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ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 792–799
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Original article

New amido derivatives as potential BKCa potassium channel activators. XI

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Received 6 April 2007; received in revised form 13 June 2007; accepted 15 June 2007
Available online 5 July 2007

Abstract

The vasorelaxing effects of exogenous activators of large-conductance calcium-activated potassium channels (BK channels) can furnish the pharmacological rational basis for the treatment of hypertension and/or other diseases related with an impaired contractility of vessels. Since in previous works some benzanilide derivatives showed BK channel-induced vasorelaxing activity, in this paper we have taken into consideration the introduction of methylene spacer(s) between the amide linker and one or both the aromatic substituents, to evaluate the pharmacological effect caused by these lengthenings and to obtain possible useful information about structure–activity relationships. Overall, the main findings of this work suggest that the introduction of one or two methylene group(s) in the amide linker exerts a negative influence on the BK-opening properties, which can be due to an excessive lengthening of the spacer between the two aromatic rings and/or to further degrees of conformational freedom.

Keywords: BK potassium channel; Benzanilide; Amide; BK-activator

1. Introduction

The large-conductance, calcium-activated potassium channels (BK, also termed BKCa, Slo, or MaxiK) are distributed in both excitable and non-excitable cells. They are involved in many cellular functions such as action potential repolarization, neuronal excitability, neurotransmitter release, hormone secretion, tuning of cochlear hair cells, innate immunity, and modulation of the tone of vascular, airway, uterine, gastrointestinal, and urinary bladder smooth muscle tissues [1–3].

BKCa channels characteristically respond to two distinct physiological stimuli, changes in membrane voltage and in cytosolic Ca^{2+} concentration. The BK channels gate-open in response to an increase in cytosolic Ca^{2+} concentration and membrane depolarization, resulting in an increase of K^+ efflux, which leads to rapid hyperpolarization of the excitatory membrane and reduces Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

Then, 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 fundamental function of such ion channels in the modulation of the muscular tone of vessels [4,5]. Consequently, the vasorelaxing effects of exogenous BK-openers can furnish the pharmacological rational basis for the treatment of hypertension and/or other diseases related with an impaired contractility of vessels (for example, coronary vasospasm) [1,2].

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doi:10.1016/j.ejmech.2007.06.005

In a previous work [6], we could observe that the synthesised 1-(2′-hydroxybenzoyl)-5-methyl-benzotriazole, showing 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 [7–9]. Of course, these reports let us foresee a further potential use of BK-activators in cardiovascular pharmacotherapy, as anti-ischemic drugs.

Our research program about potential activators of BK channels, in the beginning considered heterocyclic compounds [6,10–12] which referred to the benzimidazolone derivatives NS 004 and NS 1619 reported in the literature [13] as BK-openers. After regarding to an hypothetic simple pharmacophore (Fig. 1**A**) which consisted of two appropriately substituted phenyl rings connected by a linker, 1,2,3-triazole derivatives [14–16] and benzanilides [17–19] were synthesised and tested, in which the 1,2,3-triazole ring and the amide function, respectively, represented the linker.

In particular, after a first paper [17] which had shown a BK channel-induced vasorelaxing activity of the *N*-(2-hydroxy-5-chlorophenyl)-2-methoxy-5-chlorobenzamide (Fig. 1**B**) higher than NS 1619, the research about new compounds bearing the amide linker was advanced changing the benzanilide structure [18] by the introduction of a five or six membered aromatic heterocyclic substituent in the acid moiety of the amide, keeping unaltered the basic moiety as 2-hydroxy-5-chloro-anilide (Fig. 1**C**). In addition an aromatic or aliphatic six or five membered heterocyclic substituent was introduced in the basic moiety of the amide, keeping unaltered the acid moiety as 2-methoxy-5-chlorobenzoyl, which was then converted to the corresponding 2-hydroxy derivative (Fig. 1**D**). 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 were 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, but the presence of aliphatic heterocycles lowered the pharmacological effect. The presence of a phenolic function as a certain H-bond donor was confirmed.

Another structural change of the benzanilide pharmacophore concerned a further deepening of the structure–activity relationships of benzanilide derivatives previously studied as BK channel activators [19]. In this paper were reported several substitutions on the phenyl rings of the reference benzanilides, which possessed particular and specific properties from a mesomeric and/or steric point of view. The pharmacological results indicated that several compounds exhibited vasorelaxing effects which could not be attributed to the activation of BK channels, but two derivatives showed a clear profile of BK-activators with a vasodilator activity comparable to or slightly lower than that recorded for the reference benzimidazolone NS 1619.

In this paper we have taken into consideration the introduction of methylenic spacers between the amide linker and the two aromatic substituents which form the benzanilide pharmacophore to evaluate the pharmacological effect caused by these lengthenings.

2. Chemistry

At first a methylenic bridge was introduced in the acid moiety of the benzanilide, by the preparation of a new series of phenylacetanilides (Scheme 1). Compounds **3a–g** were prepared by reaction of 2-methoxy-phenylacetic acid chloride (**1**) with the suitable substituted aniline (**2a–g**) in toluene in the presence of triethylamine. The employed anilines had differentiated substituents as 2-hydroxy-5-chloro- (**2a**), 2-hydroxy-5-methyl- (**2b**), 2-methoxy-5-nitro- (**2c**), 2-methyl-4-nitro- (**2d**), 4-nitro- (**2e**), 2-fluoro- (**2f**) together with the unsubstituted aniline (**2g**). Thus the phenol function, which appears as one of the potential requirement for the pharmacological activity, is present in the *ortho*-position of the basic moiety of the

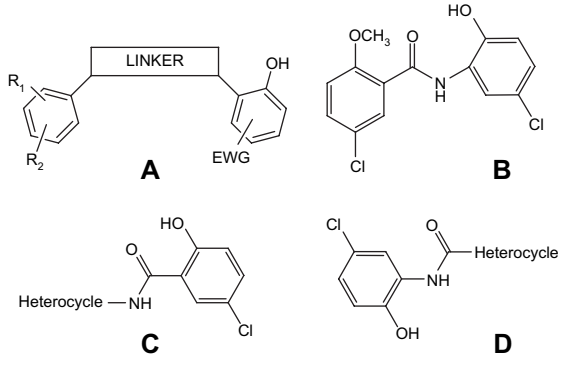
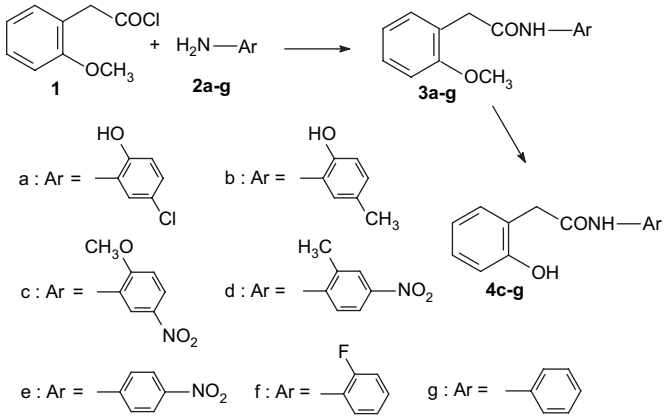


Fig. 1. Pharmacophore model of BK-activators (**A**) and chemical structures of previously synthesised compounds (**B–D**).



Scheme 1. Synthetic route for compounds **3a–g** and **4c–g**.

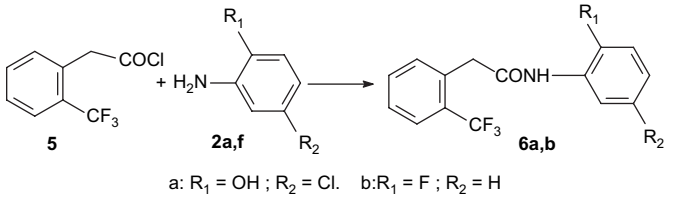
phenylacetanilides **3a** and **3b**. In any case, in the acid moiety of the phenylacetanilides, a methoxy substituent is present which can be converted to a phenol function by action of boron tribromide. Such a reaction allowed the preparation of the corresponding derivatives **4c–g**, bearing the hydroxy group on the acid moiety of the amide.

Compound **3c** underwent monodemethylation of the methoxy group on the acid moiety as confirmed by the comparison of its ^{1}H NMR spectrum (OCH_3 signals at 3.81 and 3.99 ppm) with the spectrum of **4c** (OCH_3 signal at 4.02 ppm). This result agrees with the monodemethylation of *N*-(2-methoxyphenyl)-2-methoxy-benzanilides in which the methoxy group of the acid moiety, adjacent to the carbonyl function, more easily forms a complex with boron tribromide and undergoes demethylation [16,19,20].

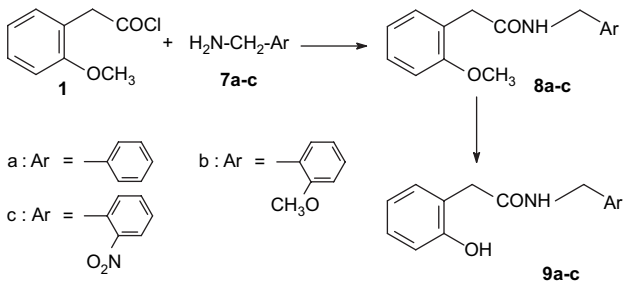
As a further change of the acid portion, two new 2-trifluoromethyl-phenylacetanilides **6a** and **6b** (Scheme 2) were also prepared and tested, obtained by reaction of 2-trifluoromethyl-phenylacetic acid chloride (**5**) with 2-hydroxy-5-chloro-aniline (**2a**) and 2-fluoro-aniline (**2f**), respectively.

Then the methylenic bridge was introduced either in the acid moiety or in the basic one of the benzanilide structure, with the preparation of a limited series of phenylacetobenzylamides (**8a–c**), which show two spacers between the amide linker and the substituted phenyls (Scheme 3). Compounds **8a–c** were obtained in the usual manner, by reaction of the same 2-methoxy-phenylacetic acid chloride (**1**) with the suitable benzylamine (**7a–c**). Therefore benzylamine (**7a**), 2-methoxy-benzylamine (**7b**) and 2-nitro-benzylamine (**7c**) were chosen for the lengthening of the basic moiety. In this case also, the presence of a methoxy group on the acid moiety of the three derivatives **8a–c** as well as on the basic moiety of **8b** allowed the obtainment of the corresponding phenol compounds **9a–c**, by cleavage of the ether function with boron tribromide. It is worth noting that, under the employed experimental conditions, compound **8b** underwent the monodemethylation of the methoxy group on the acid moiety to give the monohydroxy derivative **9b**. The structure of **9b** was hypothesized by the comparison of the ^{1}H NMR chemical shift values of the methoxy group of the available derivatives and unequivocally confirmed by the alkaline hydrolysis of **9b** which gave 2-hydroxy-phenylacetic acid and 2-methoxy-benzylamine. This result also agrees with the monodemethylation reported in the literature [16,19,20].

At last the methylenic bridge was introduced only in the basic moiety of the benzanilide, using the same benzylamines



Scheme 2. Synthetic route for compounds **6a** and **6b**.



Scheme 3. Synthetic route for compounds **8a–c** and **9a–c**.

7a–c, to prepare an analogous little series of benzobenzylamides (**11a–c**) (Scheme 4). The new compounds **11a–c** were obtained in the same manner by reaction of the suitable benzylamine (**7a–c**) with 2-methoxy-benzoic acid chloride (**10**). The cleavage of the methoxy groups with boron tribromide allowed the preparation of the corresponding phenol derivatives **12a–c**. In this case also, the dimethoxy derivative **11b**, under the employed experimental conditions, provided the monohydroxy derivative **12b**. According to the previous results of demethylation reactions, this compound maintained the methoxy group on the benzylamine moiety as shown by the ^{1}H NMR data and by the obtainment of 2-hydroxy-benzoic acid from the alkaline hydrolysis of **11b**.

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

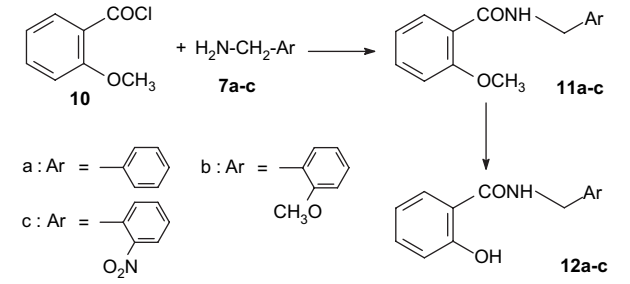
3. Pharmacology

As a preliminary indication about a possible BK-activating mechanism of action, the vasodilating effect of the new compounds was studied *in vitro* on isolated rat aortic rings precontracted with KCl (20 mM) (see later for the pharmacological details).

4. Results and discussion

The vasorelaxing efficacy and potency of the tested compounds and NS 1619 are summarised in Table 3. Many of the synthesised compounds resulted ineffective or exhibited low levels of vasorelaxing efficacy (<50%).

The first evidence emerging from the pharmacological results seems to give a clear indication about the negative impact exerted by the structural features showed by compounds **8**, **9**, **11** and **12**.



Scheme 4. Synthetic route for compounds **11a–c** and **12a–c**.

Table 1
Physico-chemical properties of compounds **3**, **4**, **6**, **8**, **9**, **11** and **12**

Compound	Yield (%)	Crystal solvent	M.p. (°C)	Analyses (C, H, N)	IR (cm^{-1})	Mass (*m/z*)	
						M^+	Base
3a	54	MeOH/H_2O	174–176	$C_{15}H_{14}ClNO_3$	1656 (CO); 3340 (NH); 3150 br (OH)	291	148
3b	51	MeOH/EtOAc	156–158	$C_{16}H_{17}NO_3$	1649 (CO); 3341 (NH); 3130 br (OH)	271	107
3c	70	MeOH/H_2O	167–170	$C_{16}H_{16}N_2O_5$	1683 (CO); 3330 (NH)		
3d	76	MeOH	153–155	$C_{16}H_{16}N_2O_4$	1667 (CO); 3278 (NH)	300	142
3e	78	MeOH	142–144	$C_{15}H_{14}N_2O_4$	1668 (CO); 3260 (NH)	287	218
3f	73	MeOH/H_2O	104–106	$C15H_{14}FNO_2$	1663 (CO); 3327 (NH)	259	148
3g	82	MeOH/H_2O	103–105	$C_{15}H_{15}NO_2$	1662 (CO); 3323 (NH)		
4c	54	MeOH/H_2O	172–174	$C_{14}H_{12}N_2O_5$	1662 (CO); 3310 (NH); 3156 (OH)		
4d	55	MeOH	145–147	$C_{15}H_{14}N_2O_4$	1669 (CO); 3430 br (NH, OH)	286	121
4e	58	MeOH	198–200	$C_{14}H_{12}N_2O_4$	1669 (CO); 3406 br (NH, OH)	272	138
4f	59	MeOH/H_2O	145–147	$C_{14}H_{12}FNO_2$	1736 (CO); 3253 br (NH, OH)		
4g	63	MeOH	153–155	[21]	1681 (CO); 3345 (NH); 3198 (OH)		
6a	80	MeOH/H_2O	173–175	$C_{15}H_{11}ClF_3NO_2$	1671 (CO); 3375 (NH); 3160 br (OH)		
6b	85	MeOH/H_2O	135–137	$C_{15}H_{11}F_4NO$	1663 (CO); 3261 (NH)		
8a	85	MeOH/H_2O	78–80	[22]	1654 (CO); 3298 (NH)	255	255
8b	66	EtOAc/hexane	97–100	$C_{17}H_{19}NO_3$	1649 (CO); 3284 (NH)		
8c	60	MeOH/H_2O	102–104	$C_{16}H_{16}N_2O_4$	1649 (CO); 3230 (NH)		
9a	58	EtOAc/hexane	98–100	$C_{15}H_{15}NO_2$	1613 (CO); 3305 (NH); 3390 br (OH)		
9b	62	EtOAc/hexane	110–112	$C_{16}H_{17}NO_3$	1649 (CO); 3384 (NH); 3000 br (OH)	271	252
9c	55	MeOH/H_2O	160 (dec.)	$C15H_{14}N_2O_4$	1627 (CO); 3334 (NH); 3000 br (OH)	286	193
11a	57	EtOAc/hexane	85–86	[23]	1630 (CO); 3306 (NH)		
11b	62	EtOAc/hexane	72–74	$C_{16}H_{17}NO_3$	1642 (CO); 3392 (NH)	271	136
11c	80	EtOAc/hexane	84–85	$C_{15}H_{14}N_2O_4$	1654 (CO); 3400 (NH)		
12a	63	MeOH/H_2O	138–140	[24]	1640 (CO); 3354 (NH); 3120 br (OH)	227	91
12b	65	H_2O	103–105	$C_{15}H_{15}NO_3$	1633 (CO); 3350 (NH); 3400 br (OH)		
12c	50	MeOH/H_2O	120–122	$C_{14}H_{12}N_2O_4$	1624 (CO); 3351 (NH); 3399 (OH)		

For the crystallization from EtOAc/hexane the solutions were cooled at −20 °C.

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

3a	3.72 (s, 2H, CH_2); 3.82 (s, 3H, OCH_3); 6.82–7.10, 7.23–7.33, 8.07(m, m, s, 7H, Ar); 9.1(NH); 10.3 (OH)
3b	2.15 (s, 3H, CH_3); 3.66 (s, 2H, CH_2); 3.80 (s, 3H, OCH_3); 6.69, 6.88–7.06, 7.20–7.27, 7.68 (s, m, m, s, 7H, Ar); 9.0 (NH); 9.6 (OH)
3c	3.75 (s, 2H, CH_2); 3.81 (s, 3H, OCH_3); 3.99 (s, 3H, OCH_3); 6.88–7.07, 7.21–7.32, 7.99, 9.03 (m, m, d, s, 7H, Ar); 9.4 (NH)
3d	2.35 (s, 3H, CH_3); 3.75 (s, 2H, CH_2); 3.79 (s, 3H, OCH_3); 6.86–7.03, 7.22–7.30, 7.93–8.13 (m, m, m, 7H, Ar); 9.5 (NH)
3e	3.70 (s, 2H, CH_2); 3.75 (s, 3H, OCH_3); 6.87–7.29, 7.85, 8.21 (m, d, d, 8H, Ar); 10.7 (NH).
3f	3.35 (s, 2H, CH_2); 3.98 (s, 3H, OCH_3); 7.12–7.37, 7.60, 7.83, 8.13 (m, d, s, m, 8H, Ar); 10.2 (NH)
3g	3.62 (s, 2H, CH_2); 3.76 (s, 3H, OCH_3); 6.86–7.09, 7.19–7.33, 7.60 (m, m, d, 9H, Ar); 10.2 (NH)
4c	2.75 (s, 2H, CH_2); 4.02 (s, 3H, OCH_3); 6.74–6.88, 7.08–7.27, 7.99, 9.05 (m, m, d, s, 7H, Ar); 9.5 s, 9.8 s (NH; OH)
4d	2.34 (s, 3H, CH_3); 3.72 (s, 2H, CH_2); 6.73–7.20, 7.98–8.12 (m, m, 7H, Ar); 9.5 (NH); 9.9 (OH)
4e	3.66 (s, 2H, CH_2); 6.70–7.16, 7.85, 8.22 (m, m, d, 8H, Ar); 9.5 (NH); 10.7 (OH)
4f	3.66 (s, 2H, CH_2); 6.72–6.83, 7.02–7.29, 7.93 (m, m, m, 8H, Ar); 9.6, 9.7 (NH, OH)
4g	3.59 (s, 2H, CH_2); 6.71–6.83, 6.98–7.34, 7.60 (m, m, d, 9H, Ar); 9.5 (NH); 10.0 (OH)
6a	4.02 (s, 2H, CH_2); 6.84–6.96, 7.42–7.73, 7.96 (m, m, s, 7H, Ar); 9.5 (NH); 10.2 (OH)
6b	4.00 (s, 2H, CH_2); 7.10–7.32, 7.47–7.88, (m, m, 8H, Ar); 10.0 (NH)
8a	3.45 (s, 2H, CH_2); 3.74 (s, 3H, OCH_3); 4.27 (d, 2H, CH_2); 6.81–6.98, 7.14–7.36 (m, m, 9H, Ar); 8.3 t (NH)
8b[a]	3.61 (s, 2H, CH_2); 3.71 (s, 3H, OCH_3); 3.74 (s, 3H, OCH_3); 4.39 (d, 2H, CH_2); 6.80–6.99, 7.10–7.33 (m, m, 8H, Ar); 6.3 br (NH)
8c[a]	3.58 (s, 2H, CH_2); 3.82 (s, 3H, OCH_3); 4.61 (d, 2H, CH_2); 6.88–6.99, 7.20–7.60, 8.03 (m, m, d, 8H, Ar); 6.7 br (NH)
9a	3.46 (s, 2H, CH_2); 4.29 (d, 2H, CH_2); 6.71–6.82, 7.01–7.11, 7.25 (m, m, m, 9H, Ar); 8.5 t (NH); 9.6 br (OH)
9b	3.47 (s, 2H, CH_2); 3.78 (s, 3H, OCH_3); 4.24 (d, 2H, CH_2); 6.70–7.20 (m, 8H, Ar); 8.2 (NH); 9.7 (OH)
9c	3.48 (s, 2H, CH_2); 4.55 (d, 2H, CH_2); 6.71–6.83, 7.00–7.11, 7.50–7.74, 8.02 (m, m, m, d, 8H, Ar); 8.5 t (NH); 8.6 (OH)
11a[a]	3.88 (s, 3H, OCH_3); 4.50 (d, 2H, CH_2); 6.99–7.52, 7.74 (m, d, 9H, Ar); 8.7 t (NH).
11b	3.86 (s, 3H, OCH_3); 3.92 (s, 3H, OCH_3); 4.47 (d, 2H, CH_2); 6.88–7.31, 7.48, 7.81 (m, t, d, 8H, Ar); 8.6 t (NH)
11c	3.93 (s, 3H, OCH_3); 4.77 (d, 2H, CH_2); 7.00–7.23, 7.46–7.84, 8.06 (m, m, d, 8H, Ar); 8.9 t (NH)
12a	4.52 (d, 2H, CH_2); 6.87–6.94, 7.35, 7.90 (m, m, d, 9H, Ar); 9.4 t (NH); 12.5 (OH)
12b	3.83 (s, 3H, OCH_3); 4.48 (d, 2H, CH_2); 6.84–7.03, 7.16–7.44, 7.94 (m, m, d, 8H, Ar); 9.2 t (NH); 12.4 (OH)
12c	4.79 (d, 2H, CH_2); 6.88–6.95, 7.37–8.08 (m, m, 8H, Ar); 9.3 t (NH); 12.1 (OH)

[a] Registered in $CDCl_3$.

Table 3
Parameters of vasorelaxing efficacy (E_{max} in %) and potency (pIC_{50}) exhibited by the synthesised compounds and by the reference drug NS 1619

Compound	E_{max} (%)	pIC_{50}
3a	37 ± 10[a]	NC
3b	61 ± 5[a]	4.61 ± 0.03[a]
3c	21 ± 3[a]	NC
3d	Ineffective	–
3e	29 ± 2[a]	NC
3f	28 ± 13[a]	NC
3g	Ineffective	–
4c	82 ± 2	4.74 ± 0.02[a]
4d + IbTX 100 nM	84 ± 2, 32 ± 8[b]	4.70 ± 0.02[a], NC
4e	79 ± 5	4.64 ± 0.03[a]
4f	43 ± 4[a]	NC
4g	40 ± 4[a]	NC
6a	50 ± 16[a]	NC
6b	55 ± 4[a]	4.56 ± 0.05[a]
8a	25 ± 2[a]	NC
8b	22 ± 2[a]	NC
8c	NT	
9a	33 ± 3[a]	NC
9b	40 ± 16[a]	NC
9c	40 ± 4[a]	NC
11a	23 ± 2[a]	NC
11b	16 ± 3[a]	NC
11c	29 ± 2[a]	NC
12a	47 ± 4[a]	NC
12b	NT	
12c	47 ± 4[a]	NC
NS 1619	91 ± 3	5.36 ± 0.04

NT: not tested; NC indicates that the potency value could not be calculated because of the low efficacy (≤50%).
[a] Significantly different from the corresponding value exhibited by NS 1619.
[b] Significantly different from the corresponding value recorded in the absence of IbTX.

In particular, these compounds, all characterised by very low levels of vasorelaxing efficacy, show the presence of a methylenic bridge at the basic side of the amide linker, which probably represents the main cause for their poor pharmacological activity. On the other hand, some of these molecules (in particular, compounds **8** and **9**) exhibit also the presence of a further methylene spacer, at the acidic side of the amide, but this structural characteristic is likely to be less responsible for their poor efficacy. In fact, this last structural feature is also present in compounds **3**, **4** and **6**. Among these phenyl acetanilide derivatives **4c–e** showed appreciable levels of vasorelaxing efficacy, although with modest potency parameters, significantly lower than that of NS 1619 ($P < 0.05$). Furthermore, the selective BK-blocker iberiotoxin (IbTX; 100 nM) caused a significant ($P < 0.05$) decrease of the vasorelaxing efficacy of compound **4d**, indicating that the activation of these potassium channels plays a major role in the vasodilator activity.

Noteworthy, all these three compounds possess a phenolic hydroxy group at the *ortho*-position of the benzene ring at the acidic side of the amide linker, which seems to represent a significant indication about the need of such a structural feature as an useful requirement for the interaction with the biological target (BK channel). This remark seems to be well supported by the observation of the pharmacological profile exhibited by the couples of analogues **4c–3c**, **4d–3d** and **4e–3e**.

On the contrary, the poor pharmacological effects of analogous compounds, bearing a phenolic hydroxyl group at the *ortho*-position of the benzene ring at the basic side of the amide linker (**3a**, **3b**, and **6a**), suggest that this structural feature plays a negligible role for the interaction with the biological target.

Furthermore, the presence of an aromatic nitro-group in the benzene ring at the basic side of the amide linker seems to be a further favourable requirement. Indeed, this group is present in compounds **4c–e** (i.e., in the most effective compounds of these series), while it is absent in compounds **4g** and **4f**, whose vasorelaxing efficacy resulted markedly decreased. In previous papers, we reported benzanilide derivatives which, in some cases, showed a satisfactory profile of BK-openers. Therefore, this work was aimed at evaluating the influence of structural variation on the biological properties of such derivatives. Overall, the main findings of this work suggest that the introduction of one or two methylene group(s) in the amide linker exerts a negative influence on the vasorelaxing responses, which can be due to an excessive lengthening of the spacer between the two aromatic rings and/or to the conferring of further degrees of conformational freedom, leading to a reduced ability of a potential interaction with the ''receptorial'' site of the BK channel.

5. Experimental section

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. Elemental analyses (C, H, N) were within ±0.4% of the theoretical values and were performed on a Carlo Erba Elemental Analyzer Mod. 1106 apparatus.

5.1.1. N-(Substituted-phenyl)-2-methoxy-phenylacetamides (3a–g)

A solution of 2-methoxy-phenylacetic acid (0.830 g, 5.0 mmol) in 10 mL of $SOCl_2$ was heated under reflux for 1 h. The reagent was distilled off and the residue, consisting of the corresponding acyl chloride **1**, was dissolved in 20 mL of anhydrous toluene. This solution was added dropwise to a solution of the suitable amine [5.0 mmol of 2-hydroxy-5-chloro-aniline (**2a**), 2-hydroxy-5-methyl-aniline (**2b**), 2-methoxy-5-nitro-aniline (**2c**), 2-methyl-4-nitro-aniline (**2d**), 4-nitro-aniline (**2e**), 2-fluoro-aniline (**2f**) or aniline (**2g**)] and TEA (0.7 mL, 5.0 mmol) in 20 mL of anhydrous toluene and the mixture was refluxed for 12 h.

For the isolation of **3a** and **3b**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration and treated with H_2O to dissolve the possible TEA

hydrochloride. The insoluble material consisted of a first portion of **3a** or **3b**. The toluene filtrate was evaporated in vacuo, the residue was dissolved in $CHCl_3$ and the new solution, after washing with 10% HCl and 5% $NaHCO_3$, was dried ($MgSO_4$) and evaporated to give a further amount of **3a** or **3b**. The combined fractions were purified by crystallization (Table 1).

For the isolation of **3e**, the toluene solution, after cooling, separated a crystalline solid which was collected by filtration and consisted of TEA hydrochloride. The filtrate, worked up as above, provided **3e** which was purified by crystallization (Table 1).

For the isolation of **3c**, **3d**, **3f** and **3g**, the toluene solution was evaporated and worked up in the usual manner (Table 1).

5.1.2. *N-(Substituted-phenyl)-2-hydroxy-phenylacetamides (**4c–g**)*

To a solution of 2.0 mmol of the suitable methoxy derivative **3c–e**, **3f** or **3g** in 100–120 mL of anhydrous CH_2Cl_2, cooled at −30 °C, a solution of BBr_3 (≈2 mL, ≈20 mmol; ≈3 mL, ≈30 mmol for **3c**) in 10 mL of anhydrous CH_2Cl_2 was added drop by drop, under stirring. After 1 h at this temperature, the reaction mixture was left at −20 °C overnight. The excess of the reagent was decomposed by cautious addition of MeOH (≈10 mL) and H_2O (≈30 mL). The organic phase, after washing with H_2O, was extracted with 10% NaOH. From the alkaline solution, cooled in an ice-bath, by acidification with 36% HCl, precipitated the product which was collected by filtration and purified by crystallization (Table 1).

The CH_2Cl_2 solution, dried ($MgSO_4$) and evaporated, left traces of solid residue.

5.1.3. *2-Trifluoromethyl-phenylacetanilides (**6a** and **6b**)*

A solution of 2-trifluoromethyl-phenylacetic acid (1.22 g, 6.0 mmol) in 10 mL of $SOCl_2$ was heated under reflux for 1 h. The reagent was distilled off and the residue, consisting of the corresponding acyl chloride **5**, was dissolved in 30 mL of anhydrous toluene. This solution was added dropwise to a solution of the suitable amine [6.0 mmol of 2-hydroxy-5-chloro-aniline (**2a**) or 2-fluoro-aniline (**2f**)] and TEA (0.85 mL, 6.0 mmol) in 50 mL of anhydrous toluene and the mixture was heated at 100 °C overnight. After cooling the solution was evaporated in vacuo and the residue was dissolved in $CHCl_3$. The chloroform solution, after washing with 10% HCl and 5% $NaHCO_3$, was dried ($MgSO_4$) and evaporated to give the title compounds as a solid residue (Table 1).

5.1.4. *N-(Substituted-benzyl)-2-methoxy-phenylacetamides (**8a–c**)*

A solution of 2-methoxy-phenylacetic acid (0.830 g, 5.0 mmol) in 10 mL of $SOCl_2$ was heated under reflux for 1 h. The reagent was distilled off and the residue, consisting of the corresponding acyl chloride **1**, was dissolved in 20 mL of anhydrous toluene. This solution was added dropwise to a solution of the suitable amine [5.0 mmol of benzylamine (**7a**), 2-methoxy-benzylamine (**7b**) or 2-nitro-benzylamine (**7c**)] and TEA (0.7 mL, 5.0 mmol) in 20 mL of anhydrous toluene and the mixture was refluxed for 12–16 h. After cooling, a crystalline precipitate of triethylamine hydrochloride was formed which was separated by filtration. The filtrate was evaporated in vacuo and the residue was dissolved in $CHCl_3$. The chloroform solution, after washing with 10% HCl and 10% NaOH, was dried ($MgSO_4$) and evaporated to give the title compounds as a residue which was purified by crystallization (Table 1).

5.1.5. *N-(Substituted-benzyl)-2-hydroxy-phenylacetamides (**9a–c**)*

A solution of 2.0 mmol of the appropriate methoxy derivative **8a**, **8b** or **8c** in 80–100 mL of anhydrous CH_2Cl_2 was cooled at −20 °C and, under stirring, a solution of BBr_3 (2 mL, ≅20 mmol; 3 mL, ≅30 mmol for **9b**) in 8–10 mL of anhydrous CH_2Cl_2 was added drop by drop. After 1 h of stirring at −20 °C, the reaction mixture was left in an ice-chamber (−20 °C) overnight. The reagent was decomposed by addition of MeOH (8 mL) followed by H_2O (30–40 mL) and the organic phase was separated from the aqueous one. The organic phase (CH_2Cl_2), after washing with H_2O, was extracted with 10% NaOH, then dried ($MgSO_4$) and evaporated to give no residue or little amounts of the starting material. Acidification of the combined alkaline extracts provided the title compounds which were collected by filtration and purified by crystallization (Table 1).

5.1.6. *N-(Substituted-benzyl)-2-methoxy-benzamides (**11a–c**)*

A solution of 2-methoxy-benzoic acid (0.761 g, 5.0 mmol) in 8 mL of $SOCl_2$ was heated under reflux for 1 h. The reagent was distilled off and the residue, consisting of the corresponding acyl chloride **10**, was dissolved in 20 mL of anhydrous toluene. This solution was added dropwise to a solution of the suitable amine [5.0 mmol of benzylamine (**7a**), 2-methoxy-benzylamine (**7b**) or 2-nitro-benzylamine (**7c**)] and TEA (1 mL, 7.2 mmol) in 20 mL of anhydrous toluene and the mixture was refluxed for 15 h. After cooling a precipitate was formed which was collected by filtration and treated with H_2O. The insoluble material consisted of a fraction of the title compounds. The filtrated toluene solution 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 fraction of the title compounds. The combined fractions of product were purified by crystallization (Table 1).

5.1.7. *N-(Substituted-benzyl)-2-hydroxy-benzamides (**12a–c**)*

A solution of 2.0 mmol of the appropriate methoxy derivative **11a**, **11b** or **11c**, in 100 mL of anhydrous CH_2Cl_2 was cooled at −20 °C and, under stirring, a solution of BBr_3 (2 mL, ≅20 mmol; 3 mL, ≅30 mmol for **12b**) in 8–10 mL of anhydrous CH_2Cl_2 was added drop by drop. After 1 h of stirring at −20 °C, the reaction mixture was left in an ice-chamber (−20 °C) overnight. The reagent was decomposed by addition of MeOH (8 mL) followed by H_2O (40 mL) and

the organic phase (CH_2Cl_2) after washing with H_2O was extracted with 10% NaOH, then dried ($MgSO_4$) and evaporated to give no residue (or little amounts of the starting material). Acidification of the combined alkaline extracts provided the title compounds which were collected by filtration and purified by crystallization (Table 1).

5.2. 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 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; $MgSO_4$ 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 as 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, 3-fold increasing concentrations (10 nM–30 μM) of the tested compounds or of the reference drug NS 1619 (a well-known BK-activator) were added cumulatively. For the selected compound **4d**, the same protocol was also carried out in the presence of the selective BK-blocker iberiotoxin (IbTX; 100 nM), which was incubated for 20 min before the addition of KCl (20 mM). 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. IbTX was diluted in bidistilled water. 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 of 30 μM (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).

References

[1] V. Calderone, Curr. Med. Chem. 9 (2002) 1385–1395.
[2] S.-N. Wu, Curr. Med. Chem. 10 (2003) 649–661.
[3] S. Ghatta, D. Nimmagadda, X. Xu, S. O'Rourke, Pharmacol. Ther. 110 (2006) 103–116.
[4] J.E. Brayden, M.T. Nelson, Science 256 (1992) 532–535.
[5] Y. Tanaka, K. Koike, L. Toro, J. Smooth Muscle Res. 4 and 5 (2004) 125–153.
[6] G. Biagi, I. Giorgi, O. Livi, V. Scartoni, P.L. Barili, V. Calderone, E. Martinotti, Farmaco 56 (2001) 827–834.
[7] Y. Shintani, K. Node, H. Asanuma, S. Sanada, S. Takashima, Y. Asano, Y. Liao, M. Fujita, A. Hirata, Y. Shinozaki, Tà Fukushima, Y. Nagamachi, H. Okuda, J. Kim, H. Tomoike, M. Hori, M. Kitakaze, J. Mol. Cell. Cardiol. 37 (2004) 1213–1218.
[8] M. C-Cao, Q. Xia, Q. Gao, M. Chen, T.-M. Wong, J. Pharmacol. Exp. Ther. 312 (2005) 644–650.
[9] T. Sato, T. Saito, N. Saegusa, H. Nakaya, Circulation 111 (2005) 198–203.
[10] G. Biagi, V. Calderone, I. Giorgi, O. Livi, V. Scartoni, B. Baragatti, E. Martinotti, Eur. J. Med. Chem. 35 (2000) 715–720.
[11] B. Baragatti, G. Biagi, V. Calderone, I. Giorgi, O. Livi, E. Martinotti, V. Scartoni, Eur. J. Med. Chem. 35 (2000) 949–955.
[12] G. Biagi, V. Calderone, I. Giorgi, O. Livi, V. Scartoni, B. Baragatti, E. Martinotti, Farmaco 56 (2001) 841–849.
[13] S.P. Olesen, E. Munch, P. Moldt, J. Drejer, Eur. J. Pharmacol. 251 (1994) 53–59.
[14] G. Biagi, V. Calderone, I. Giorgi, O. Livi, E. Martinotti, A. Martelli, A. Nardi, Farmaco 59 (2004) 397–404.
[15] V. Calderone, I. Giorgi, O. Livi, E. Martinotti, A. Martelli, A. Nardi, Farmaco 60 (2005) 367–375.
[16] V. Calderone, I. Giorgi, O. Livi, E. Martinotti, E. Mantuano, A. Martelli, A. Nardi, Eur. J. Med. Chem. 40 (2005) 521–528.
[17] G. Biagi, I. Giorgi, O. Livi, A. Nardi, V. Calderone, A. Martelli, E. Martinotti, O. LeRoy Salerni, Eur. J. Med. Chem. 39 (2004) 491–498.
[18] V. Calderone, F.L. Fiamingo, I. Giorgi, M. Leonardi, O. Livi, A. Martelli, E. Martinotti, Eur. J. Med. Chem. 41 (2006) 761–767.
[19] V. Calderone, F.L. Fiamingo, I. Giorgi, M. Leonardi, O. Livi, A. Martelli, E. Martinotti, Eur. J. Med. Chem. 41 (2006) 1421–1429.

[20] F.M. Dean, J. Goodchild, L.E. Houghton, J.A. Martin, R.B. Morton, B. Parton, A.W. Price, N. Somvichien, Tetrahedron Lett. 7 (1966) 4153–4159.
[21] T. Yamana, A. Tsuji, Y. Mizukami, Chem. Pharm. Bull. 21 (1973) 721–728.
[22] V.C. Agwada, J. Chem. Eng. Data 27 (1982) 481–483.
[23] A.F. Burchat, J.M. Chong, N. Nielsen, J. Org. Chem. 61 (1996) 7627–7630.
[24] K. Waisser, M. Perina, J. Kunes, V. Klimesova, J. Kaustova, Farmaco 58 (2003) 1137–1149.