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Original article

Structural modifications of benzanilide derivatives, effective potassium channel openers. X.

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Abstract

Large-conductance calcium-activated potassium (BK) channels are involved in many fundamental cell functions. Consistently, the ability to activate BK channels by exogenous compounds is considered as a promising pharmacodynamic pattern for the potential treatment of several pathologies. In this perspective, the development of new and selective BK-openers can be considered as an actual field of research. This paper reports the synthesis and pharmacological evaluation of new benzanilides, useful for deepening the comprehension of the structure–activity relationships, emerged in previous studies on this class of BK-activators. From a structural point of view, these benzanilides belong to a general class of BK-activators, showing a common pharmacophoric model, consisting of two aryl groups linked through an appropriate ''spacer'' and the almost obligatory presence of a phenolic hydroxyl. In particular, a new series of benzanilides, in which the phenyl rings have been widely changed both on the acidic portion and the basic one of the amide spacer, were synthesised. Their vasorelaxing effects, induced through the activation of BK channels, were also evaluated. Although many compounds exhibited effects which could not be attributed to the activation of BK channels, two derivatives showed a clear profile of BK-activators with vasodilator activity comparable to or slightly lower than that recorded for the reference benzimidazolone **NS1619**. A further molecular modelling approach allowed us to obtain a molecular electrostatic potential feature which suggests a suitable interaction with the receptor site of the BK channel, from a tri-dimensional point of view. This approach seems to represent a further contribution for the development of new BK-activators, designed on the basis of the pharmacophoric model above-mentioned.

Keywords: BK channel; Benzanilide; BK-activator; Molecular modelling

1. Introduction

The large-conductance calcium-activated potassium (BK) channels 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; also, 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 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, membrane hyperpolarisation and reduction of the cellular excitability. Conversely, the availability of exogenous compounds capable of activating BK channels can guarantee an innovative pharmacological tool for the clinical management of many pathological states, due to cell hyperexcitability,

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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 synthesised 1-(2′-hydroxybenzoyl)-5-methyl-benzotriazole, showing structural analogies with the reference BK-openers **NS004** and **NS1619** (Fig. 1A) and exhibiting vasorelaxing effects probably due to the activation of vascular BK channels, was able to confer significant protection to 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 **NS1619**, could reduce 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 the synthesis and pharmacological experimentation of new compounds such 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. High pharmacological activity was also detected in some appropriately substituted benzanilide derivatives [15].

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

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

Thus, considering that *N*-(2-hydroxy-5-chloro-phenyl)-2-methoxy-5-chloro-benzamide (Fig. 2A) was the most active compound as BK-opener, we began [17] with a modification of the acid moiety of the benzanilide, introducing a heterocyclic ring (furan, thiophene, pyrrole and pyridine) in place of the phenyl ring but leaving unaltered the basic anilino substituent (Fig. 2B). The next analogous structural modification considered the basic moiety of benzanilide, by the introduction of a heterocyclic ring (pyridine, thiazole, morpholine and pyrrolidine) in place of the aniline, leaving unaltered the benzoic acid substituent. In this case the useful phenol function was introduced by cleavage of the *ortho*-methoxy substituent on the acid moiety (Fig. 2C). The pharmacological results indicated that the presence of nitrogen heterocycles on the acid side of the amide linker seems to be a negative requirement, while furan and thiophene rings are well tolerated. On the contrary, the introduction of unsaturated 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. The presence of a phenolic function as a probable H-bond donor was confirmed.

In this new paper, concerning a further deepening of the structure–activity relationships of benzanilide derivatives previously studied as BK channel activators, some substitutions on the acid or basic moiety of the reference benzanilides, showing particular and specific properties from a mesomeric and/or steric point of view, have been taken into consideration.

2. Chemistry

Scheme 1 reports the preparation of three benzanilides **4a–c**, corresponding to the compounds previously prepared [5,15], in whose acid moiety a chlorine atom was introduced to increase the acid property of the phenolic function, whilst the substituents present on the basic moiety were maintained. Thus the 2-methoxy-5-chloro-benzoyl chloride (**1**) reacted with 2-nitro-4-methyl- (**2a**), 2-nitro-4-methoxy- (**2b**) or 2-methoxy-4-nitro-aniline (**2c**) in refluxing toluene in the presence of triethylamine, to give the corresponding benzanilides **3a–c** in good yield. The next reactions of demethylation of the methoxy substituent, carried out with excess of boron tribromide in dichloromethane at −20 °C overnight, caused the cleavage of the methoxy groups present in the *ortho* position of the benzoic moiety of the amides **3a–c**, because adjacent

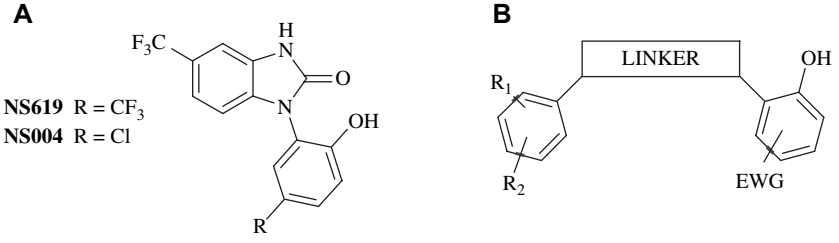


Fig. 1. A – Benzimidazolones **NS004** and **NS1619**. B – Generic pharmacophoric model for a BK-opener, consisting of two aryl rings, spaced by an appropriate heterocyclic or acyclic linker. EWG = electron-withdrawing group; R^1 and R^2 represent various kinds of possible substituents.

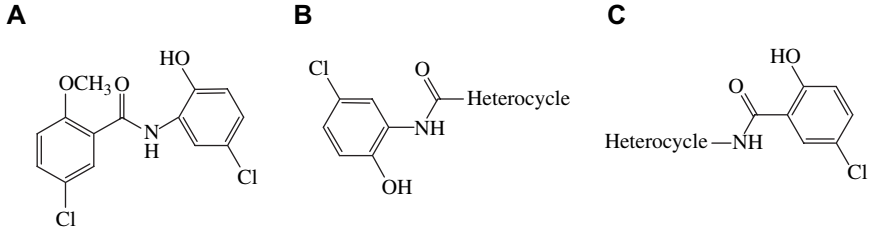


Fig. 2. A – Previously synthesised compounds. (A) The most active benzanilide as BK-opener; (B) and (C) modified benzanilides by introduction of heterocyclic substituents.

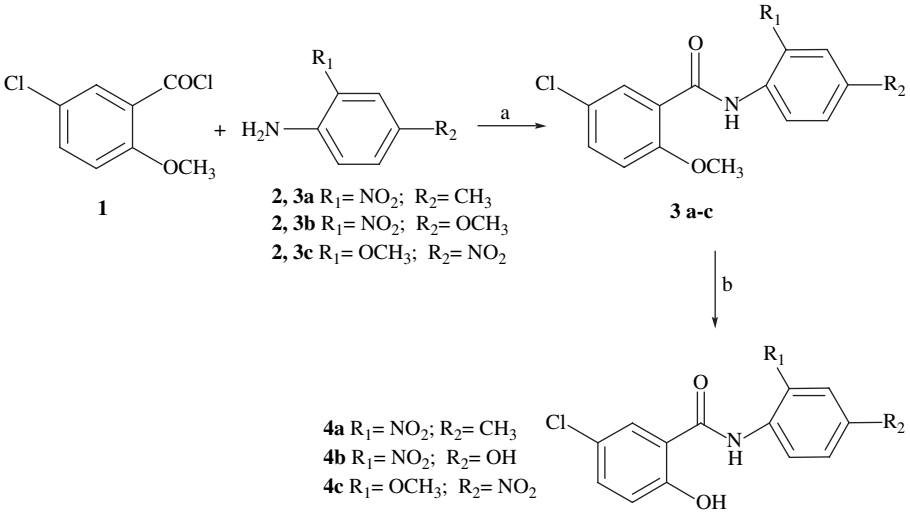
to a carbonyl function which can stabilise the complex with boron tribromide [15,18]. The methoxyl in the *para* position of the anilino moiety was also demethylated in the same manner to give the dihydroxy derivative **4b**, but under these experimental conditions, the methoxyl in the *ortho* position of the anilino substituent of **3c** was left unaltered.

In Scheme 2 is reported the preparation of a series of benzanilides **7a–h**, where the substituent in the *ortho* position of the benzoic moiety was changed increasing gradually its steric hindrance, so that the contribution to the pharmacological activity of this structural pattern could be evaluated. In addition, to better evaluate the possible role of the oxygen atom as a hydrogen bond acceptor, in the methoxy and ethoxy derivatives, the *ortho*-fluoro derivatives **7i** and **7l** were considered. The basic moiety of the anilides was exactly similar to that of previously synthesised active compounds. Therefore 2-hydroxy-5-chloroaniline (**6a**) and 2-hydroxy-5-methylaniline (**6b**) were employed. They reacted in the usual manner with benzoic (**5a**), 2-methylbenzoic (**5b**), 2-methoxybenzoic (**5c**), 2-ethoxybenzoic (**5d**) or 2-fuorobenzoic (**5e**) acyl chlorides to give the corresponding benzanilides **7a–l**.

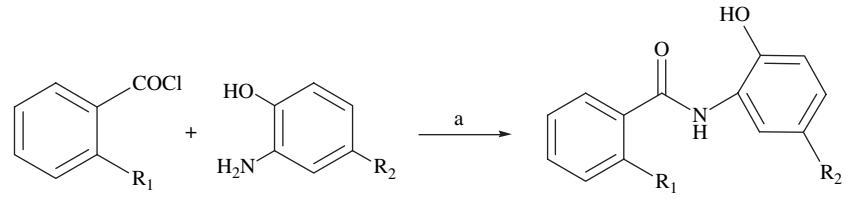
In Scheme 3 a further modification of the acid moiety of the benzanilide is reported, corresponding to the removal of the substituent in the *ortho* position and to the introduction of an electron-withdrawing group in the *meta* position, to give compounds **9a–d**. The chlorine and iodine atoms were chosen as substituents, to differentiate the mesomeric and steric effects. The basic moiety of the new benzanilides was kept corresponding to that of the more active derivatives, employing again 2-hydroxy-5-chloroaniline (**6a**) and 2-hydroxy-5-methylaniline (**6b**). Thus starting from 3-chlorobenzoyl chloride (**8a**), by reaction with the anilines **6a** and **6b**, the corresponding amides **9a** and **9b** were prepared, while by the reaction of 3-iodobenzoyl chloride (**8b**) with the same anilines, the derivatives **9c** and **9d**, respectively, were obtained.

Finally, in order to evaluate the effect of a further displacement of the substituent on the aromatic ring of the acid moiety of the benzanilide, from the *meta* to the *para* position, in Scheme 4 the synthesis of the benzanilides **11a–d** is reported. As new substituents a fluorine atom, a strong electron-withdrawing group with a few steric hindrance, and a methoxy group, with different electronic properties but with a larger steric hindrance, were utilised. The basic moiety of the amide was kept unaltered utilising the pharmacologically effective anilines **6a** and **6b** again. The usual synthetic route for the new benzanilides involved the 4-fluorobenzoyl chloride (**10a**) and the 4-methoxybenzoyl chloride (**10b**) which, on reaction with **6a** and **6b**, provided the derivatives **11a**, **b** and **11c**, **d**, respectively.

The structures of all the prepared compounds were easily assigned on the basis of reaction mechanisms and were confirmed by analytical and spectroscopic data (Tables 1 and 2).



Scheme 1. Synthesis of **3a–c** and **4a–c**. (a) Δ, toluene, NEt_3; (b) CH_2Cl_2, BBr_3, −30 °C.



5a R_1= H
5b R_1= CH_3
5c R_1= OCH_3
5d R_1= OCH_2CH_3

6a R_2= Cl
6b R_2 = CH_3

7a R_1= H; R_2= Cl
7b R_1= H; R_2= CH_3.
7c R_1= CH_3;R_2= Cl
7d R_1= CH_3; R_2= CH_3
7e R_1= OCH_3; R_2= Cl
7f R_1= OCH_3; R_2= CH_3
7g R_1= OCH_2CH_3; R_2= Cl.
7h R_1= OCH_2CH_3; R_2= CH_3
7i R_1= F; R_2= Cl
7l R_1= F; R_2= CH_3

Scheme 2. Synthesis of **7a–l**. (a) Δ, toluene, NEt_3.

In particular, the structure of the monomethoxy derivative **4c**, arising from the demethylation with boron tribromide of the dimethoxy derivative **3c**, was assigned by comparison with an analogous monomethoxy derivative obtained under the same reaction conditions [15].

3. Results and discussion

3.1. Pharmacology

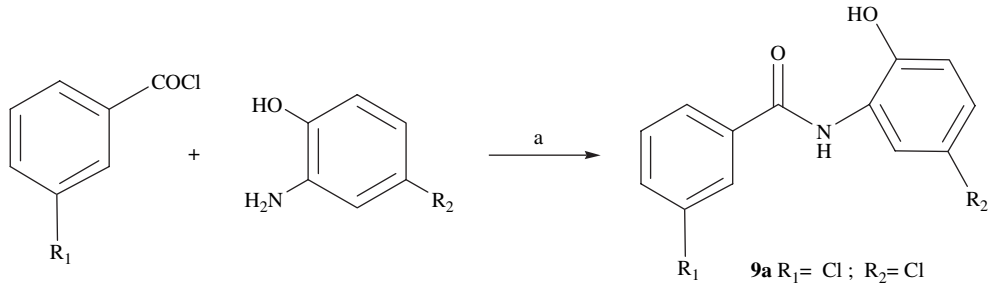
In order to characterise a potential BK-activator profile, all the synthesised compounds were submitted to preliminary screening through the evaluation of their possible vasorelaxing activity on endothelium-denuded rat aortic rings (Table 3).

Compounds **4a–c**, **7a**, **7g**, **9a** and **11a–b** exhibited a full or nearly full vasorelaxing efficacy ($E_{max} > 80\%$), while, compounds **9c** and **9d** showed a completely ineffective vasorelaxing profile. All the remaining compounds (**7b–f**, **7h–l**, **9b** and **11c–d**) showed partial vasorelaxing efficacy, with E_{max} parameters ranging from 30 to 80%.

As concerns the potency indexes, all the compounds with an efficacy value > 50% showed potency parameters ranging around the 10 μM order of magnitude, except for compound **4c** which exhibited a potency value in the micromolar range.

The non-selective K^+-channel blocker NEt_3 was not able to antagonise the vasorelaxing effects of **4a** and **4c**. Therefore, these effects cannot be ascribed to an activation of NEt_3-sensitive potassium channels (such as the BK one). The vasorelaxing activity of **4b** was significantly antagonised by NEt_3, however, the BK-selective blocker IbTX failed to exert any significant antagonism. Thus, the effects of **4b** are likely to be due to an activation of NEt_3-sensitive potassium channel types, different from the BK ones.

As concerns compound **7**, the mechanism of action was investigated only for compound **7g**, which exhibits the highest level of efficacy and an appreciable index of potency (comparable with that of **NS1619**). Both NEt_3 and IbTX were able to produce a significant antagonism against the vasorelaxing effects of this derivative, indicating that the activation of BK channels plays a significant role in the mechanism of the vasorelaxing action. With regard to some possible structure–activity relationships, it is possible to observe that a Cl atom in *para* position with respect to the phenolic hydroxyl is preferable to a methyl group. In fact, except for the couple **7e–f**, in all the other couples of analogous compounds (sharing the R^1 substituent) the Cl atom in R^2 determines an improvement in vasorelaxing efficacy. Analysing the influence of the R^1 substituent in the **7** series, it was evident that a F atom in R^1 (compounds **7i–l**) determines a dramatic reduction in efficacy. This negative impact cannot be due to a steric hindrance. Indeed, compounds **7c–h** (all bearing a steric hindrance in R^1), as well as compounds **7a–b** (bearing a H atom in R^1), exhibited vasorelaxing efficacy higher than that exhibited by their respective analogues bearing a F atom in R^1. Thus, the

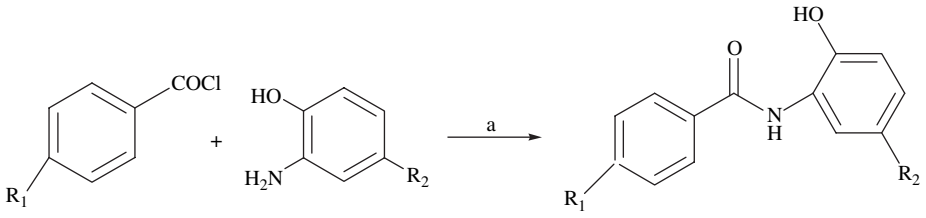


8a R_1= Cl
8b R_1= I

6a R_2= Cl
6b R_2= CH_3

9a R_1= Cl ; R_2= Cl
9b R_1= Cl ; R_2= CH_3
9c R_1= I ; R_2= Cl
9d R_1= I ; R_2= CH_3

Scheme 3. Synthesis of **9a–d**. (a) Δ, toluene, NEt_3.



10a R_1= F
10b R_1= OCH_3

6a R_2= Cl
6b R_2= CH_3

11a R_1= F; R_2= Cl.
11b R_1= F; R_2= CH_3
11c R_1= OCH_3; R_2= Cl
11d R_1= OCH_3; R_2= CH_3

Scheme 4. Synthesis of **11a–d**. (a) Δ, toluene, NEt_3.

negative influence of the F atom might be ascribed to other reasons: (i) an electronic influence on the aromatic system (F is the only electron-withdrawing substituent in R^1 of this group of compounds); (ii) the possible establishment of an intramolecular H-bond with one of the available H atoms, thus forcing the molecule into an unfavourable conformation and/or subtracting a requisite useful for the interaction with the receptor; (iii) the presence of a polar feature giving unfavourable interactions with some residues of the channel.

The observation that compounds **11a–b**, bearing a F atom in *para* position, exhibit a more satisfactory vasorelaxing effect, excludes the hypothesis of the importance of the electronic influence of F on the aromatic system. However, it should be taken into account that **11a–b** bearing a F atom in R^1, represent the most effective compounds in the **11** series (characterised by an R^1 substituent in *para* position) but show either NEt_3-insensitive effects (**11a**) or NEt_3-sensitive but IbTX-insensitive activity (**11b**), suggesting that BK channels are not involved in their mechanism of vasodilation.

In the **9** series, characterised by the R^1 substituent in *meta* position, the preference for a Cl atom in R^2 position with respect to a methyl group was again confirmed. For example, compound **9a**, showing a Cl atom both in R^1 and in R^2, showed a NEt_3- and IbTX-sensitive vasorelaxing activity, typical of a BK-opener profile, with a potency index not much lower than **NS1619**.

Therefore, among the molecules observed, it appears evident that for some of them the vasorelaxing effect was clearly due to the interaction with the BK channel, in particular compounds **7g** and **9a**.

3.2. Molecular modelling

On the basis of such results, the structure of compounds **7g** and **9c** was investigated by molecular modelling studies. The

Table 1
Physico-chemical properties of compounds **3**, **4**, **7**, **9** and **11**

Compounds	Yield (%)	Crystallisation solvent	m.p. (°C)	Analyses (C, H, N)	IR (cm^{-1})
3a	69	Iso-PrOH	190–192	$C_{15}H_{13}ClN_2O_4$	1680 (CO); 3284 (NH)
3b	59	MeOH	185–187	$C_{15}H_{13}ClN_2O_5$	1672 (CO); 3309 (NH)
3c	84	AcOEt	235–237	$C_{15}H_{13}ClN_2O_5$	1666 (CO); 3292 (NH)
4a	85	MeOH	191–193	$C_{14}H_{11}ClN_2O_4$	1642 (CO); 3250 (NH, OH)
4b	62	AcOEt/hexane	224–226	$C_{13}H_9ClN_2O_5$	1646 (CO); 3213 (OH); 3388 (NH)
4c	68	AcOEt/hexane	233–235	$C_{14}H_{11}ClN_2O_5$	1654 (CO); 3253 (OH); 3392 (NH)
7a	78	MeOH/H_2O	232–234	$C_{13}H_{10}ClNO_2$	1648 (CO); 3080 b (OH); 3422 (NH)
7b	72	MeOH	135–137	$C_{14}H_{13}NO_2$	1645 (CO); 3410 b (NH, OH)
7c	80	MeOH/H_2O	225–227	$C_{14}H_{12}ClNO_2$	1648 (CO); 3100 b (OH); 3406 (NH)
7d	75	MeOH	125–127	$C_{15}H_{15}NO_2$	1647 (CO); 3090 b (OH); 3407 (NH)
7e	83	MeOH	234–236	$C_{14}H_{12}ClNO_3$	1644 (CO); 3145 b (OH); 3298 (NH)
7f	75	MeOH/H_2O	165–167	$C_{15}H_{15}NO_3$	1642 (CO); 3180 b (OH); 3320 (NH)
7g	70	MeOH/H_2O	170–172	$C_{15}H_{14}ClNO_3$	1637 (CO); 3380 b (NH, OH)
7h	80	MeOH	133–135	$C_{16}H_{17}NO_3$	1640 (CO); 3100 b (OH); 3353 (NH)
7i	82	MeOH	236–238	$C_{13}H_9ClFNO_2$	1650 (CO); 3392 (NH); 3120 b (OH)
7l	79	MeOH/H_2O	195–197	$C_{14}H_{12}FNO_2$	1649 (CO); 3389 (NH); 3200 b (OH)
9a	78	MeOH/H_2O	220–222	$C_{13}H_9Cl_2NO_2$	1648 (CO); 3116 (OH); 3415 (NH)
9b	65	MeOH/H_2O	190–192	$C_{14}H_{12}ClNO_2$	1638 (CO); 3220 b (NH, OH)
9c	70	MeOH	235–237	$C_{13}H_9Cl\ INO_2$	1637 (CO); 3210 b (NH, OH)
9d	85	MeOH	228–230	$C_{14}H_{12}INO_2$	1636 (CO); 3230 b (NH, OH)
11a	90	MeOH	228–230	$C_{13}H_9ClFNO_2$	1644 (CO); 3100 b (OH); 3412 (NH)
11b	52	AcOEt/hexane	189–190	$C_{14}H_{12}FNO_2$	1650 (CO); 3070 b (OH); 3414 (NH)
11c	58	MeOH	203–205	$C_{14}H_{12}ClNO_3$	1649 (CO); 3070 b (OH); 3415 (NH)
11d	78	MeOH/H_2O	129–131	$C_{15}H_{15}NO_3$	1639 (CO); 3080 (OH); 3369 (NH)

Table 2
^{1}H NMR spectra in DMSO-d_6, δ values

3a	2.38 (s, 3H, CH_3); 4.05 (s, 3H, OCH_3); 7.30–8.46 (m, 6H, Ar.); 10.1 (NH).
3b	3.85 (s, 3H, OCH_3); 4.04 (s, 3H, OCH_3); 7.28–8.40 (m, 6H, Ar.); 11.4 (NH).
3c	4.06 (s, 3H, OCH_3); 4.08 (s, 3H, OCH_3); 6.97–8.11(m, 6H, Ar.); 10.8 (NH).
4a	2.39 (s, 3H, CH_3); 7.03–8.40 (m, 6H, Ar.); 10.9 (NH); 11.9 (OH).
4b	7.02–8.16 (m, 6H, Ar.); 10.3 (NH); 11.4 (OH); 12.1 (OH).
4c	4.04 (s, 3H, OCH_3); 7.06–8.73 (m, 6H, Ar.); 11.3 (NH); 12.3 (OH).
7a	6.90–7.97 (m, 8H, Ar.); 9.5 (NH); 10.1 (OH).
7b	2.22 (s, 3H, CH_3); 6.82–7.98 (m, 8H, Ar.); 9.5 (NH, OH).
7c	2.51 (s, 3H, CH_3); 6.90–7.91 (m, 7H, Ar.); 9.4 (NH); 10.2 (OH).
7d	2.22 (s, 3H, CH_3); 2.41 (s, 3H, CH_3); 6.81–7.54 (m, 7H, Ar.); 9.4 (NH); 9.5 (OH).
7e	4.04 (s, 3H, OCH_3); 6.91–8.46 (m, 7H, Ar.); 10.6 (NH); 10.7 (OH).
7f	2.22 (s, 3H, CH_3); 4.03 (s, 3H, OCH_3); 6.74–8.21(m, 7H, Ar.); 9.9 (NH); 10.5 (OH).
7g	1.52, 4.30 (t, 3H, q, 2H, CH_3CH_2O); 6.87–8.51 (m, 7H, Ar.); 10.5 (NH); 10.6 (OH).
7h	1.52, 4.29 (t, 3H, q, 2H, CH_3CH_2O); 2.22 (s, 3H, CH_3); 6.70–8.21 (m, 7H, Ar.); 10.0 (NH); 10.4 (OH).
7i	6.88–8.21 (m, 7H, Ar.); 9.5 (NH); 10.5 (OH).
7l	2.23 (s, 3H, CH_3); 6.74–7.92 (m, 7H, Ar.); 9.4 (NH); 9.8 (OH).
9a	6.92–8.01 (m, 7H, Ar.); 9.7 (NH); 10.1 (OH).
9b	2.22 (s, 3H, CH_3); 6.82–8.01 (m, 7H, Ar.); 9.4 (NH); 9.7 (OH).
9c	6.90–8.30 (m, 7H, Ar.); 9.7 (NH); 9.8 (OH).
9d	2.22 (s, 3H, CH_3); 6.83–8.32 (m, 7H, Ar.); 9.4 (NH); 9.8 (OH).
11a	6.91–8.08 (m, 7H, Ar.); 9.5 (NH); 10.1 (OH).
11b	2.23 (s, 3H, CH_3); 6.83–8.08 (m, 7H, Ar.); 9.4(NH); 9.5 (OH).
11c	3.84 (s, 3H, OCH_3); 6.94–7.97 (m, 7H, Ar.); 9.4 (NH); 10.2 (OH).
11d	2.22 (s, 3H, CH3); 3.82 (s, 3H, OCH3); 6.82–7.98 (m, 7H, Ar.); 9.4 (NH); 9.5 (OH).

Table 3
Pharmacological results

Compounds	E_{max}	pIC_{50}
4a	94.6 ± 5.0	5.39 ± 0.07
4b	96.7 ± 4.4	5.28 ± 0.04
4c	97.4 ± 0.1	6.16 ± 0.04
7a	96.4 ± 9.4	4.87 ± 0.03
7b	72.4 ± 7.1	5.03 ± 0.26
7c	62.2 ± 4.6	4.52 ± 0.03
7d	48.4 ± 8.2	N.C.
7e	67.5 ± 8.8	4.73 ± 0.06
7f	77.3 ± 8.7	4.90 ± 0.12
7g	100[a]	5.51 ± 0.06
7h	67.9 ± 3.2	4.95 ± 0.07
7i	52.6 ± 4.2	4.52 ± 0.03
7l	39.7 ± 10.6	N.C.
9a	100[a]	4.93 ± 0.03
9b	33.7 ± 10.5	N.C.
9c	Ineffective[b]	N.C.
9d	Ineffective[b]	N.C.
11a	81.4 ± 12.6	4.76 ± 0.04
11b	84.0 ± 6.1	4.95 ± 0.04
11c	45.7 ± 8.5	N.C.
11d	46.0 ± 1.8	N.C.
NS1619	100[a]	5.31 ± 0.08

The values of potency (pIC_{50}) could not be calculated (N.C.) for those compounds exhibiting an efficacy (E_{max}) lower than 50.

[a] At the concentration of 30 μM, the compound evoked a 100% vasorelaxing effect, in all the experiments.

[b] At the concentration of 30 μM, the compound evoked a mean vasorelaxing effect lower than 10% and therefore it was considered almost ineffective.

molecules were chosen as representative structures of an effective opener (**7g**) and an almost completely ineffective molecule (**9c**). A Molecular Mechanic (MM) approach was carried out on these selected molecules and on **NS1619**, which was chosen as a representative moiety to be subjected to an exhaustive conformational search. The molecules were built using the molecular modelling package Insight (Accelrys, San Diego, CA). Different plausible conformers were generated for **NS1619** and solvated with a 5 Å layer of water. The conformers were subjected to energy minimisation using the cff91 Force Field implemented in the Discover program (Accelrys, San Diego, CA). The conjugate gradient algorithm was exploited for energy minimisation and the convergence criterion for the energy gradient was 0.01 kcal mol^{-1} Å^{-1}. All calculations were performed on an SGI Octane R12000 workstation. A reasonably good sampling of the potential energy surface was obtained by selecting the optimised conformer for **NS1619** (Fig. 3A) characterised by the lowest energy value. The structures of the other selected molecules were then optimised on the template of **NS1619** using a restraining force of 100 kcal mol^{-1} Å^{-2}. The two aromatic nuclei and the OH function were chosen as critical features for the restrain, since they constitute the major pharmacophoric points of the hypothesised pharmacophore (Fig. 1B). After that, molecular electrostatic potentials (MEPs) were calculated on the optimised structures of **7g** and **9c** (Fig. 3B,C) using the program VEGA [19].

The *ortho* substitution in the R^1 aromatic ring of the vasorelaxing molecule **7g** with respect to the ineffective **9c** is highlighted by the electrostatic potential surface profile in Fig. 3B,C. This observation allows us to hypothesise that some favourable interaction between bulky non-polar groups (such as methoxy or ethoxy) in *ortho* position of one of the aromatic rings and the hydrophobic residues of the receptor could account for the observed activity of **7g**. On the contrary, the ineffective compound **9c** is lacking in the ethoxy substituent and bears an I atom as substituent in *meta* position which could fill different regions of the receptor unable to accommodate it. Thus, the inefficacy of **9c** could be attributed both to the lack of the bulky susbtituent and to the role of I in a different position of the molecule, thus inducing an unfavourable interaction with the receptor. Although the limited number of compounds studied does not allow us to consider it an exhaustive modelling and SAR study, the hypothesis about the importance of a bulky non-polar substituent on one of the two aromatic ring confirms some data previously obtained in a work by us [9] and seems to be in accord with the observations of Ohwada et al.[20].

4. Conclusion

Summing up, the synthesis of the series of substituted benzanilides and the structure–activity relationship (SAR) study performed in this work confirm the pharmacophoric features described above. The presence of two aromatic nuclei and

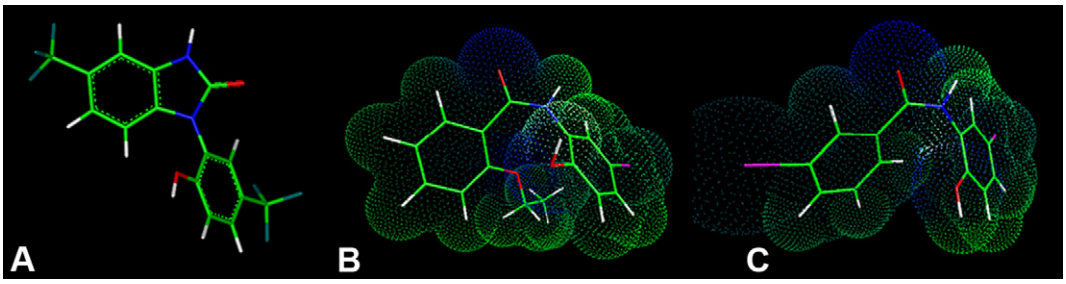


Fig. 3. Three-dimensional structure of **NS1619** (A). Molecular electrostatic potential (MEP) surface calculated projecting the atomic charge on the surface for **7g** (B), **9c** (C). In blue, the polar influence of the phenolic OH and the carbonyl group in the amide function of the linker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a phenolic OH is essential, even if they cannot be considered the unique elements accounting for the activity of the compounds. Furthermore, a Cl atom in *para* position with respect to the phenolic hydroxyl is preferable to a methyl group, according to the observation of Biagi et al. [15] and Hewawasam et al.[21].

With regard to the compounds of the series **7**, bearing the OH function in the anilino moiety of the benzanilide, it can be observed that the presence of an appropriate substituent in *ortho* position of the acid moiety of the benzanilide might be able to give favourable interactions with a specific region of the channel. Indeed, the vasorelaxing efficacy increases in compounds **7a–h** with the steric hindrance of *ortho* substituent R^1; whereas it is dramatically reduced by the introduction of the F atom in the same position.

The evaluation of this spatial region of the pharmacophore and the role of the linker could be worth of further investigation in future perspectives of synthesis.

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 an internal standard. Mass spectra were performed with a Trace GC Q plus, thermo quest Finnigan. Elemental analyses (C, H, N) were within $\pm$ 0.4% of the theoretical values and were performed on a Carlo Erba Elemental Analyzer Mod. 1106 apparatus.

5.1.1. N-(2,4-Substituted-phenyl)-2-methoxy-5-chloro-benzamides (*3a–c*)

A solution of 2-methoxy-5-chloro-benzoic acid (0.930 g, 5.0 mmol) in 5–10 mL of $SOCl_2$ was heated under reflux for 45 min. The solvent was evaporated *in vacuo* and the liquid residue consisting of the corresponding acid chloride (**1**) was dissolved in 20 mL of anhydrous toluene. This solution was added drop by drop to a solution of the appropriate substituted aniline (5.0 mmol of **2a**, **2b** or **2c**) and triethylamine (NEt_3) (1.0 mL, 7.2 mmol) in 20 mL of anhydrous toluene. The mixture was refluxed overnight and, after cooling, a solid precipitate was formed which was collected by filtration and treated with H_2O. The insoluble material consisted of the crude title compounds **3a–c** in variable amounts. The toluene filtrate was evaporated *in vacuo* and the residue was dissolved in $CHCl_3$. The chloroform solution, after washing with 10% NaOH and 10% HCl, was dried ($MgSO_4$) and evaporated to give a further amount of the title compounds. The two solid fractions of each compound **3a**, **3b** or **3c** were combined and purified by crystallisation (Table 1).

5.1.2. N-(2,4-Substituted-phenyl)-2-hydroxy-5-chloro-benzamides (*4a–c*)

A solution of 2.00 mmol of methoxyderivative **3a**, **3b** or **3c** in 150 mL of anhydrous CH_2Cl_2 was cooled at −30 °C and, under stirring, a solution of BBr_3 in anhydrous CH_2Cl_2 was added drop by drop [1.5 mL (≅ 15 mmol) in 5 mL of solvent for **3a**; 3 mL (≅ 30 mmol) in 10 mL of solvent for **3b** and **3c**]. After the addition the mixture was stirred at −30 °C for 1 h, then kept at −20 °C overnight. The CH_2Cl_2 solution was cooled in an ice-bath and the excess of the reagent was decomposed by the addition of MeOH and H_2O.

For the isolation of **4a**, 6 mL of MeOH followed by 30 mL of H_2O were added, the organic layer was separated and, after washing with H_2O, was extracted with 10% NaOH. Addition of the alkaline solution caused precipitation of a red solid (consisting of **4a** sodium salt) in the interface of the two liquid phases, which was collected by filtration. This solid was dissolved in H_2O and the solution was acidified with 10% HCl to give **4a** as a yellow precipitate. The two liquid phases were separated and the alkaline extracts acidified to give a further amount of **4a**, which was combined with the previous fraction and purified by crystallisation (Table 1).

For the isolation of **4b**, 8 mL of MeOH followed by 60 mL of H_2O were added, the organic layer was separated and, after washing with H_2O, was extracted with 10% NaOH. The alkaline extracts were combined and acidified to precipitate **4b** which was collected by filtration and purified by crystallisation (Table 1). For the isolation of **4c**, 8 mL of MeOH followed by 40 mL of H_2O were added, the organic layer was separated and, after washing with H_2O, was extracted with 10% NaOH. Addition of the alkaline solution caused precipitation of a solid (consisting of **4c** sodium salt) in the interface of the two liquid phases, which was collected by filtration. This solid was

suspended in H_2O then acidified to give **4c** which was collected and purified by crystallisation (Table 1).

Acidification of the alkaline layer, after separation from the organic phase, did not give a further precipitate.

*5.1.3. 2-Substituted benzoyl chlorides (**5a–e**)*

A solution of 3.00 mmol of the suitable benzoic acid [benzoic (**5a**), 2-methylbenzoic (**5b**), 2-methoxybenzoic (**5c**), 2-ethoxybenzoic (**5d**) or 2-fluorobenzoic (**5e**)] in 5–10 mL of $SOCl_2$ was heated under reflux for 45–60 min. The solvent was evaporated *in vacuo* and the liquid residue was dissolved in 15 mL of anhydrous toluene to be employed in the following reactions.

*5.1.4. N-(2-Hydroxy-5-chloro-phenyl)-2-substituted-benzamides (**7a, 7c, 7e, 7g and 7i**)*

To a solution of 2-hydroxy-5-chloro-aniline (**6a**) (0.430 g, 3.00 mmol) and NEt_3 (0.5 mL, 3.6 mmol) in 20 mL of anhydrous toluene, a solution of the suitable acyl chloride (**5a**, **5b**, **5c**, **5d** or **5e**) was added drop by drop and the mixture was refluxed overnight.

For the isolation of **7a**, **7c** and **7i**, after cooling, a solid precipitate was formed which was collected by filtration and treated with H_2O to give a main fraction of the title compounds as insoluble material. The toluene filtrate was evaporated, the residue dissolved in $CHCl_3$ and the chloroform solution, after washing with 5% $NaHCO_3$ and 10% HCl, was dried ($MgSO_4$) and evaporated to give a further fraction of **7a**, **7c** and **7i** (Table 1).

For the isolation of **7e**, after cooling, a solid precipitate was not present, whilst for **7g** a solid precipitate was formed but was completely soluble in H_2O. Therefore the toluene solutions were evaporated, the residues were dissolved in $CHCl_3$ and the chloroform solutions, after washing with 5% $NaHCO_3$ and 10% HCl, were dried ($MgSO_4$) and evaporated to provide the title compounds **7e** and **7g** in good yield (Table 1).

*5.1.5. N-(2-Hydroxy-5-methyl-phenyl)-2-substituted-benzamides (**7b, 7d, 7f, 7h and 7l**)*

To a solution of 2-hydroxy-5-methyl-aniline (**6b**) (0.370 g, 3.00 mmol) and NEt_3 (0.5 mL, 3.6 mmol) in 20 mL of anhydrous toluene, a solution of the suitable acyl chloride (**5a**, **5b**, **5c**, **5d** or **5e**) was added drop by drop and the mixture was refluxed overnight.

For the isolation of **7b**, **7d**, **7h** and **7l**, after cooling, a solid precipitate was formed and the reactions were worked up as described for **7a** (Table 1).

For the isolation of **7f**, after cooling, a solid precipitate was not present, therefore the toluene solution was worked up as described for **7e** (Table 1).

*5.1.6. N-(2-Hydroxy-5-substituted-phenyl)-3-chloro-benzamides (**9a, b**)*

A solution of 3-chlorobenzoic acid (0.783 g, 5.0 mmol) in 5–10 mL of $SOCl_2$ was heated under reflux for 45–60 min. The solvent was evaporated *in vacuo* and the residue, consisting of the acyl chloride **8a**, was dissolved in 20 mL of anhydrous toluene. This solution was added drop by drop to a solution of the substituted aniline **6a** or **6b** (5.0 mmol) and NEt_3 (1.0 mL, 7.2 mmol) in 20 mL of anhydrous toluene. The mixture was refluxed overnight, the solvent evaporated *in vacuo* and the residue dissolved in $CHCl_3$. The chloroform solution, after washing with 5% $NaHCO_3$ and 10% HCl, was dried ($MgSO_4$) and evaporated to give the title compounds as a solid residue which was purified by crystallisation (Table 1).

*5.1.7. N-(2-Hydroxy-5-substituted-phenyl)-3-iodo-benzamides (**9c, d**)*

A solution of 3-iodobenzoic acid (1.24 g, 5.0 mmol) in 5–10 mL of $SOCl_2$ was heated under reflux for 45–60 min. The solvent was evaporated *in vacuo* and the residue, consisting of the acyl chloride **8b**, was dissolved in 20 mL of anhydrous toluene. This solution was added drop by drop to a solution of the substituted aniline **6a** or **6b** (5.0 mmol) and NEt_3 (1.0 mL, 7.2 mmol) in 20 mL of anhydrous toluene. The mixture was refluxed overnight and, after cooling, a solid precipitate was formed which was collected by filtration and treated with H_2O. The insoluble material consisted of the title compounds **9c** or **9d**. The toluene filtrate, worked up in the usual manner, gave only a further moderate amount of **9c**. The crude solids obtained were purified by crystallisation (Table 1).

*5.1.8. N-(2-Hydroxy-5-substituted-phenyl)-4-fluoro-benzamides (**11a, b**)*

A solution of 4-fluorobenzoic acid (0.700 g, 5.0 mmol) in 5–10 mL of $SOCl_2$ was heated under reflux for 45–60 min. The solvent was evaporated *in vacuo* and the residue, consisting of the acyl chloride **10a**, was dissolved in 20 mL of anhydrous toluene. This solution was added drop by drop to a solution of the substituted aniline **6a** or **6b** (5.0 mmol) and NEt_3 (1.0 mL, 7.2 mmol) in 20 mL of anhydrous toluene. The mixture was refluxed overnight and, after cooling, a solid precipitate was formed which was collected by filtration and treated with H_2O. The insoluble material consisted of the title compounds **11a** or **11b**. The toluene filtrate, worked up in the usual manner, gave a further moderate amount of product. The solid fractions were combined and purified by crystallisation (Table 1).

*5.1.9. N-(2-Hydroxy-5-substituted-phenyl)-4-methoxy-benzamides (**11c, d**)*

A solution of 4-methoxybenzoic acid (0.761 g, 5.0 mmol) in 5–10 mL of $SOCl_2$ was heated under reflux for 45–60 min. The solvent was evaporated *in vacuo* and the residue, consisting of the acyl chloride **10b**, was dissolved in 20 mL of anhydrous toluene. This solution was added drop by drop to a solution of the substituted aniline **6a** or **6b** (5.0 mmol) and NEt_3 (1.0 mL, 7.2 mmol) in 20 mL of anhydrous toluene. The mixture was refluxed overnight and, after cooling, a solid precipitate was formed which was collected by filtration and treated with H_2O. The insoluble material consisted of the title compounds **11c** or **11d**. The toluene filtrate, worked up in the usual manner, gave a further moderate amount of product. The solid fractions were combined and purified by crystallisation (Table 1).

5.2. Pharmacology

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

To determine a possible vasodilator mechanism of action, the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g).

The rats were sacrificed by cervical dislocation under light ether anaesthesia and bled. The aortae were immediately excised and freed of extraneous tissues. The endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five-millimeter 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; $MgSO_4 \cdot 7H_2O$ 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 to a preamplifier (Buxco Electronics) and to a software of data acquisition (BIOPAC Systems Inc., MP 100).

After an equilibration period of 60 min, endothelial removal 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 $\geq 10\%$ (i.e. significant presence of the 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 contraction reached a stable *plateau*, 3-fold increasing concentrations of the tested compounds or of the reference drug **NS1619** (a well-known BK-activator) were added cumulatively in the concentration range 10 nM–30 μM.

Preliminary experiments showed that the KCl (20 mM)-induced contractions remained in a stable tonic state for at least 40 min.

In other sets of experiments, the non-selective potassium channel blocker tetraethylammonium chloride (NEt_3 10 mM) or the BK-selective blocker Iberiotoxin (IbTX, 100 nM) were added, after the KCl (20 mM)-induced contraction, followed by the administration of selected compounds.

The reference drug **NS1619** (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 and NEt_3 were both dissolved in Tyrode solution. All the synthesised derivatives were dissolved (10 mM) in DMSO; they were all further diluted in Tyrode solution. All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicle.

Vasorelaxing efficacy was evaluated as a maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 20 mM. The parameter of efficacy (E_{max}) represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 20 mM, evoked by the limit concentration 0.1 mM (the highest concentration, which could be administered). Those compounds exhibiting an E_{max} lower than 10% were considered as ineffective. The parameter of potency was expressed as pIC_{50}, calculated as a negative Logarithm of the molar concentration of the tested compounds, evoking a half-reduction of the contractile tone induced by KCl 20 mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as mean ± standard error, for 5–10 experiments. Student-*t* test was selected as 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 4.0).

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