santagati, G. Romeo, L. Materia, *et al.*, *16th National Meeting of the Medicinal Chemistry Division of the Italian Chemical Society, Sorrento, Italy, September 18 – 22*, **2002**.

[21] M. Modica, M. Palkó, G. Romeo, L. Materia, *et al.*, *21st European Colloquium on Heterocyclic Chemistry, Sopron, Hungary, September 12 – 15,* **2004**.

[22] A. Cappelli, M. Anzini, S. Vomero, L. Mennuni, *et al.*, *Curr. Top. Med. Chem.* **2002**, *2*, 599 – 624, and references therein.

[23] K. Gewald, E. Schinke, H. Böttcher, *Chem. Ber*. **1966**, *99*, 94 – 100.

[24] F. J. Tinney, W. A. Cetenko, J. J. Kerbleski, D. T. Connor, *et al.*, *J. Med. Chem.* **1981**, *24*, 878 – 882.

[25] F. J. Tinney, D. T. Connor, W. A. Cetenko, J. J. Kerbleski, R. J. Sorenson, U.S. Patent 4230707, 1980 [Chem. Abstr. **1980**, 94, P84162].

[26] M. R. Briejer, L. M. Akkermans, J. A. Schuurkes, *Arch. Int. Pharmacodyn. Ther.* **1995**, *329*, 121 – 133.

[27] P. Joshi, A. Suryanarayanan, E. Hazai, M. K. Schulte, *et al.*, *Biochemistry* **2006**, *45*, 1099 – 1105.

[28] G. Maksay, Zs. Bikádi, M. Simonyi, *J. Recept Signal Transduct. Res.* **2003**, *23*, 255 – 270.

[29] G. Maksay, M. Simonyi, Zs. Bikádi, *J. Comput. Aided Mol. Des.* **2004**, *18*, 651 – 664.

[30] B. Vitális, L. Sebestyén, M. Sike, S. Sólyom, L. G. Hársing Jr., *Pharmacol. Res.* **2001**, *43*, 291 – 299.

[31] Z. Yang, A. Ney, B. A. Cromer, H. L. Ng, *et al.*, *J. Neurochem.* **2007**, *100,* 758 – 769.

[32] M. Morales, S. Wang, *J. Neurosci.* **2002**, *22*, 6732 – 6741.

[33] K. Miller, E. Weisberg, P. W. Fletcher, M. Teitler, *Synapse* **1992**, *11*, 58 – 66.

[34] C. J. Grossman, G. J. Kilpatrick, K. T. Bunce, *Br. J. Pharmacol*. **1993**, *109*, 618 – 624.

[35] A. De Lean, P. J. Munson, D. Rodbard, *Am. J. Physiol.* **1978**, *235*, E97 – E102.

[36] Y. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099 – 3108.

[37] N. Unwin, *J. Mol. Biol.* **2005**, *346*, 967 – 989.

[38] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, *et al.*, *J. Comput. Chem.* **1998**, *19*, 1639 – 1692.

