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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · JULY 2006

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Original article

Heterocyclic analogs of benzanilide derivatives as potassium channel activators. IX

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Accepted 9 March 2006
Available online 19 April 2006

Abstract

On the basis of our previous works, addressed to synthesise new activators of BK potassium channels, and of many suggestions from the international literature, a simple pharmacophoric model, consisting of two suitably substituted phenyl rings bound to various kinds of linkers, was hypothesised. In particular, the effectiveness of the amidic linker was demonstrated, since several benzanilide derivatives showed interesting BK-opener properties. As a development of these benzanilides, in this work we introduced heterocyclic substituents, replacing the aryl ring on the acid side or on the basic one of the amide linker of the above pharmacophore.

The pharmacological results indicated some relevant remarks about the structural requirements, needed for a satisfactory BK-opener activity. In particular, the presence of a phenolic function, with a possible H-bond donor role, has been confirmed. Furthermore, the presence of nitrogen heterocycles on the acid side of the amide linker seems to be a negative requirement, while furan and thiophene were well tolerated. On the contrary, the introduction of insaturated heterocyclic rings (pyridine and thiazole) on the basic side of the amide linker, led to satisfactory biological activity, while the presence of aliphatic heterocycles lowered the pharmacological effect.

Keywords: Potassium channels; Potassium channel openers; BK-activators; Benzanilides; Heterocyclic amides

1. Introduction

The large conductance calcium-activated potassium channels (BK) are expressed in excitable as well as in non-excitable cells. They control several cell functions: in the nervous system, BK channels contribute to the shaping of action potential and modulate the neuronal excitability and the release of neurotransmitters; moreover, BK channels play a fundamental role in the regulation of the tone of smooth muscle cells [1,2].

The physiological activation of BK channels, induced mainly by two triggering signals, such as the rise of the intracellular free calcium ions and membrane depolarisation, ensures the massive flow of potassium ions (with a single channel conductance of 150–300 pS) to the extracellular side of the plasmalemma, the membrane hyperpolarisation and the reduction of the cellular excitability. Conversely, the availability of exogenous compounds able to activate BK channels can guarantee an innovative pharmacological tool for the clinical management of many pathological states, due to a cell hyperexcitability, such as asthma, urge incontinence and bladder spasm, gastric hypermotility, neurological and psychiatric disorders [1,2].

As concerns the cardiovascular system, it is now widely accepted that BK channels ensure the predominant component of the outward K^+ current in vascular smooth muscle cells, accounting for the fundamental function of such ion channels in the modulation of the muscular tone of vessels [3,4]. Consequently, the vasorelaxing effects of exogenous BK-openers can furnish the pharmacological rational basis for the treatment of hypertension and/or other diseases related to an impaired contractility of vessels (for example, coronary vasospasm) [1,2].

In a previous work [5], we could observe that the synthetised 1-(2′-hydroxybenzoyl)-5-methyl-benzotriazole, showing

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doi:10.1016/j.ejmech.2006.03.009

structural analogies with the reference BK-openers NS-004 and NS-1619 and exhibiting vasorelaxing effects probably due to the activation of vascular BK channels, was able to confer a significant protection of the myocardial function, in isolated rat hearts submitted to ischemia/reperfusion cycles. This result (originally unexpected) can be now explained thanks to more recent experimental evidence showing that the activation of cardiac calcium-activated potassium channels could be involved in the cardioprotective mechanisms of "ischemic preconditioning" and that the administration of BK-openers, such as NS-1619, could reduce the cardiac injury following an ischemic event [6–8]. Of course, these reports let us foresee a further potential use of BK-activators in cardiovascular pharmacotherapy, as anti-ischemic drugs.

Some years ago, we undertook a research program concerning synthesis and pharmacological experimentation of new compounds as BK channel openers. As a consequence 1,2,3-triazole derivatives [9–12], benzimidazoles [13,14] and benzotriazoles [5,13,14] were tested, providing good and encouraging results. A high pharmacological activity was also detected in some appropriately substituted benzanilide derivatives [15].

Upon the basis of these results and of the suggestions reported in the literature [16], a simple pharmacophoric model consisting of two suitably substituted phenyl rings bound to various kinds of linkers, was hypothesised (Fig. 1).

In order to support this pharmacophoric model and, in particular, the effectiveness of the amidic linker, by closer investigation of the structure–activity relationships, we decided to continue our research program developing the simple structures of benzanilide derivatives.

Thus, considering that the N-(2-hydroxy-5-chloro)-2-methoxy-5-chloro-benzanilide (Fig. 2) was the more active compound as a BK-opener, we began with a modification of the acid moiety of the benzanilide, introducing a heterocyclic ring in the place of the phenyl one but leaving unaltered the basic anilino substituent. Three five-membered rings (furan, thiophene, pyrrole) and the pyridine ring bound to give the three available substitution isomers (*ortho*, *meta* and *para* regarding to the heterocycle) were employed.

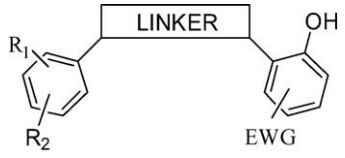


Fig. 1. Generic pharmacophoric model for a BK-opener, consisting of two aryl rings, spaced by an appropriate heterocyclic or acylcic linker. EWG = electron withdrawing group; R1 and R2 represent various kinds of possible substituents.

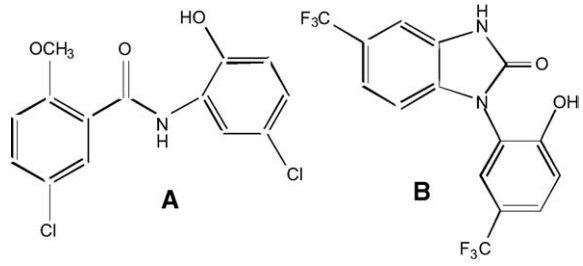


Fig. 2. Chemical structures of the previously synthesised benzanilide BK-opener (**A**), fitting the pharmacophoric model shown in Fig. 1, and of the reference benzimidazolone NS 1619 (**B**).

Furthermore, the heterocyclic substituent was introduced in the basic moiety of the benzanilide, employing the same three pyridine substituents and thiazole, morpholine and pyrrolidine rings.

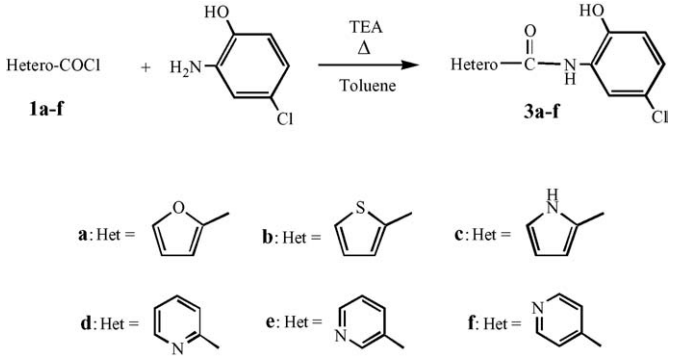
2. Chemistry

The usual procedure for amide synthesis (Scheme 1), by heating the appropriate heterocyclic acid chloride (**1a**–**f**) with 2-hydroxy-5-chloro-aniline (**2**) in toluene in the presence of triethylamine, provided the expected derivatives **3a**–**f** in good yields.

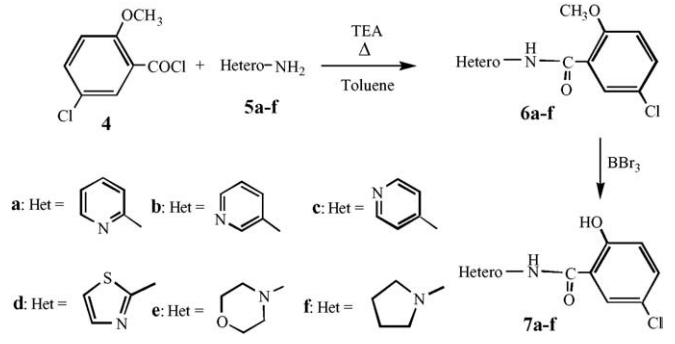
On the contrary (Scheme 2), the next analogous structural modification concerned the basic moiety of the benzanilide, by the introduction of a heterocyclic ring in the place of the aniline, leaving unaltered the acid benzoic substituent.

The pyridine ring, utilised according to the three available substitution isomers (*ortho*, *meta* and *para* with respect to the nitrogen atom), was chosen as a heterocyclic pattern. In fact the pyridine ring, when introduced in the acid moiety of the anilides, resulted more active than the experimented five-membered heterocycles.

Thus, by reaction of 2-methoxy-5-chloro-benzoic acid chloride (**4**) with the appropriate aminopyridine (**5a**–**c**) under the known experimental conditions, the new N-pyridyl-benzamides **6a**–**c** were prepared in good yield.



Scheme 1. Synthetic route for the target compounds **3a**–**f**.



Scheme 2. Synthetic route for the target compounds **7a**–**f**.

Further modifications of the basic moiety of the anilide consisted of the introduction of a thiazole ring (compound **6d**), a five-membered heterocycle bearing two heteroatoms and of the introduction of an aliphatic amine in place of the aniline. The compounds **6e** and **6f**, bearing a morpholine and pyrrolidine ring, were selected as patterns of six- and five-membered aliphatic heterocycles, respectively.

As seen [15,16], one of the requirements for the opening of the BK channels would appear to be the presence of an acid hydroxylic function on at least one of the aromatic rings of the pharmacofore. Therefore the methoxy group present on the acid moiety of the N-pyridyl-benzamides **6a–c** and of the other benzamides **6d–f** underwent a cleavage with boron tribromide in dichloromethane at –40 °C to give the corresponding phenol amides **7a–f**.

The structures of all the prepared compounds were confirmed by analytical and spectroscopic methods (Tables 1 and 2).

3. Pharmacology

The vasodilating effect of the novel potential BK-openers on the vascular contractile function was studied in vitro using isolated rat aortic rings precontracted with KCl 20 mM (see later for the pharmacological details).

4. Results and discussion

Since a vasorelaxing activity on endothelium-denuded rat aortic rings is a pharmacodynamic property of BK-activators [17], this experimental model was chosen for the preliminary screening of the target compounds **3a–f, 6a–f** and **7a–f** (Table 3).

Compounds **6e**, **6f** and **7e** were almost ineffective and **3d**, **6b** and **7f** showed weak vasorelaxing efficacy (< 50%), while compounds **3c**, **3e**, **3f**, **6a**, **6c**, **6d** and **7a** exhibited a partial vasorelaxing efficacy (ranging between 50% and 90%). Finally, compounds **3a**, **3b**, **7b**, **7c** and **7d** exhibited almost full vasorelaxing efficacy (≥ 95%). All the compounds exhibiting significant vasorelaxing efficacy (>50%) showed a potency index slightly lower than that exhibited by the reference benzimidazolone NS1619.

The pharmacological results allow us to make some initial observations concerning the structure–activity relationships. Compounds **3c–f**, all exhibiting a nitrogen heterocycle in the acidic side of the amide spacer, showed only a partial vasorelaxing efficacy. On the contrary, the insertion of other heterocycle moieties in the acidic side of the amide linker, such as furan and thiophene (compounds **3a** and **3b**, respectively), seems to be well tolerated, because **3a** and **3b** resulted full vasodilators. This evidence seems to indicate that the presence of a nitrogen atom in the heterocycle at the acidic side of the amide spacer causes a detrimental impact.

Instead, the introduction of pyridine cycles in the basic side of the amide linker does not seem to determine, per se, negative effects, as compounds **7b** and **7c** showed full vasorelaxing efficacy. The lower efficacy exhibited by compound **7a** is probably due to the position *ortho* of the nitrogen atom of the pyridine ring. The data actually available do not allow us an exhaustive explanation for the negative effect exerted by the nitrogen atom in position *ortho*, however, the following observations might suggest a possible key of interpretation.

According to our previous studies [10,11,15] and to a generally accepted pharmacophore model [1], the presence of the hydroxy group, as a H-bond donor, seems to be an important structural requirement for the interaction with BK channels. This hypothesis is well supported by the observation of the vasorelaxing effects exerted by compounds **7b** and **7c** (full va-

Table 1
Physico-chemical properties

Compounds	Heterocyclic substituent	Yield (%)	Crystallisation solvent	M.p. (°C)	IR (cm^{-1})	Mass *m/z* M^+	base
3a	2-Furyl	74	MeOH	230–232	1648 (CO), 3388 (NH)	237	95
3b	2-Thienyl	80	MeOH/H_2O	213–215	1636 (CO), 3375 (NH)	253	100
3c	2-Pyrrolyl	74	MeOH/H_2O	210–212	1626 (CO), 3402 (NH)	236	94
3d	2-Pyridyl	77	MeOH/H_2O	122–125	1691 (CO), 3325 (NH)	248	106
3e	3-Pyridyl	75	MeOH/H_2O	227–230	1648 (CO), 3331 (NH)	248	106
3f	4-Pyridyl	70	MeOH/H_2O	252–256	1676 (CO), 3408 (NH)	248	106
6a	N-(2-pyridyl)	72	MeOH	142–145	1667 (CO), 3341 (NH)	262	169
6b	N-(3-pyridyl)	79	MeOH/H_2O	169–171	1668 (CO), 3330 (NH)	262	169
6c	N-(4-pyridyl)	75	MeOH/H_2O	166–168	1669 (CO), 3328 (NH)	262	169
6d	N-(2-thiazolyl)	86	MeOH/H_2O	139–141	1655 (CO), 3313 (NH)	268	169
6e	N-(morpholine)	48	MeOH/H_2O	145–148	1669 (CO), 3301 (NH)	271	169
6f[a]	N-(pyrrolidine)	36	EtOAc/hexane	103–105	1647 (CO), 3313 (NH)	255	169
7a	N-(2-pyridyl)	63	MeOH/H_2O	227–230	1690 (CO), 3340 (NH) combinatorial bands	248	155
7b[b]	N-(3-pyridyl)	50	MeOH	267–270	1648 (CO), combinatorial bands	248	155
7c[c]	N-(4-pyridyl)	54	MeOH	285–287	1680 (CO), combinatorial bands	248	155
7d	N-(2-thiazolyl)	66	MeOH	272–273	1671 (CO), 3253 (NH and OH)	254	155
7e	N-(morpholine)	56	H_2O	187–188	1636 (CO), 3203 (NH), 3063 (OH)	256	86
7f	N-(pyrrolidine)	47	EtOAc/hexane	200–202	1637 (CO), 3257 (NH and OH)	240	85

[a] Characterised as hydrochloride.
[b] isolated and characterised as hydrobromide.
[c] isolated and characterised as hydrobromide, melting point of free base 162–164 °C.

Table 2
^{1}H-NMR in DMSO-d_6, ppm from TMS

3a ($C_{11}H_8ClNO_3$): (6.72 m, 7.32 d, 7.95 d, 3 H, furyl); (6.93 d, 7.02 d, 7.98 s, 3 H, arom.); 9.1 (NH); 10.5 (OH)
3b ($C_{11}H_8ClNO_2S$): (7.21 d, 7.68 d, 7.86 d, 3 H, thienyl); (6.92 d, 7.07 d, 7.99 s, 3H, arom.); 9.5 (NH); 10.1 (OH)
3c ($C_{11}H_9ClN_2O_2$): (6.18 m, 6.89 d, 7.00 d, 3 H, pyrryl); 6.92 d, 7.03 d, 7.82 s, 3 H, arom.); 9.0 (NH); 10.2 (OH); 11.8 (NH)
3d ($C_{12}H_9ClN_2O_2$): (7.74 m, 8.17 m, 8.44 d, 8.77 d, 4 H, 2-pyridyl); (6.92 d, 7.09 d, 8.05 s, 3H, arom.); 10.4 (NH); 10.6 (OH)
3e ($C_{12}H_9ClN_2O_2$): (7.59 m, 8.29 d, 8.76 d, 9.10 s, 4 H, 3-pyridyl); 56.92 d, 7.10 d, 7.77 s, 3 H, arom.); 9.9 broad (NH, OH)
3f ($C_{12}H_9ClN_2O_2$): (7.84 d, 8.76 d, 4 H, 4-pyridyl); (6.92 d, 7.11 d, 7.75 s, 3 H, arom.); 10.0 broad (NH, OH)
6a ($C_{13}H_{11}ClN_2O_2$): 3.96 (s, 3H, OMe); [7.17 d, 7.87 d, 8.20 d, 8.37 d, 4 H, N-(2-pyridyl)]; (7.27 d, 7.60 d, 7.78 s, 3 H, arom.); 10.5 (NH)
6b ($C_{13}H_{11}ClN_2O_2$): 3.88 (s, 3 H, OMe); [7.39 q, 8.16 d, 8.34 d, 8.84 s, 4 H, N-(3-pyridyl)]; (7.22 d, 7.57 d, 7.64 s, 3 H, arom.); 10.4 (NH)
6c ($C_{13}H_{11}ClN_2O_2$): 3.87 (s, 3 H, OMe); [7.68 d, 8.46 d, 4 H, N-(4-pyridyl)]; (7.22 d, 7.55 d, 7.59 s, 3 H, arom.); 10.5 (NH)
6d ($C_{11}H_9ClN_2O_2S$): 3.92 (s, 3 H, OMe); [7.33 d, 7.56 d, 2 H, N-(2-thiazolyl)]; (7.25 d, 7.62 d, 7.69 s, 3 H, arom.); 4.12 broad (NH)
6e ($C_{12}H_{15}ClN_2O_3$): 3.82 (s, 3 H, OMe); (2.84 m, 3.64 m, 8 H, morpholine); (7.17 d, 7.46 s, 7.47d, 3 H, arom.); 9.8 broad (NH)
6f ($C_{12}H_{15}ClN_2O_2$): 3.85 (s, 3 H, OMe); (2.01 t, 3.56 t, 8 H, pyrrolidine); (7.22 d, 7.59 d, 7.64 s, 3 H, arom.); 11.7 (NH)
7a ($C_{12}H_9ClN_2O_2$): [7.18 m, 7.86 t, 8.24 d, 8.36 d, 4 H, N-(2-pyridyl)]; (7.08 d, 7.49 d, 7.97 s, 3 H, arom.); 10.9 (NH); 12.1 (OH)
7b ($C_{12}H_9ClN_2O_2$.HBr): [8.05 m, 8.68 d, 8.71 d, 9.34 s, 4 H, N-(3-pyridyl)]; (7.10 d, 7.50 d, 7.82 s, 3 H, arom.); 11.0 (NH); 11.5 broad (OH); 13.4 b (H, hydrobromide)
7c ($C_{12}H_9ClN_2O_2$.HBr): [7.72 d, 8.48 d, 4 H, N-(4-pyridyl)]; (7.00 d, 7.47 d, 7.81 s, 3 H, arom.); 10.8 (NH); 3.8 broad (OH)
7d ($C_{10}H_7ClN_2O_2S$): [7.27 d, 7.58 d, 2 H, N-(2-thiazolyl)]; (7.01 d, 7.46 d, 7.91 s, 3 H, arom.)]; 12.6 broad (NH, OH)
7e ($C_{11}H_{13}ClN_2O_3$): (2.88 m, 3.66 m, 8 H, morpholine); (6.95 d, 7.42 d, 7.84 s, 3 H, arom.); 9.8 broad (NH); 12.2 broad (OH)
7f ($C_{11}H_{13}ClN_2O_2$): (1.81 s, 2.94 s, 8 H, pyrrolidine); 6.91 d, 7.37 d, 7.82 s, 3 H, arom.); 9.6 broad (NH); 12.3 broad (OH)

Table 3
Experimental results: efficacy (Emax %) and potency values of the tested compounds

Compounds	Emax %	pIC_{50}
3a	100	4,82 ± 0.06
3b	95 ± 11	4,93 ± 0.05
3c	75 ± 4	4,87 ± 0.08
3d	41 ± 16	N.C.
3e	61 ± 2	4,99 ± 0.17
3f	55 ± 10	4,57 ± 0.26
6a	67 ± 12	4,77 ± 0.09
6b	37 ± 14	N.C.
6c	84 ± 2	4,91 ± 0.02
6d	51 ± 14	4,51 ± 0.07
6e	Ineffective	N.C.
6f	Ineffective	N.C.
7a	52 ± 15	4,51 ± 0.07
7b	99 ± 1	4,82 ± 0.03
7c	98 ± 2	4,93 ± 0.03
7d	97 ± 3	4,80 ± 0.02
7e	Ineffective	N.C.
7f	45 ± 13	N.C.
NS1619	100	5.23 ± 0.07

N.C. indicates that the parameter of potency could not be calculated because of the low efficacy (<50%).

sodilators) and of the reduction of the vasorelaxing effects found in the analogous methoxylated compounds **6b** and **6c**. On the contrary, the weak vasorelaxing efficacy of compound **7a** (bearing an OH group) is not lowered in the methoxylated analogue **6a**. In other words, the presence of the hydroxy group in compound **7a** does not seem to represent a useful requirement, while it is important in compounds **7b** and **7c**.

It can be suggested that the position *ortho* of the nitrogen atom in the pyridine ring could ensure an intramolecular interaction with the phenolic hydroxy group, so that this hydroxy group appears less available for a H-bond interaction with the receptor site.

The introduction of a thiazole ring in the basic side of the amide linker seems to be well tolerated, since compound **7d** showed full vasorelaxing effects. The replacement of the hydroxy group of **7d** with a methoxy substituent (compound **6d**) caused a negative impact on the vasorelaxing activity. Thus, the presence of the nitrogen atom in position *ortho* of the thiazole ring of compound **7d** does not seem to determine a deleterious impact, as above observed for compound **7a**. This different behaviour could be probably explained by the basic properties of the thiazole ring (very lower than those of the pyridine ring), which could not be sufficient to ensure a significant interaction with the phenolic hydroxy group.

The introduction of morpholine and pyrrolidine heterocycles in the basic side of the amide spacer (compounds **7e** and **7f**, respectively), led to ineffective (**7e**) or weakly effective (**7f**) vasodilators. Again, the weak vasorelaxing effects of **7f** were completely abolished in the methoxy analogue **6f**.

As a preliminary explanation for the fall in vasorelaxing activity of **7e** and **7f**, it can be observed that the heterocyclic moieties of these compounds (morpholine and pyrrolidine) are devoid of any insaturation and this absence of olephynic bonds could be the cause of the inadequate function of such substituents. Indeed, as described in literature [19–21] and in our previous reports [12,15], an aromatic ring (often substituted with a electron withdrawing group) is a fundamental element for the activity, because of its possible involvement in π–π stacking interaction with a receptorial area.

To date, our studies on these derivatives do not allow us to recognise whether the influence of the amide linker is due to the presence of such a specific function or to the ability to ensure a correct 3D arrangement of the molecule according to the receptorial site.

Future synthetic efforts will be addressed to further modification of the amide structure in order to define the pharmacophoric model and the role of the amide linker.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Kofler hot-stage and are uncorrected. IR spectra in nujol mulls were recorded on a Mattson Genesis series FTIR spectrometer. ^{1}H NMR spectra were recorded with a Varian Gemini 200 spectrometer in

DMSO-d_6 or $CDCl_3$, in δ units, using TMS as internal standard. Mass spectra were performed with a Trace GC Q plus, thermo quest Finnigan. TLC data were obtained with Merck silica gel 60 F_{254} aluminium sheets, using the elution mixture reported for the flash-chromatographies. Elemental analyses (C, H, N) were within ± 0.4% of the theoretical values and were performed on a Carlo Erba Analyser Mod. 1106 apparatus.

*5.1.1. Heterocyclic 2-hydroxy-5-chloro-anilides (**3a–f**)*

A solution of 6.0 mmoles of the suitable carboxylic acid **1a**–**f** (2-furoic, 2-thienyl, 2-pyrryl, 2-pyridyl, 3-pyridyl and 4-pyridyl, respectively) in 6 ml of $SOCl_2$ was heated under reflux for 30 min. The solution was evaporated in vacuo and the residue was dissolved in 40 ml of anhydrous toluene. This solution was then added to a solution of 2-hydroxy-5-chloro-aniline (**2**) (0.860 g, 6.0 mmol) and triethylamine (0.9 ml, 6.5 mmol) in 40 ml of anhydrous toluene and the mixture was heated under reflux for 6 h (compound **3c** heating at 80 °C).

For the isolation of **3a**, the solvent was evaporated in vacuo and the solid residue obtained was dissolved in $CHCl_3$. This solution was washed with 10% HCl, 5% $NaHCO_3$, then it was dried ($MgSO_4$) and evaporated to give **3a** which was purified by crystallisation (Table 1).

For the isolation of **3b**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration. Crystallisation of this precipitate from MeOH/H_2O provided a fraction of the expected amide. The filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution was washed with 10% HCl, 5% $NaHCO_3$ and dried ($MgSO_4$). Evaporation of the solvent gave another corresponding fraction of **3b** (Table 1).

For the isolation of **3c**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration. Crystallisation of this precipitate from MeOH/H_2O provided a solid polymeric material which was rejected. The filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution was extracted with 10% HCl, 20% NaOH and dried ($MgSO_4$). Evaporation of the solvent left a negligible residue. Neutralisation of the alkaline extract provided a bulky precipitate which was collected by filtration. This precipitate was treated with 5% $NaHCO_3$ and the insoluble material, consisting of **3c**, was collected and purified by crystallisation (Table 1).

For the isolation of **3d**, **3e** and **3f** the toluene solution, after cooling, separated a crystalline solid which was collected by filtration. Crystallisation of this precipitate from MeOH/H_2O provided the expected compound (Table 1); compound **3e** was characterised as hydrochloride. The filtrate worked up in the usual manner (evaporation, solution in $CHCl_3$, washings, …) did not give other products.

*5.1.2. N-heterocyclic 2-methoxy-5-chloro-benzamides (**6a–f**)*

A solution of 5-chloro-2-methoxy-benzoic acid (**4**) (0.653 g, 3.50 mmol) in 4 ml of $SOCl_2$ was heated under reflux for 50 min. The solvent was evaporated in vacuo and the solid residue was dissolved in 20 ml of anhydrous toluene. This solution was then added to a solution consisting of 3.50 mmoles of the appropriate amine **5a**–**f** (2-aminopyridine, 3-aminopyridine, 4-aminopyridine, 2-aminothiazole, N-aminomorpholine and N-aminopyrrolidine, respectively) and triethylamine (0.70 ml, 5.1 mmol for compounds **6a**–**c**; 0.48 ml, 3.5 mmol for compounds **6d**–**f**) in 20 ml of anhydrous toluene and the mixture was heated under reflux for a night.

For the isolation of **6a**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration and consisted of triethylamine hydrochloride. The filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution was extracted with 5% $NaHCO_3$ and 10% HCl. After drying ($MgSO_4$) and evaporation of the solvent, the chloroform solution did not leave a residue. The alkaline solution by acidification gave traces of solid residue. From the acid extract, by neutralisation, **6a** precipitated as a white solid which was collected by filtration and purified by crystallisation (Table 1).

For the isolation of **6b** and **6c**, the toluene solution, after cooling, separated a bulky precipitate which was collected by filtration and treated with H_2O and 5% $NaHCO_3$. The insoluble material, consisting of **6b** or **6c**, was purified by crystallisation (Table 1). The toluene filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution was extracted with 5% $NaHCO_3$ and 10% HCl. From the acid solution, by neutralisation, a further amount of **6b** or **6c** precipitated (Table 1). It is worth noting that sometimes the extraction of the chloroform solution with 10% HCl caused precipitation of the corresponding hydrochlorides to the interface of the two liquid phases.

For the isolation of **6d**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration and consisted of triethylamine hydrochloride. The filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution was extracted with 5% $NaHCO_3$ and 10% HCl. Acidification of the alkaline solution and neutralisation of the acid solution did not give precipitates. The chloroform solution, after drying ($MgSO_4$) and evaporation of the solvent, provided the amide **6d** which was purified by crystallisation (Table 1).

For the isolation of **6e** and **6f**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration and consisted of triethylamine hydrochloride. For **6f**, sometimes the treatment of this crystalline precipitate with H_2O left a moderate amount of insoluble material consisting of **6f** hydrochloride. The toluene filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution was extracted with H_2O and 5% $NaHCO_3$. The chloroform layer, dried ($MgSO_4$) and evaporated, provided **6e** or **6f** as a crystalline residue which was purified by crystallisation (Table 1).

*5.1.3. N-heterocyclic-2-hydroxy-5-chloro-benzamides (**7a–f**)*

To a solution of 2.0 mmoles of the suitable methoxy derivative **6a**–**f** in 120 ml of anhydrous CH_2Cl_2, cooled at –10 °C, a solution of BBr_3 (1.9 ml, 20 mmol) in 8 ml of anhydrous

CH_2Cl_2 was added drop by drop, under stirring. After 1 h at this temperature, the reaction mixture was transferred at –20 °C and left for a night. The excess of the reagent was decomposed by cautious addition of MeOH (10 ml) and H_2O (30 ml), then the organic phase was separated and washed with portions of H_2O (30 ml) which were combined with the aqueous phase.

For the isolation of **7a** and **7d**, which is reported in the literature prepared by another route [18], the organic phase was extracted with 10% NaOH from which, by neutralisation, precipitated the product which was collected by filtration and purified by crystallisation (Table 1). From the original acid aqueous phase, by neutralisation with 10% NaOH, further product precipitated as a suspension and it was isolated by extraction with $CHCl_3$ and evaporation of the solvent (Table 1).

For the isolation of **7b**, when the organic phase (CH_2Cl_2) was washed with H_2O, a solid precipitated to the interface of the two liquid phases, which was collected by filtration. Crystallisation from MeOH gave **7b** hydrobromide (Table 1).

For the isolation of **7c**, the organic phase (CH_2Cl_2), dried ($MgSO_4$) and evaporated, left only traces of solid residue. The original acid aqueous phase, by neutralisation with 10% NaOH, separated a light precipitate (thickening) which had to be isolated by extraction with $CHCl_3$. But the $CHCl_3$ addition caused precipitation of a solid to the interfaces of the two liquid phases, consisting of **7c** hydrobromide, which was collected by filtration (Table 1). A portion of the previous hydrobromide was treated with 5% $NaHCO_3$ and the insoluble material, consisting of **7c**, was collected and purified (Table 1).

For the isolation of **7e**, the organic phase (CH_2Cl_2) was extracted with 10% NaOH. From the combined alkaline extracts, by neutralisation, **7e** precipitated very slowly as a white crystalline solid which was collected by filtration (Table 1). The acid aqueous phase, by neutralisation, did not give further product.

For the isolation of **7f**, the organic phase was extracted with 10% NaOH from which, by neutralisation, precipitated the product as a light suspension which had to be isolated by extraction with $CHCl_3$ and evaporation of the solvent (Table 1). From the original acid aqueous phase, by neutralisation, further product separated as a suspension which had to be isolated by extraction with $CHCl_3$ (Table 1).

5.1.4. Pharmacology

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. A possible vasodilator mechanism of action was investigated by testing the effects of the compounds on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, the rats were sacrificed by cervical dislocation and bleeding. The aortae were immediately excised and freed of extraneous tissues. The endothelial layer was removed by gently rubbing the intima surface of the vessels with a hypodermic needle. Five millimetres wide aortic rings were suspended, under a preload of 2 g, in 20 ml organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with a unirecord microdynamometer (Buxco Electronics).

After an equilibration period of 60 min, endothelial integrity was confirmed by the administration of acetylcholine (Ach, 10 μM) to KCl (20 mM)-precontracted vascular rings. A relaxation < 10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs showing a relaxation ≥ 10% (i.e. significant presence of endothelium), were discarded. From 30 to 40 min after confirmation of the endothelium removal, the aortic preparations were contracted by treatment with a single concentration of KCl (20 mM) and, when the contraction reached a stable plateau, threefold increasing concentrations of the tested compounds or of the reference drug NS 1619 (a well-known BK-activator) were added cumulatively. Each compound was tested in 5–10 experiments. Preliminary experiments showed that the KCl (20 mM)-induced contractions remained in a stable tonic state for at least 40 min. The reference drug NS 1619 (Sigma) was dissolved (10 mM) in EtOH 95% and further diluted in Tyrode solution. Acetylcholine chloride (Sigma) was dissolved (100 mM) in EtOH 95% and further diluted in bidistilled water whereas KCl was dissolved in Tyrode solution. All the synthesised derivatives were dissolved (10 mM) in DMSO, and then diluted in Tyrode solution. All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the vehicles. The vasorelaxing efficacy was evaluated as maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 20 mM. When the limit concentration 30 mM (the highest concentration, which could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 20 mM, evoked by this limit concentration. The parameter of potency was expressed as $pIC_{50,}$ calculated as a negative Logarithm of the molar concentration of the compounds tested, evoking a half reduction of the contractile tone induced by KCl 20 mM. The pIC_{50} value could not be calculated for those compounds that showed an efficacy value < 50%. Compounds exhibiting an efficacy level < 20% were considered as ineffective. The parameters of efficacy and potency were expressed as mean ± standard error, for 5–10 experiments. Student's *t*-test was selected as a statistical analysis, $P < 0.05$ was considered representative of a significant statistical difference. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 3.0).

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