

## Building a model of interaction at the NK-2 receptors: Polycondensed heterocycles containing the pyrimidoindole skeleton<sup>†</sup>

A Santagati<sup>1\*</sup>, J Longmore<sup>2</sup>, S Guccione<sup>1</sup>, T Langer<sup>3</sup>, E Tonnel<sup>3\*\*</sup>, M Modica<sup>1</sup>,  
M Santagati<sup>1</sup>, L Monsù Scolaro<sup>4</sup>, F Russo<sup>1</sup>

<sup>1</sup>Dipartimento di Scienze Farmaceutiche, Università di Catania, Viale Andrea Doria 6, Ed 12,  
Città Universitaria, I-95125 Catania, Italy;

<sup>2</sup>Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR, UK;

<sup>3</sup>Institut für Pharmazeutische Chemie, Universität Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria;

<sup>4</sup>Istituto Chimica e Tecnologia dei Prodotti Naturali (ICTPN), Sezione di Messina, Dipartimento di Chimica Inorganica,  
Analitica e Struttura Molecolare, Università di Messina, Villaggio S Agata, Salita Sperone 31, I-98166 Messina, Italy

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**Summary** — The pyrazolopyrimidothiazole ring system (compound N, table II) has been previously reported by us as a new competitive antagonist (apparent  $pA_2 = 7.3$  equiv to 55 nM) at NK2-receptors. As part of our investigation on polycondensed heterocycles containing the pyrimidine ring as antagonists of G-protein coupled receptors, pyrimidoindole derivatives were prepared and tested in order to probe the topography of the NK2-receptors and ascertain the pattern of frameworks that result in optimum affinity and specificity. The title indole derivatives **5**, **11a–d**, **12c**, **13a,b** and **14b** were 'de novo' designed or selected from our chemical archives and prepared by up-to-date synthetic routes, thus exploring new synthetic methodologies. According to the established graphic computer model, none of the tested substances exhibited activity as a consequence of the violation of an excluded volume area due the unfavourable position of the aromatic substituents.

indole derivatives / neurokinin receptors / graphic computer model

### Introduction

G-protein mediated signalling pathways have generated a great deal of attention because of the many physiological and pharmacological events that are

modulated by these mechanisms [1]. The antagonist–receptor binding interactions are likely to be different for each compound at the G-protein coupled receptors, even for two members of closely related chemical families. This suggests a direct interaction between the non-peptide compound and specific amino acid side chains in the identified domain [2–6]. Aromatic and heteroaromatic features are common structural elements in G-protein coupled receptors antagonists [7–10].

A logical corollary of the pharmacophoric concept is replacement of the chemical scaffold holding the pharmacophoric groups with retention of activity. Indeed, it has been previously reported by these laboratories that sites for secondary interactions provided for by the heteroaromatic moiety and its substituent(s) may exert a marked influence [11–15]. Moreover, recent studies reported the possibility of allosteric interactions between ligands and the competitive agent at the G-protein coupled receptors [16].

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\*Correspondence and reprints

\*\*Present address: Laboratoire de Chimie Thérapeutique ENSCM, Université de Montpellier, 8 rue de l'Ecole Normale, F-34000 Montpellier, France

Remarkably, the compounds of our investigation are achiral. This is in marked contrast to the stereochemical requirements observed in other series of G-protein coupled receptor antagonists [9, 17, 18].

Recently, intense interest has been focused on discovering antagonists for the neurokinin receptors [18]. The neurokinin substance P, neurokinin A and neurokinin B (SP, NKA, and NKB) form a family of neuropeptides sharing a common carboxyl-terminal sequence Phe-X-Gly-Leu-Met-NH<sub>2</sub>. They mediate their biological effects by interaction with three structurally related receptors, ie, NK-1, NK-2, and NK-3. The preceding N-terminal portion of the receptor (including the extracellular N-terminal domain) is predicted [19] to be involved in recognition of the address sequence, thus providing the subtype-specific characteristic of each neurokinin receptor. Recent studies have led to the hypothesis that NK-1 and NK-2 receptor subtypes might also exist [17–19].

The actions of SP and NKA at NK-1 and NK-2 receptors, respectively, have been implicated in neurogenic inflammation, transmission of pain, vasodilation, airway smooth muscle contraction, and the regulation of the immune response. Consequently, a neurokinin receptor antagonist may be of therapeutic use in the threatment of chronic pain, rheumatoid arthritis and bowel disease [17–19]. Recently, Barnes and Lundberg have proposed an involvement of substance P in the pathology of asthma [21, 22].

The introduction of novel ligands has a central role in defining the distribution, physiological roles and pathophysiological significance of this family of neurotransmitters and their receptors [23, 24].

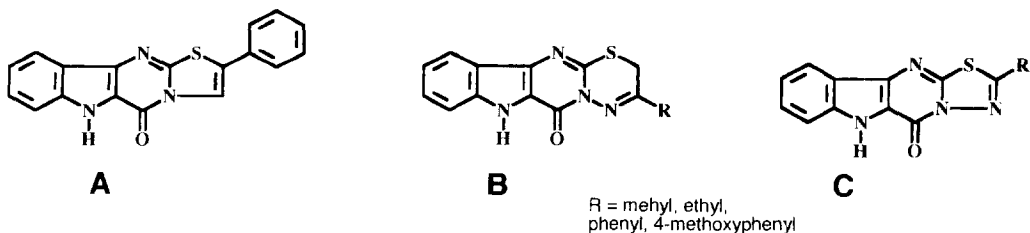
In cases where the receptor structure is not known, 3D SARs should be investigated by superposing the 3D structure of active and inactive molecules. Compounds capable of presenting the pharmacophoric pattern, but incapable of binding, are useful in order to help determine the location of receptor-occupied space in relation to the pharmacophore (receptor-mapping), ie, the volumes that are common, different or occupied by at least one molecule. Molecules with diverse structural features are especially useful, even if their activities are not highly potent. In this

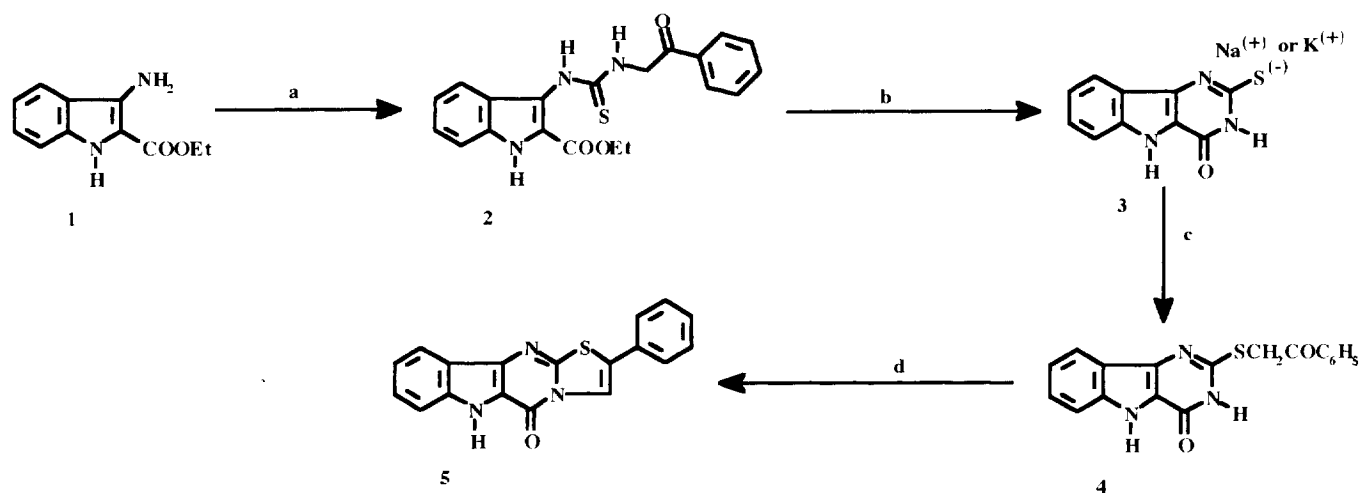
case further superpositioning of molecules with low potency or without activity can help to determine volume areas that are obstructive to binding.

The aim of this investigation was to explore the steric interference of the binding of thiazolo-, thiadiazino- and thiadiazolopyrimidoindoles in comparison with a pyrazolopyrimidothiazole lead **N** (table II) already reported by us [15], providing further information to improve or rebuild a model of interaction at NK-2 receptors through iterations of chemical synthesis and biological evaluation.

Overall, the receptor affinity of neurokinin antagonists seems to be governed by steric parameters. Two correctly oriented aromatic rings seem to be a prerequisite for the biological activity, as also shown by the 1,6-diphenylpyrazolopyrimidothiazole NK-2 receptor antagonist **N** previously reported by us [15]. To date, the effect of substitutions on the aromatic rings of non-peptide ligands escapes a rational relationship also when they are arranged on very active molecules. Therefore 'steric criteria', ie, those affecting the spatial orientation of the seemingly pharmacophoric phenyls, substantially directed our current design and proved that other reversible binding interactions (eg, electrostatic, hydrophobic) as a consequence of the different heterocycle should play a role. The benzene moiety of the synthetically accessible pyrimidoindole derivatives should allow us to get insight into the effect of a less flexible junction (ie, more constrained) of the phenyl ring in comparison with that of the more freely rotating *N*-phenylpyrazole, so defining the potential of the latter aromatic moiety as 'conformational selector' to collide with the binding site in the correct conformation and orientation to form the binding complex. The premise implicitly made is that the loss in activity is a result of not being capable to adopt the 'active' conformation energetically available to the active ligands.

The syntheses (schemes 1 and 2) and the biological evaluation of the title thiazolo-, thiadiazino- and thiadiazolopyrimidoindoles **5**, **11a–d**, **12c**, **13a,b** and **14b** (formulas A–C) as well as the preliminary results of ongoing CoMFA studies are reported in this work.



**Reagents and conditions:**

(a)  $\text{SCNCOCH}_2\text{C}_6\text{H}_5$ , acetone,  $\Delta$ ; (b)  $\text{EtOH} / \text{KOH}$ ,  $\Delta$ ; (c)  $\text{Hal-CH}_2\text{COC}_6\text{H}_5$ ,  $\text{EtOH}$ ,  $\Delta$ ; (d) 98%  $\text{H}_2\text{SO}_4$ , 25 °C, 72 h (slight modification of the literature procedure ( $\text{CH}_3\text{COOH} / 98\% \text{H}_2\text{SO}_4$ ,  $\Delta$ ) [25]).

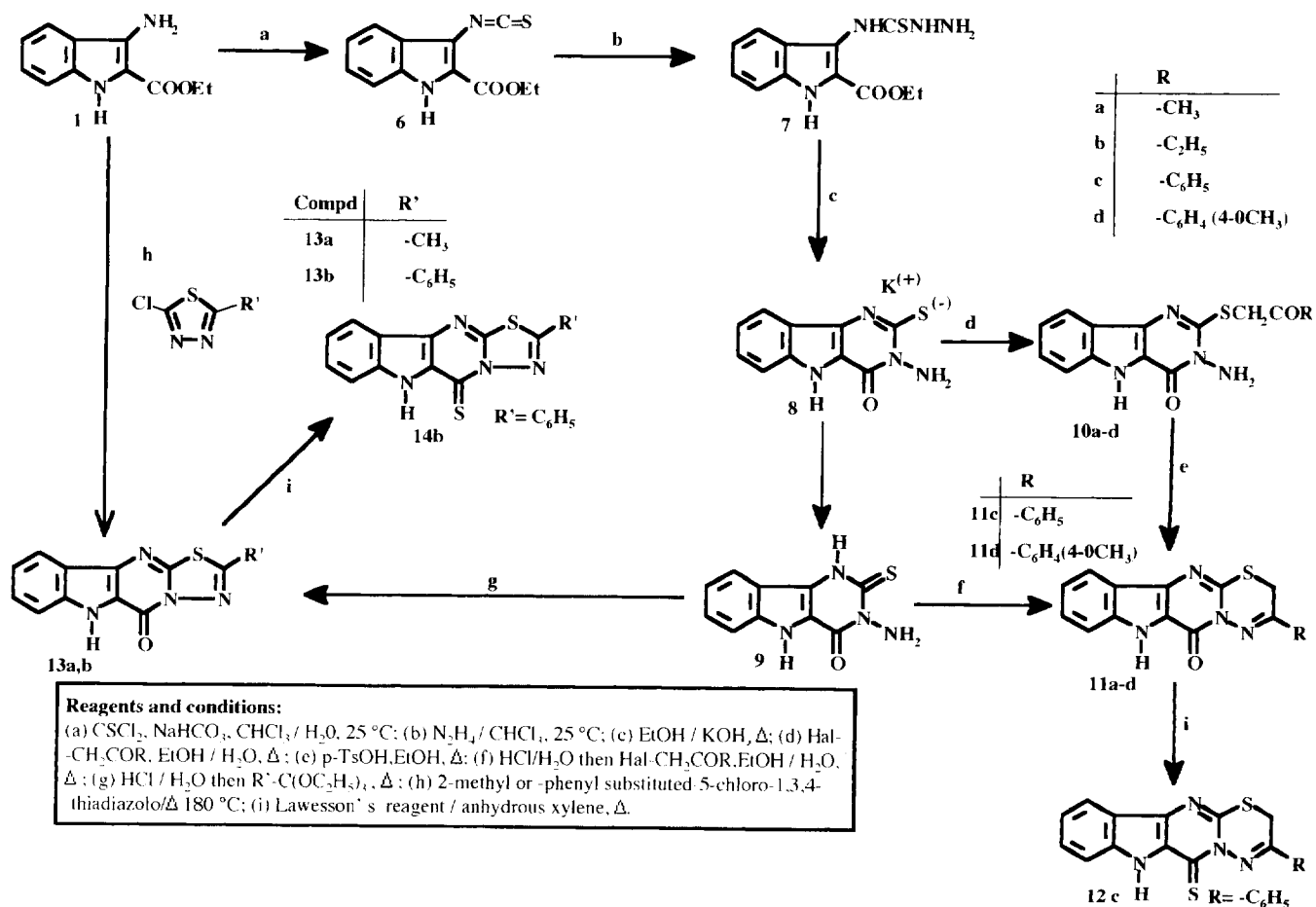
**Scheme 1.** Preparation of 3-phenyl-6H-thiazolo[3',2':1,2]-5-oxypyrimido[5,4-*b*]indole **5**.

## Chemistry

The synthetic routes to prepare the indole derivatives **5**, **13a,b** [25, 26] and **14b** by up-to-date methods and the derivatives of the new pyrimidothiadiazinoindole ring system **11a–d** and **12c** are outlined in schemes 1 and 2. The common starting 3-amino-1H-indole-2-carboxylic acid ethyl ester **1** was prepared according to the method of Unangst [27] with a slight modification improving the yield to 75%, ie, the use of sodium *tert*-butoxide as a cyclizing agent in place of potassium salt by alkaline (KOH/ethanol) ring closure reaction of *N*-(2-carboethoxyindol-3-yl)-*N'*-(benzoyl) thiourea **2**. As such it was used to obtain the intermediate **4** by reaction with  $\alpha$ -bromoacetophenone in ethanol. Starting *N*-(2-carboethoxyindol-3-yl)-*N'*-(benzoyl) thiourea

**2** was obtained by reaction of 3-amino-1H-indole-2-carboxylic acid ethyl ester **1** with an equimolar amount of benzoyl isothiocyanate, either commercially available or prepared in situ, in acetone at reflux for 2 h [15, 28, 29].

Derivatives **11a–d**, **12c**, **13a,b** and **14b** (scheme 2) were prepared starting from the isothiocyanate **6**, obtained by reaction of 3-amino-1H-indole-2-carboxylic acid ethyl ester **1** [27] with 97% thiophosgene in the presence of sodium bicarbonate, with chloroform–water as a heterogeneous two-phase solvent system. Addition of the isothiocyanate **6** to a stirred solution of hydrazine hydrate in chloroform at room temperature gave the thiosemicarbazide derivative **7**. The latter was cyclized by alkaline (KOH/ethanol) ring closure reaction to the versatile 1H-pyrimido[5,4-*b*]indolo-3-amino-2-thioxo-4H,5H one-potassium salt **8** easily converted into the corresponding acid **9**. The synthetic intermediate **8** can be used to prepare the pyrimidothiadiazinoindoles **11a–d** by reaction with the appropriate  $\alpha$ -haloketones at room temperature followed by cyclodehydration with *p*-toluenesulphonic acid (*p*-TsOH) as a catalyst in ethanol at reflux. In an alternative synthetic route, derivatives **11c,d** were obtained by ‘one-step’ reaction refluxing the amino-thioxo derivative **9** with the appropriate 2-bromoaceto-



**Scheme 2.** Synthetic routes to 3-alkyl or -aryl-substituted-1,3,4-thiadiazolo[2,3:2',1']pyrimido[5,4-*b*]indol-6(7H)-ones **11a-d** or thione **12c**, 3-alkyl or -aryl-substituted-1,3,4-thiadiazolo[3',2':2,1]pyrimido[5,4-*b*]indol-5(6H)-ones **13a,b** and **14b**.

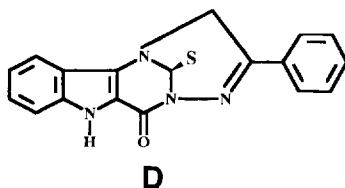
phenone in ethanol–water ( $\phi = 20\%$ ). The bioisosteric thione analogs **12c** and **14b** were obtained by stirring compound **11c** and **13b** with an excess of Lawesson's reagent, in anhydrous xylene at reflux for 30 min and 4 h respectively, according to the procedure previously reported by us [15, 25, 26]. The identification of the thio group ( $\text{C}=\text{S}$ ) stretching of compound **12c** and **14b** is difficult. Based on the superimposition of the registered spectrum with the ones of strictly related compounds, the subject stretching may be hypothesized at  $1560\text{ cm}^{-1}$ . In consideration of the reported relationship  $\nu_{\text{C}=\text{O}}/\nu_{\text{C}=\text{S}} = 1.5$  [30] it may be identified for compound **12c** and **14b** at  $1115$  and  $1100\text{ cm}^{-1}$

( $\nu_{\text{C}=\text{O}}/\nu_{\text{C}=\text{S}} = 1.51$ ), respectively. Indeed the IR spectrum did not show the peak at  $1690$  and  $1685\text{ cm}^{-1}$  attributable to the  $\text{C}=\text{O}$  group as in the starting compound **11c** and **13b**.

Compound **13a** and **13b** were obtained by reaction of the aminothioxo derivative **9** with triethyl orthoacetate or triethyl orthobenzoate at reflux, respectively. The structures of thiadiazolopyrimidoindoles **13a,b** and **14b** were furthermore defined using as a comparison samples obtained according to the methods previously reported by Russo et al [26], based on the condensation of aminoester **1** with 5-methyl or -phenyl substituted 2-chloro-1,3,4-thiadiazole. Therefore, the

structure of derivatives **8**, **9** was confirmed and the formation of dimeric structures in the reaction between the isothiocyanate **6** with hydrazine hydrate was excluded, instead of the bifunctional character of this latter reactant (scheme 2).

All the spectral data (IR,  $^1\text{H}$  and  $^{13}\text{C}$ -NMR) listed below are in accordance with the assigned structures. Particularly, the absence of peaks concerning the NH or  $\text{NH}_2$  groups except in the case of the indole ring NH (regions 12.20–12.10) and the presence of those in the region 4.33–3.32 (methylene protons in the position 2 of the thiadiazine ring) in the proton NMR spectrum of the thiadiazinopyrimidoindoles **11a–d**, rules out other possible isomeric structures and clearly confirms the presence of the 3,4-unsaturated thiadiazine ring. The latter structural characterization was further supported by the results of theoretical studies using semi-empirical MO (molecular orbital) calculations (table I). Considering charge and orbital control of the reaction as outlined in the frontier molecular concept [31], from homo/lumo energy comparison as well as from atom partial charge estimation (fig 1), it is obvious that the first attack of a carbenium ion is more likely to take place at the sulphur atom than at the NH group. Moreover, from estimation of the heat of formation, it becomes evident that a hypothetical isomer containing the triazine ring (formula D) in

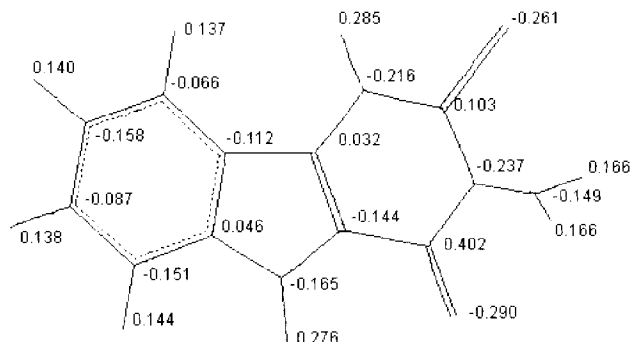


place of the thiadiazine one is thermodynamically less favored ( $\Delta H_f = 38.0216$  Kcal/mol) (table I). The mp of the crude tetracyclic compounds and their synthetic intermediates were within  $\pm 3^\circ\text{C}$ , if compared with the pure product. Therefore, the synthetic intermediates can be used without purification.

**Table I.** Summary of AM1 calculations.

Compound	Heat of formation (Kcal/mol)
<b>9</b>	84.26
<b>11c</b>	137.73
<b>D<sup>a</sup></b>	175.75

<sup>a</sup>Hypothetic product.



**Fig 1.** Charge density calculation on AM1 optimized structure of compound **9**.

### CoMFA

When atomic and substructural properties do not provide a reliable means to reveal QSARs, an analysis of fields surrounding the molecules can explain the biological effect. In this context comparative molecular field analysis (CoMFA) has been successfully used [32]. A training set of 14 ligands covering an activity range of almost 5 orders of magnitude ( $\text{EC}_{50}$  10 nM – 80  $\mu\text{M}$ ) was chosen (table II) [15, 33].

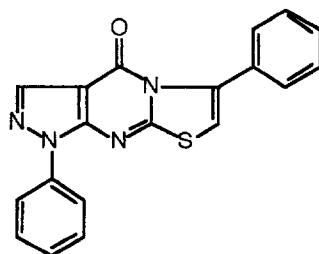
To define an alignment rule for the 3D-QSAR study, common features of all molecules were searched and defined. In all the investigated compounds, two phenyl rings and at least one carbonyl dipole were present and were therefore defined as a pharmacophore working hypothesis. As all the peptide compounds [33] under investigation show a high degree of conformational freedom, an exhaustive conformational analysis was performed using a simulated annealing procedure (20 cycles of 1000 ps MD simulation at 700 K and 1000 ps annealing using an exponential function to 200 K; conformers were saved from the low-temperature simulation periods and minimized further). The analysis of a geometric parameter (distance between the two centroids of the phenyl rings) revealed a mean distance of 8–10 Å for all the investigated compounds (fig 2), the alignment of which is given in figure 3.

For interaction energy matrix calculation, a three-dimensional box was automatically created (size: 28 x 36 x 34 Å, 4284 points with 2 Å grid spacing in x, y, and z directions) making sure that every grid protruded in all directions at least 4 Å beyond the shape of each molecule.

Steric and electrostatic interaction energies at the lattice intersections were generated with a probe atom

**Table II.** Compounds of the training set investigated in the CoMFA study.

Compound	Structure	EC <sub>50</sub> (nM)
A <sup>a</sup>	Ava-Phe-Phe-Gly-Leu-Met-NH <sub>2</sub>	1190
B <sup>a</sup>	Ava-Phe-Phe-Ala-Leu-Met-NH <sub>2</sub>	1860
C <sup>a</sup>	Ava-Phe-Phe-D-Ala-Leu-Met-NH <sub>2</sub>	452
D <sup>a</sup>	Ava-Phe-Phe-Pro-Leu-Met-NH <sub>2</sub>	31500
E <sup>a</sup>	Ava-Phe-Phe-D-Pro-Leu-Met-NH <sub>2</sub>	67
F <sup>a</sup>	Ava-Trp-Phe-Gly-Leu-Met-NH <sub>2</sub>	12500
G <sup>a</sup>	Ava-D-Trp-Phe-Gly-Leu-Met-NH <sub>2</sub>	7530
H <sup>a</sup>	Ava-Phe-Phe-Gly-Leu-D-Met-NH <sub>2</sub>	50000
I <sup>a</sup>	Ava-Phe-Phe-Gly-[(R)-γ-lactam]-Leu-Met-NH <sub>2</sub>	9990
J <sup>a</sup>	Ava-Phe-Phe-Gly-[(R)-spiro lactam]-Leu-Met-NH <sub>2</sub>	1390
K <sup>a</sup>	Ava-Phe-Phe-Gly-[(R)-spiro lactam]-Leu-Met-NH <sub>2</sub>	80000
L <sup>a</sup>	Ava-Phe-Phe-Gly-[(R)-fused lactam]-Leu-Met-NH <sub>2</sub>	15
M <sup>a</sup>	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH <sub>2</sub>	167

N<sup>b</sup>

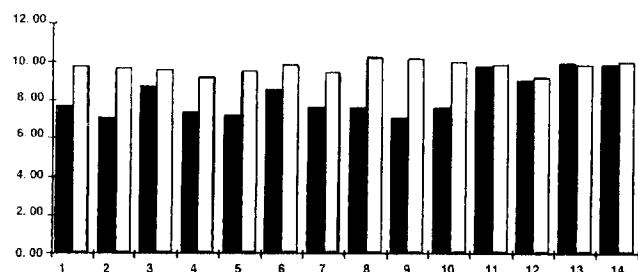
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<sup>a</sup>Brown et al [33]; <sup>b</sup>Guccione et al [15].

that had the Van der Waals properties of sp<sup>3</sup> carbon and a charge of +1.0.

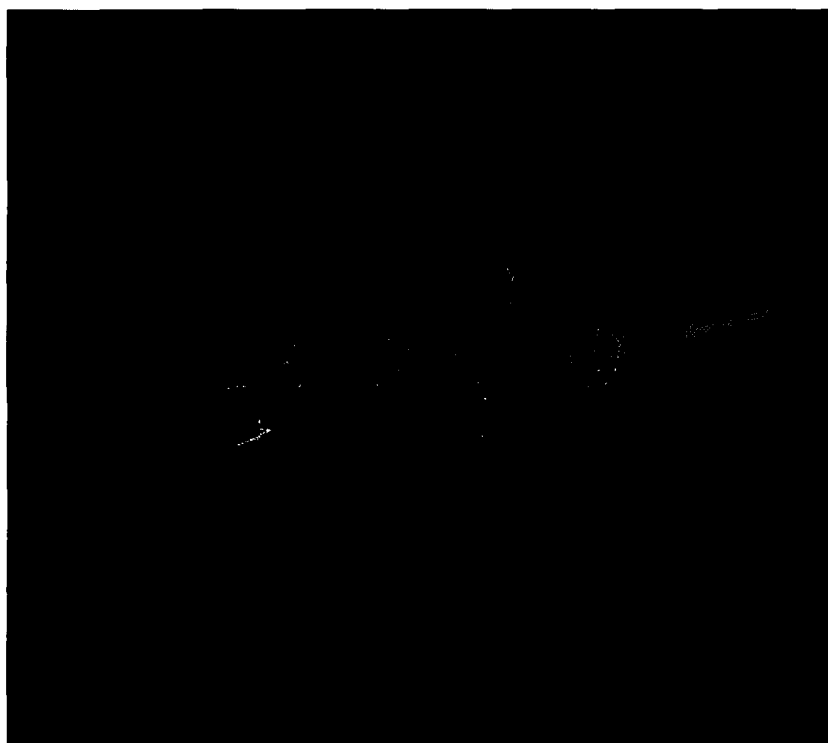
The steric and electrostatic energy values were truncated to 30 Kcal/mol. The electrostatic energy term was ignored at the lattice intersections yielding maximal (30 Kcal/mol) steric values. The obtained interaction energy values were scaled using the CoMFA standard method.

The standard deviation threshold for the PLS analysis was set to 2.0. Cross validation was performed by

**Fig 2.** Compounds **5**, **11c** and **13b** superimposed on the previously reported 1,6-diphenyl-pyrazolopyrimidothiazole [15].

means of the 'leave-one-out' validation technique, ie, the number of cross-validation groups was the same as the number of molecules in the training set. This type of cross-validation makes it possible to maximize the information capacity of the training set. Preliminary analyses were carried out with a maximum of five components. Subsequently, the analyses were repeated using a number of components at which the difference between the cross-validated  $r^2$  value and the next was less than 0.05. The first run was performed in order to determine the predictivity of the model. A  $r^2_{cv}$  of 0.447 ( $S_{press} = 1.123$ ; 5 components) was found, showing a satisfactory quality of the 3D-QSAR model. The final analysis was performed without cross-validation using 4 components:  $r^2 = 0.995$ ,  $s = 0.101$ ,  $F = 438.366$  (table III, fig 4).

The interpretation of the CoMFA results can easily be reached by observing the contour graphs resulting from the final analysis (fig 5a and 5b) for displaying favourable and unfavourable regions of the different fields analyzed. The steric contour map exhibits green and yellow contours for regions where more bulk would lead to compounds with a higher target value and for regions where less steric interaction would enhance the affinity, respectively (fig 5a).



**Fig 3.** Training set of the CoMFA model. Green structure: 1,6-diphenyl-pyrazolopyrimidothiazole [15, 32]. Magenta structure: indole derivatives **5**, **11c** and **13b**. The arrows are highlighting both regions where the aromatic moieties of the training set of the compounds are located.

In the electrostatic map, more positive or more negative charges are favourable within the blue and the red contour regions, respectively as shown in figure 5b. From the comparison of less active or inactive analogues with the active compound **N** (table III) [15] a 'forbidden' volume can be defined, as depicted in figure 6 (green: compound **N** [15]; red: less active or inactive analogues).

## Conclusion

None of the aryl-substituted indole derivatives **5**, **11c,d**, and **13b** show superposition of the aromatic substituent in the 'common volumes' of the active antagonists (figs 3 and 6). Compound **5** shows a right superposition of the aromatic ring in the putative 'message area', but steric interference with receptor binding (essential volume violation) is in the indole moiety area. Only small alkyl substituents placed on the C3 (pyrazole ring) (fig 6) [15] should make a more productive binding [unpublished data].

All the tested substances exhibited no activity (table IV), thus suggesting that correctly arranged aromatic portions are a rigorous prerequisite to make the NK-2 receptors interact. A motif in the amino acid sequence of reported peptide NK-2 receptor antagonists is the presence of a common Trp-Phe. The two aromatic groups of Trp-Phe may play a crucial role for interaction with conserved regions of the receptor protein [18]. Obviously, the alkyl-substituted terms **11a,b**, **13a** and the thione derivatives **12c**, **14b** are completely inactive up to a concentration of 10  $\mu$ M (table IV).

The result is self-evident and the data support our model in progress, showing that overall, the receptor affinity of this type of antagonists seems to be governed by steric parameters (figs 2, 3).

Each of the inactive compounds required extra volume not required by the active compounds as shown by the superposition of active NK-2 ligands comprehensive of the 1,6-diphenyl-pyrazolopyrimidothiazole (apparent  $pA_2 = 7.3$  equiv to 55 nM) previously reported by us in figure 3 [15, 32]. Further

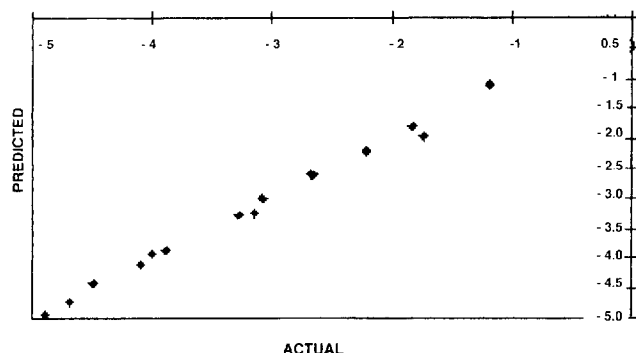
**Table III.** Predicted pEC<sub>50</sub>.

Compound	Log(1/EC <sub>50</sub> ) (nM)
A	-2.998
B	-3.264
C	-2.609
D	-4.414
E	-1.802
F	-4.122
G	-3.852
H	-4.748
I	-3.917
J	-3.248
K	-4.950
L	-1.096
M	-2.208
N	-1.955

superposition of the potent NK-2 antagonist SR-48968 [17] (pA<sub>2</sub> = 9.0 equiv to 1 nM in our tests) showed two of the aromatic rings in the same region as depicted in figure 3.

Based on our results, the condensed pyrazolopyrimido portion may provide the subtype specificity at the NK-2 receptors [15], raising the intriguing suggestion of a message-address hypothesis at the NK-2 receptors [18]. The latter 'aromatic guidance' (address) [34] may represent a putative recognition site for the preceding *N*-terminal portion of the receptor (including the extracellular *N*-terminal domain). Amino-aromatic interactions are believed to play an important role in G proteins coupled receptor ligand binding [11–15, 35].

Optimization, full explanation of SARs and 3D-SARs along with comparison of volume maps [36, 37] for different receptors allowing insight into

**Fig 4.** Predicted versus actual pEC<sub>50</sub> values of the training set.

G-protein coupled receptors and the optimization of activity at one receptor with respect to another, are part of our program and will be subsequently reported.

## Experimental protocols

### Chemical methods

Melting points were determined on a Büchi capillary apparatus and are uncorrected. IR spectra of compounds **2–5** (scheme 1) and **6–9**, **10a–d**, **11a–d**, **12c**, **13a,b** and **14b** (scheme 2) were obtained with potassium bromide discs on a Perkin-Elmer 281 spectrophotometer.

<sup>1</sup>H- and <sup>13</sup>C-NMR spectra were obtained on a Bruker AMX-R 300 spectrometer equipped with a broadband probe operating at 300.13 and 75.5 MHz respectively. Sample solutions in DMSO-*d*<sub>6</sub> were referenced to TMS. In each case, chemical shifts (δ) are reported in ppm downfield from TMS, and coupling constants in Hz. All the measurements were performed at 303 K. Elemental combustion analyses were performed on a Carlo Erba Mod EA 1108 Analyzer instrument by Dr S Di Marco of the Microanalysis Laboratory of the Dipartimento di Scienze Farmaceutiche, Università di Catania. Analyses indicated by the symbols of the elements or functions were within ± 0.40% of theoretical values.

Reactions were routinely followed by thin-layer chromatography on silica gel plates (60 F254 Merck), ethyl acetate **2–5**, **6–13a,b**, **14b** ethyl acetate–cyclohexane (φ = 50%) **6–8**, **10a–d**, **13a,b** as an eluent. Similarly the purity of each compound was checked. The spots were detected by UV irradiation at 254–365 nm. All chemicals were purchased from Aldrich, Fluka, Merck and Carlo Erba Chemical Co and were used without further purification.

### Synthesis of *N*-(2-carboethoxyindol-3-yl)-*N'*-(benzoyl) thiourea **2**

To a stirred solution of commercially available or prepared in situ benzoylisothiocyanate (7.3 mmol) [28, 29] in anhydrous acetone (10 mL when commercially available benzoylisothiocyanate was used), a saturated 3-amino-1*H*-indole-2-carboxylic acid ethyl ester **1** solution (7.3 mmol) [27] in anhydrous acetone (20 mL) was slowly added drop by drop and the mixture was refluxed for 2 h. The precipitate was collected by filtration, repeatedly washed with cold water and dried. Compound **2** can be processed without recrystallization. An analytically pure sample can be obtained by recrystallization from ethanol (white powder).

Yield: 32%; mp 191–193 °C; IR (KBr) ν (neat): 3320 cm<sup>-1</sup> NH; 1680 cm<sup>-1</sup> C=O. TLC system: ethyl acetate. Anal C<sub>19</sub>H<sub>17</sub>N<sub>3</sub>O<sub>3</sub>S (C, H, N, S).

### Synthesis of 2-(phenacylthio)-3,5-dihydro-4*H*-pyrimido-[5,4-*b*]indol-4-one **4**

Prepared by a modification of the literature procedure [25] as follows: to a solution of potassium hydroxide (15.5 mmol) in absolute ethanol (39 mL) thiourea derivative **2** (7.7 mmol) was added and the mixture was refluxed under stirring for 3 h then filtered and the solid material **3** dried (yield: 99%; mp > 300 °C) and processed without purification.

To a suspension of the collected solid material **3** in ethanol (45 mL) α-bromoacetophenone was added then the mixture was refluxed for 8 h. After cooling the precipitate **4** was collected and recrystallized from acetic acid–water (yield 39%, lit [25] 75%).





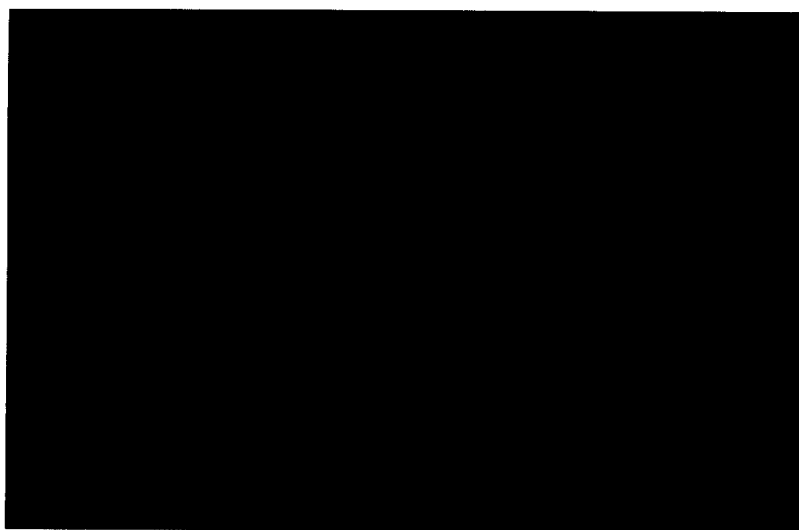
**Fig 5.** (left) Steric CoMFA contour plot. (right) Electrostatic CoMFA contour plot.

3,5-Dihydro-2-mercapto-4H-pyrimido[5,4-*b*]indol-4-one monopotassium salt **3** was easily converted with acetic acid into its parent acid, physicochemical data of which are consistent with a sample obtained according to the method of Unangst [27].

Physicochemical data of compound **4** are consistent with the literature data whereas the yield is lower than that previously reported by Russo et al [25].

*Synthesis of 3-isothiocyanato-1H-indole-2-carboxylic acid ethyl ester 6*

To a stirred suspension of chloroform (50 mL), water (30 mL), sodium hydrogencarbonate (50.7 mmol) and 97% thiophosgene (25 mmol), a solution of 3-amino-1H-indole-2-carboxylic acid ethyl ester **1** (24.07 mmol) [27] in chlorophorm (85 mL) was dropwise added during 20 min. The mixture was stirred at room temperature for an additional 2 h, then it was repeatedly



**Fig 6.** Volume maps of inactive (red) compounds.

**Table IV.** Pharmacological evaluation 'in vitro'.

Compound	Type of assay	Result ( $K_a$ $\mu$ M)
<b>5</b>	NK-1	No effect at 1 $\mu$ M ( $n = 3$ )
	NK-2	No effect at 1 $\mu$ M ( $n = 4$ )
<b>11a</b>	NK-1	No effect at 10 $\mu$ M ( $n = 3$ )
	NK-2	No effect at 10 $\mu$ M ( $n = 3$ )
<b>11b</b>	NK-1	No effect at 10 $\mu$ M ( $n = 3$ )
	NK-2	No effect at 10 $\mu$ M ( $n = 3$ )
<b>11c</b>	NK-1	No effect at 1 $\mu$ M ( $n = 4$ )
	NK-2	$1.37 \pm 0.72$ tested at 10 $\mu$ M ( $n = 4$ )
<b>11d</b>	NK-1	No effect at 1 $\mu$ M ( $n = 4$ )
	NK-2	No effect at 10 $\mu$ M ( $n = 4$ )
<b>13a</b>	NK-1	No effect at 1 $\mu$ M ( $n = 2$ )
	NK-2	No effect at 10 $\mu$ M ( $n = 2$ )
<b>13b</b>	NK-1	$0.42 \pm 0.25$ tested at 1 $\mu$ M ( $n = 4$ )
	NK-2	No effect at 10 $\mu$ M ( $n = 4$ )

extracted with chlorophorm (10 x 50 mL). The pooled organics were washed with water, dried over anhydrous sodium sulphate, filtered through silica gel and rotary evaporated in vacuo. Compound **6** can be processed without recrystallization. An analytically pure sample can be obtained by recrystallization from cyclohexane or dioxane (yellow needles).

Yield: 42%; mp 213–215 °C. IR (KBr)  $\nu$  (neat): 3290  $\text{cm}^{-1}$  NH (br); 2130  $\text{cm}^{-1}$  N=C=S; 1680  $\text{cm}^{-1}$  C=O. TLC: ethyl acetate. Anal  $\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}_2\text{S}$  (C, H, N, S).

*Synthesis of 3-[(hydrazinethioxomethyl)amino]-1H-indole-2-carboxylic acid ethyl ester 7*

To a stirred solution of 98% hydrazine hydrate (19.6 mmol) in chloroform (50 mL) a solution of isothiocyanate **6** (13.00 mmol) in chloroform (370 mL) was dropwise added. The suspension was stirred at room temperature for an additional two hours then the solid was collected, washed with chloroform and recrystallized from ethanol (yellow needles).

Compound **7** can be processed without recrystallization.

Yield: 60%; mp 188–189 °C (dec). IR (KBr)  $\nu$  (neat): 3300, 3190 (br)  $\text{cm}^{-1}$  NH; 1680 C=O  $\text{cm}^{-1}$ .  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ):  $\delta$  11.62 (br s, 1H, NH), 9.67 (br s, 1H, NH), 9.17 (br s, 1H, NH), 7.72 (br m, 1H, arom), 7.39 (m,  $^3J_{\text{av}} = 8.2$  Hz, 1H, arom), 7.23 (t,  $^3J_{\text{av}} = 7.2$  Hz, 1H, arom), 7.01 (t,  $^3J_{\text{av}} = 7.6$  Hz, 1H, arom), 4.82 (br s, 2H,  $\text{NH}_2$ ), 4.31 (q,  $^3J_{\text{HH}} = 7.1$  Hz, 2H,  $\text{CH}_2$ ), 1.33 (t,  $^3J_{\text{HH}} = 7.1$  Hz, 3H,  $\text{CH}_3$ ).  $^{13}\text{C-NMR}$  ( $\text{DMSO}-d_6$ ):  $\delta$  180.8 (1C), 160.9 (1C), 135.1 (1C), 124.7 (1C), 123.3 (1C), 123.0 (1C), 122.9 (1C), 121.7 (1C), 118.9 (1C), 112.3 (1C), 60.3 (1C), 14.2 (1C). TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi = 50\%$ ). Anal  $\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_3\text{S}$  (C, H, N, S).

*Synthesis of 3-amino-1,2,3,5-tetrahydro-2-thioxo-4H-pyrimido[5,4-b]indol-4-one monopotassium salt 8*

To a solution of potassium hydroxide (14.28 mmol) in hot ethanol (160 mL) compound **7** (14.28 mmol) was added and the mixture was stirred under reflux for two hours. After this time the solid was collected and purified by washing three times with warm dioxane (white amorphous solid).

Yield: 98%; mp > 300 °C. IR (KBr)  $\nu$  (neat): 3460, 3220 (br)  $\text{cm}^{-1}$  NH; 1660  $\text{cm}^{-1}$  C=O. Anal  $\text{C}_{10}\text{H}_7\text{N}_4\text{OSK}$  (C, H, N, S).

Converted into the corresponding acid 3-amino-1,2,3,5-tetrahydro-2-thioxo-4H-pyrimido[5,4-b]indol-4-one **9** by dropwise acidification under stirring of a suspension in water (100 mL) of **8** (6.1 mmol) with stoichiometric 37% hydrochloric acid (0.5 mL, 6.1 mmol). After 30 min the collected solid material was treated with 5%  $\text{NaHCO}_3$  and recrystallized from dioxane (yellow microcrystalline solid).

Yield: 50%; mp 285 °C (dec). IR (KBr)  $\nu$  (neat): 3280 (br)  $\text{cm}^{-1}$ ; 1670  $\text{cm}^{-1}$  C=O.  $^1\text{H-NMR}$  ( $\text{DMSO}-d_6$ ):  $\delta$  13.75 (br s, 1H, NH), 12.11 (br s, 1H, NH), 8.16 (m, 1H, arom), 7.43 (m, 2H, arom), 7.17 (m, 1H, arom), 6.47 (br s, 2H,  $\text{NH}_2$ ).  $^{13}\text{C-NMR}$  ( $\text{DMSO}-d_6$ ):  $\delta$  165.6 (1C), 138.4 (1C), 127.5 (1C), 126.0 (1C), 121.0 (1C), 120.1 (1C), 116.0 (1C), 114.1 (1C), 112.7 (1C). TLC system: ethyl acetate. Anal  $\text{C}_{10}\text{H}_8\text{N}_4\text{OS}$  (C, H, N, S).

*General procedures*

*Synthesis of 3-amino-4,5-dihydro-2-[2-alkyl- or aryl-2-oxoethylthio]pyrimido[5,4-b]indol-4(3H)-ones 10a,b*

To a stirred suspension of 3-amino-1,2,3,5-tetrahydro-2-thioxo-4H-pyrimido[5,4-b]indol-4-one monopotassium salt **8** (2.30 mmol) in ethanol (50 mL) an equimolar amount of the appropriate  $\alpha$ -haloketone was added and the mixture was gently heated under stirring. After cooling, the mixture was poured into water (100 mL) and the solid was collected and recrystallized from ethanol (amorphous solid).

**10a (methyl):** Yield: 66%; mp 257–259 °C (dec). IR (KBr)  $\nu$  (neat): 3320, 3180 (br)  $\text{cm}^{-1}$  NH; 1725, 1680  $\text{cm}^{-1}$  C=O. TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi = 50\%$ ). Anal  $\text{C}_{13}\text{H}_{12}\text{N}_4\text{O}_2\text{S}$  (C, H, N, S).

**10b (ethyl):** Yield: 79%; mp 229–233 °C (dec). IR (KBr)  $\nu$  (neat): 3320, 3180 (br)  $\text{cm}^{-1}$  NH; 1720, 1680  $\text{cm}^{-1}$  C=O. TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi = 50\%$ ). Anal  $\text{C}_{14}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$  (C, H, N, S).

*Synthesis of 3-amino-4,5-dihydro-2-[2-alkyl- or aryl-2-oxoethylthio]pyrimido[5,4-b]indol-4(3H)-ones 10c,d*

These intermediates were prepared from **8** and the appropriate 2-bromoacetophenone, similarly as **10a,b** stirring at room temperature and pouring the mixture into water (**10c**: 300 mL; **10d**: 100 mL).

**10c (phenyl):** Yield: 93%; mp 252–254 °C (dec). IR (KBr)  $\nu$  (neat): 3320, 3200 (br)  $\text{cm}^{-1}$ ; 1680  $\text{cm}^{-1}$  C=O. TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi = 50\%$ ). Anal  $\text{C}_{18}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$  (C, H, N, S).

**10d (4-methoxyphenyl):** Yield: 82%; mp 259–262 °C (dec). IR (KBr)  $\nu$  (neat): 3320, 3200 (br)  $\text{cm}^{-1}$  NH; 1680  $\text{cm}^{-1}$  C=O. TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi = 50\%$ ). Anal  $\text{C}_{19}\text{H}_{16}\text{N}_4\text{O}_3\text{S}$  (C, H, N, S).

*General procedures*

Compounds **11a–d** were prepared from the corresponding 3-amino-4,5-dihydro-2-[2-alkyl- or aryl-2-oxoethylthio]pyri-

mido[5,4-*b*]indol-4(3H)-ones **10a–d** by cyclodehydration with (*p*)-toluenesulphonic acid (*p*-TsOH). Compounds **11c,d** were also prepared by a one-step reaction from 3-amino-1,2,3,5-tetrahydro-2-thioxo-4H-pyrimido[5,4-*b*]indol-4-one **9** with the appropriate  $\alpha$ -haloketone (Method B).

*Synthesis of 3-alkyl-1,3,4-thiadiazino[2,3:2',1']pyrimido[5,4-*b*]indol-6-(7H)-one 11a,b (Method A)*

To a suspension of compound **10a,b** (1.04 mmol) in hot ethanol (25 mL) *p*-TsOH (0.13 mmol) was added. The obtained solution was refluxed for 5 h then cooled (**11a**) or frozen (**11b**). The solid material was collected, washed with warm ethanol and recrystallized from ethanol (pale yellow needles (**11a**) or pale yellow microcrystalline solid (**11b**)).

**11a (methyl):** Yield: 64%; mp 261–263 °C (dec). IR (KBr)  $\nu$  (neat): 3220 (br)  $\text{cm}^{-1}$  NH; 1690  $\text{cm}^{-1}$  C=O.  $^1\text{H-NMR}$  (DMSO- $d_6$ ):  $\delta$  12.13 (br s, 1H, NH), 7.94 (m, 1H, arom), 7.49 (m, 2H, arom), 7.20 (m, 1H, arom), 3.32 (s, 2H,  $\text{CH}_2$ ), 2.37 (s, 3H,  $\text{CH}_3$ ).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ ):  $\delta$  159.8 (1C), 152.1 (1C), 144.7 (1C), 139.1 (1C), 134.9 (1C), 127.3 (1C), 120.3 (1C), 120.1 (1C), 112.7 (1C), 25.0 (1C), 23.4 (1C). TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi$  = 50%). Anal  $\text{C}_{13}\text{H}_{10}\text{N}_4\text{OS}$  (C, H, N, S).

**11b (ethyl):** Yield: 58%; mp 242–244 °C (dec). IR (KBr)  $\nu$  (neat): 3220 (br)  $\text{cm}^{-1}$  NH; 1690  $\text{cm}^{-1}$  C=O.  $^1\text{H-NMR}$  (DMSO- $d_6$ ):  $\delta$  12.20 (br s, 1H, NH), 7.94 (m, 1H, arom), 7.49 (m, 2H, arom), 7.20 (m, 1H, arom), 3.77 (s, 2H,  $\text{CH}_2$ ), 2.67 (q,  $^3J_{\text{HH}} = 7.4$  Hz, 2H,  $\text{CH}_2$ ), 1.22 (t,  $^3J_{\text{HH}} = 7.4$  Hz, 3H,  $\text{CH}_3$ ).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ ):  $\delta$  163.4 (1C), 152.2 (1C), 145.0 (1C), 139.1 (1C), 134.9 (1C), 127.2 (1C), 120.3 (1C), 120.2 (1C), 120.0 (1C), 112.7 (1C), 30.2 (1C), 23.7 (1C), 10.0 (1C). TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi$  = 50%). Anal  $\text{C}_{14}\text{H}_{12}\text{N}_4\text{OS}$  (C, H, N, S).

*Synthesis of 3-aryl-1,3,4-thiadiazino[2,3:2',1']pyrimido[5,4-*b*]indol-6-(7H)-one 11c,d (Method A)*

To a suspension of compound **10c** (1.43 mmol) or **10d** (0.64 mmol) in hot ethanol (50 mL) *p*-TsOH (0.260 mmol) was added. The obtained solution was refluxed for 4 h then rapidly filtered, leaving the filtrate at room temperature for 16 h. The pooled solid materials were recrystallized from the appropriate solvent as yellow needles.

**11c (phenyl):** Yield: 36.4%; mp 265–266 °C (dec). IR (KBr)  $\nu$  (neat): 3200 (br)  $\text{cm}^{-1}$  NH (broad); 1690  $\text{cm}^{-1}$  C=O.  $^1\text{H-NMR}$  (DMSO- $d_6$ ):  $\delta$  12.20 (br s, 1H, NH), 8.05 (m, 2H, arom), 7.98 (m, 1H, arom), 7.58–7.45 (m, 5H, arom), 7.22 (m, 1H, arom), 4.33 (s, 2H,  $\text{CH}_2$ ).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ ):  $\delta$  156.4 (1C), 152.3 (1C), 144.8 (1C), 139.1 (1C), 134.9 (1C), 133.7 (1C), 131.5 (1C), 128.8 (2C), 127.5 (2C), 127.3 (1C), 120.4 (1C), 120.2 (1C), 120.1 (1C), 112.8 (1C), 22.5 (1C). TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi$  = 50%). Anal  $\text{C}_{18}\text{H}_{12}\text{N}_4\text{OS}$  (C, H, N, S).

**11d (4-methoxyphenyl):** Yield: 94%; mp 270–272 °C (dec). IR (KBr)  $\nu$  (neat): 3190 (br)  $\text{cm}^{-1}$  NH; 1670  $\text{cm}^{-1}$  C=O.  $^1\text{H-NMR}$  (DMSO- $d_6$ ):  $\delta$  12.17 (br s, 1H, NH), 8.02 (d,  $^3J_{\text{av}} = 8.8$  Hz, 2H, phenyl), 7.97 (m, 1H, arom), 7.51–7.44 (m, 2H, arom), 7.22 (m, 1H, arom), 7.11 (d,  $^3J_{\text{av}} = 8.8$  Hz, 2H, phenyl), 4.29 (s, 2H,  $\text{CH}_2$ ), 3.84 (s, 3H,  $\text{OCH}_3$ ).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ ):  $\delta$  162.0 (1C), 156.0 (1C), 152.2 (1C), 145.1 (1C), 139.2 (1C), 134.9 (1C), 129.3 (2C), 127.3 (1C), 125.8 (1C), 120.3 (1C), 120.2 (1C), 114.3 (2C), 112.7 (1C), 55.4 (1C), 22.3 (1C). TLC system: ethyl acetate, ethyl acetate–cyclohexane ( $\phi$  = 50%). Anal  $\text{C}_{19}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$  (C, H, N).

*Synthesis of 3-aryl-1,3,4-thiadiazino[2,3:2',1']pyrimido[5,4-*b*]indol-6-(7H)-one 11c,d (Method B)*

A suspension of compound **9** (6.0 mmol) and the appropriate 2-bromoacetophenone (6.3 mmol) in an ethanol–water mixture ( $\phi$  = 20%) was refluxed until no more of the starting material could be detected by TLC (7–8 h). After cooling, the solid material was collected, triturated with 5%  $\text{NaHCO}_3$ , washed with water and recrystallized from ethanol (yellow needles).

**11c (phenyl):** Yield: 36.4%.

**11d (4-methoxyphenyl):** Yield: 41%.

*Synthesis of 3-phenyl-1,3,4-thiadiazino[2,3:2',1']pyrimido[5,4-*b*]indol-6-(7H)-thione 12c*

To a hot solution of compound **11c** (0.60 mmol) in anhydrous xylene (10 mL) an excess of Lawesson's reagent (1.20 mmol) was added. The mixture was refluxed for 30 min under stirring then cooled. The solid material was collected, washed with cold ethanol and recrystallized from dioxane–water as yellow needles.

**12c:** Yield: 60%; mp 226–228 °C (dec). IR (KBr)  $\nu$  (neat): 3240  $\text{cm}^{-1}$  NH; 1560, 1115  $\text{cm}^{-1}$  C=S. TLC system: ethyl acetate–cyclohexane ( $\phi$  = 50%). Anal  $\text{C}_{18}\text{H}_{12}\text{N}_4\text{S}_2 + \text{C}_4\text{H}_8\text{O}_2$  (dioxane) (C, H, N, S).

Compounds **13a,b** were prepared by a new synthetic route as follows.

*Synthesis of 2-methyl-1,3,4-thiadiazolo[3',2']pyrimido[5,4-*b*]indol-5-(6H)-one 13a*

A mixture of compound **9** (3.02 mmol) and 97% triethyl orthoacetate (84.80 mmol) was refluxed for 13–14 h. After cooling the solid material was collected, washed with dioxane and recrystallized from acetic acid (white powder).

Yield: 90.5% (lit [26] 51.8%).

*Synthesis of 2-phenyl-1,3,4-thiadiazolo[3',2']pyrimido[5,4-*b*]indol-5-(6H)-one 13b*

Similarly prepared as **13a** from compound **9** (2.30 mmol) and triethyl orthobenzoate (63 mmol).

Yield: 41% (dimethylformamide) as yellow microcrystalline solid (lit [26] 60%).

Physico-chemical data of compounds **13a,b** are consistent with the ones of samples prepared by direct condensation of 3-amino-1H-indole-2-carboxylic acid ethyl ester **1** with 2-chloro-5-alkyl or -aryl substituted 1,3,4-thiadiazole according to Russo et al [26].

*Synthesis of 2-phenyl-1,3,4-thiadiazolo[3',2']pyrimido[5,4-*b*]indol-5-(6H)-thione 14b*

To a suspension of compound **13b** (0.47 mmol) in anhydrous xylene (10 mL) an excess of Lawesson's reagent (0.94 mmol) was added. The mixture was refluxed for 4 h under stirring then cooled. The solid material was collected, washed with cold ethanol and recrystallized from ethanol–water as a yellow powder.

**14b:** Yield: 25%; mp > 300 °C. IR (KBr)  $\nu$  (neat): 3320  $\text{cm}^{-1}$  NH; 1540, 1115  $\text{cm}^{-1}$  C=S. TLC system: ethyl acetate–cyclohexane ( $\phi$  = 50%). Anal  $\text{C}_{17}\text{H}_{10}\text{N}_4\text{S}_2 \times 0.5$  ethanol (C, H, N, S).

*Modelling study (semiempirical MO calculations and CoMFA)*

The entire study was performed using the Sybyl 6.2 molecular modeling software [38–40] installed on a SGI Indy workstation

Silicon Graphics 4400XZ and on a Silicon Graphics Power Challenge XL. The molecules were built starting from geometrically optimized standard fragments of the Tripos library. The charges were calculated using the Gasteiger–Marsili method [41].

The potential energy of each structure was fully refined using the MAXIMIN procedure within Sybyl. A short MD (molecular dynamics) simulation (10 ps at 300 K) was done and the lowest conformer was chosen for further minimization.

## Pharmacology

### *Assessments of potential antagonist effects at NK-1 and NK-2 receptors*

Compounds **11a–d**, **12c** and **13a,b** were tested as NK<sub>1</sub>- and NK<sub>2</sub>-receptor antagonists according to the method of Longmore et al [42, 43]. The effects of these compounds at NK-1 receptors were tested using guinea-pig ileum LM/MP and substance P O-methyl ester (SPOMe) as the agonist, and their effects at NK<sub>2</sub>-receptors were tested using guinea-pig trachea and Nle<sup>10</sup>NKA(4-10) as the agonist (table IV).

## Methods

### *NK<sub>1</sub>-receptor assay*

Strips of ileum longitudinal muscle/myenteric plexus (LM/MP) were obtained from male Dunkin Hartley guinea-pigs (250 g). The tissues were mounted for isometric tension recording in siliconised organ baths containing Krebs physiological salt solution (PSS, mM: NaCl 118, KCl 4.8, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 11.1), maintained at 37 °C, pH 7.4 and aerated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The PSS also contained methysergide maleate (1 µM), mepyramine (1 µM), indomethacin (1 µM) and atropine sulphate (1 µM). Under these conditions, contractile responses to SPOMe are mediated via direct activation of NK-1 receptors located on the smooth muscle cells. An initial resting tension of 1 g was placed on the tissues. Following a 60-min equilibration period, a sequential concentration–effect curve was obtained using SPOMe (control curve). Tissues were exposed to each concentration of agonist until a peak contractile response was obtained (approximately 3–5 min) and this was then followed by 5- and 10-minute wash off and re-equilibration periods, respectively. Following a 30 min equilibration period with the appropriate test compound, the concentration–effect curve to SPOMe was repeated. Appropriate vehicle tissues were used.

### *NK<sub>2</sub>-receptor assay*

Tracheal rings (approximately 3–5 mm in length) were obtained from male Dunkin Hartley guinea-pigs (250 g). The tissues were mounted for tension recording in siliconised organ baths containing Krebs physiological salt solution (PSS, mM: NaCl 118, KCl 4.8, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 11.1), maintained at 37 °C, pH 7.4 and aerated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The PSS also contained methysergide (1 µM), mepyramine (1 µM) and indomethacin (1 µM) to eliminate any indirect effects of the agonist and CP 99994 (0.1 µM) to eliminate any possible contractions due to NK<sub>1</sub>-receptor activation. The tissues were placed under an initial 1 g tension. Following a 60 min equilibration period, enzyme inhibitors (bestatin 1 µM, thiorphan 1 µM and bacitracin 0.04 g L<sup>-1</sup>) were added. A further 15 min later a cumulative

concentration–effect curve to Nle<sup>10</sup>NKA(4-10) was obtained (control curve). The tissues were then washed until the baseline tension was re-established. The test compound was then added directly to the appropriate organ bath. Following a 30 min equilibration period, the concentration–effect curve to Nle<sup>10</sup>NKA(4-10) was repeated in the presence of the enzyme inhibitors (see above). Appropriate vehicle tissues were used.

### *Analysis of results*

Responses were expressed as the percentage of the maximum of the initial concentration–effect curve (control).

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