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Design and synthesis of novel sulfonamide-containing bradykinin hB_2 receptor antagonists. Synthesis and structure-relationships of α,α-tetrahydropyranylglycine

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ABSTRACT

A series of α,α-cycloalkylglycine sulfonamide compounds of general formula **1** has previously been identified by our group as selective human B_2(hB_2) receptor antagonists. Here we report the in vitro and in vivo BK antagonist activity of a further evolution of the series, consisting in compounds of the general formula **2**, containing either an alkyl piperazine or a 4-alkyl piperidine ring bearing various positively charged groups (R′). These studies unexpectedly revealed quite a flat nanomolar/subnanomolar SAR for the binding affinity, while differences were seen in the in vitro functional activities. We propose that variations in the residence time may explain these results.

1. Introduction

Bradykinin (BK) is a nonapeptide (Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9]) involved in a variety of pathophysiological responses, such as pain and inflammation. Its biological effects are mediated through activation of two distinct cell surface receptors: B_1 and B_2.[1] B_1 receptors are inducible and poorly expressed under normal physiological conditions, while the B_2 receptors are constitutively expressed in several cell types and mediate the effects of BK.[2] A potential therapeutic role for kinin B_2 receptor antagonists could be the treatment of airway inflammatory pathologies associated with hyperresponsiveness to BK, such as chronic bronchial asthma,[3] or with the release of BK, such as perennial and seasonal allergic rhinitis.[4] In humans the B_2 receptor, besides being present in the upper and lower airways, is also expressed in the cardiovascular system, where kinins have been proven to exert protective effects.

On the basis of the above considerations, our group has followed a strategy to block the B_2 receptors in the airways without affecting them at the cardiovascular level via the development of antagonists aimed at local administration.

We previously reported how we developed a new series of B_2 antagonists, containing cycloalkylglycine sulfonamides of general formula **1** (Fig. 1).[5] These compounds were able, upon intratracheal delivery, to selectively antagonize the effect of BK on the airways. This selectivity may be due to the high polarity of the molecules, which could facilitate them remaining localized within the respiratory tract.

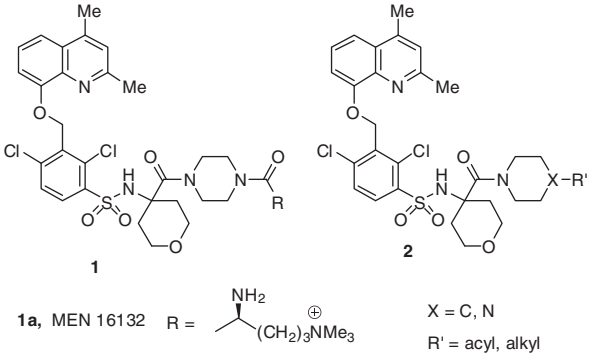

Figure 1. General formula for the series of α,α-cycloalkyl glycine sulfonamides **1** and α,α-tetrahydropyranyl glycine sulfonamide compounds **2**.

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Within this series MEN 16132 (**1a**) showed excellent activity both in vitro and in vivo. However, we wished to investigate further the effect of several factors on biological activity. Firstly could the chiral primary amine be replaced by an amino group or a second ammonium group within the alkyl chain, or even eliminated altogether? What would happen if the 'rigidifying' effect of the amide bond linking the polar side-chain to the rest of the molecule was removed? Finally, what effect would replacement of the piperazine ring with opportunely derivatized 4-alkylpiperidines have on activity?

These issues were addressed by preparing a series of compounds of general formula **2**, where various 4-alkyl piperidines and 4-alkyl piperazines were introduced onto the carboxy group of the tetrahydropyranyl glycine.

The compounds described in this study are shown in Tables 1–3 and the synthetic methods for their preparation are outlined in Schemes 1–4.[6]

All new compounds were tested for their ability to inhibit the binding of tritiated BK to the hB_2R (pK_i), and for antagonist potency (pA_2), using a functional assay in CHO cells expressing the hB_2R. The molecule (**16**) with the best overall activity in these assays was first evaluated for functional activity on the guinea pig receptor and then evaluated in vivo against **1a**.

2. Chemistry

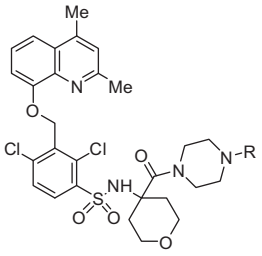
The *N*-acyl piperazine derivatives **12**–**25** were prepared by coupling acid **3** with Boc-piperazine to obtain **4**, followed by Boc cleavage and coupling of the resulting amine **5** with acids **6**–**11**, which are commercially available or were prepared according to general literature procedures. Where necessary, an additional Boc deprotection with HCl in dioxane was carried out, as shown in Scheme 1.

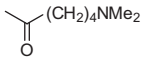
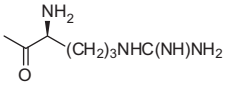
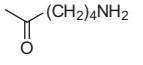
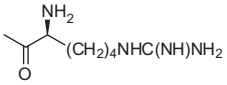
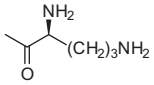
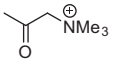
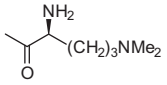
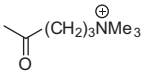
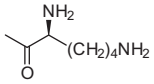
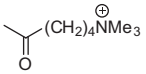
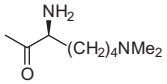
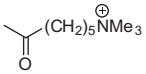
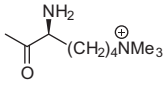
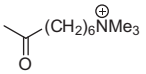
N-alkyl piperazine compounds **28**–**35** were prepared by coupling the suitably functionalized piperazine derivative **26** with acid **3**, followed by exhaustive alkylation of the terminal amino group and the piperazine nitrogen with MeI to produce the corresponding bis-ammonium salts (Scheme 2).

The 4-alkyl piperidine derivatives **38**–**44** were prepared by coupling the *N*-alkylammonium-4-aminomethyl piperidines **36** and the 4-alkylammonium piperidines **37** with acid **3** as shown in Scheme 3.

The synthesis of the spiro-analogs is shown in Scheme 4. The *N*-alkylhydroxy-piperazines **45** were coupled with acid **3**. the hydroxyl group was converted into the corresponding mesylate and spiro-cyclization either took place directly at room temperature or upon heating. The tertiary amino group of compound **49** was converted into the ammonium salt with MeI affording compound **50**.

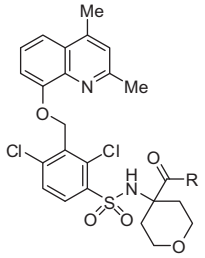
Table 1
Binding and in vitro functional activity on the hB_2 receptor of compounds **12**–**25**

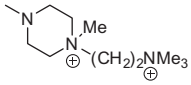
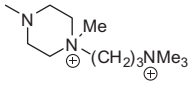
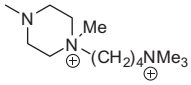
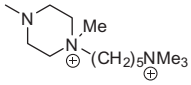
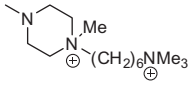
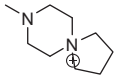
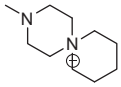
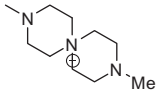
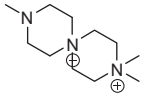


Compd	R	pK_i[a]	pA_2[b]	Compd	R	pKi[a]	pA_2[b]
1a		10.3	10.3	**19**		10	9.36
12		9.9	/	**20**		10.1	9.32
13		10.2	/	**21**		10.3	9.14
14		10.1	9.1	**23**		10.3	9.91
15		9.9	9.1	**22**		10	9.4
16		10.1	9.5	**24**		10.2	/
17		9.9	9.1	**25**		9.6	9.4
18		9.7	8.9				

[a] pK_i for the inhibition of specific binding of [^{3}H]-BK to hB_2 receptor in stably transfected CHO cells membrane preparations.
[b] pA_2 for the BK mediated accumulation of inositolphosphates in stably transfected CHOdhfr-/hB_2R cells. For details see Section 5.

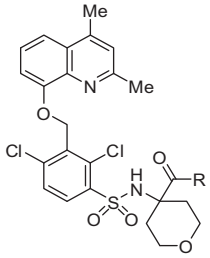
Table 2
Binding and in vitro functional activity on the hB_2 receptor of compounds and **28–32** and **47–50**

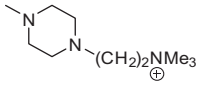
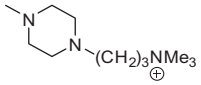
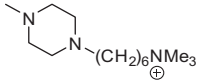
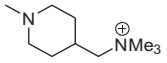
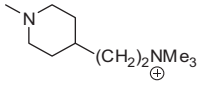
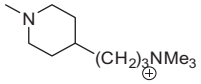
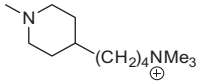
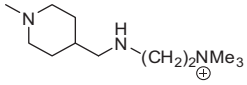
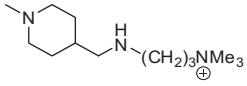
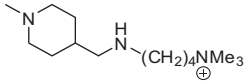


Compd	R	pK_i[a]	pA_2[b]
28		9.6	8.5
29		10	9
30		10.1	8.7
31		10.3	8.6
32		10.4	8.5
47		9.2	8.5
48		9.4	8.58
49		9	8.8
50		9.6	8.3

[a] pK_i for inhibition of specific binding of [^{3}H]-BK to hB2 receptor in stably transfected CHO cells membrane preparations.
[b] pA_2 for the BK mediated accumulation of inositolphosphates in stably transfected CHOdhfr-/hB_2R cells. For details see Section 5.

Table 3
Binding and in vitro functional activity on the hB_2 receptor of compounds **33–35** and **38–44**



Compd	R	pK_i[a]	pA_2[b]
33		9.1	/
34		9.5	/
35		10.2	9.1
38		9.4	8.3
39		9.1	8.4
40		9.3	8.8
41		9.2	8.4
42		9.7	8.6
43		9.8	8.6
44		10.3	8.6

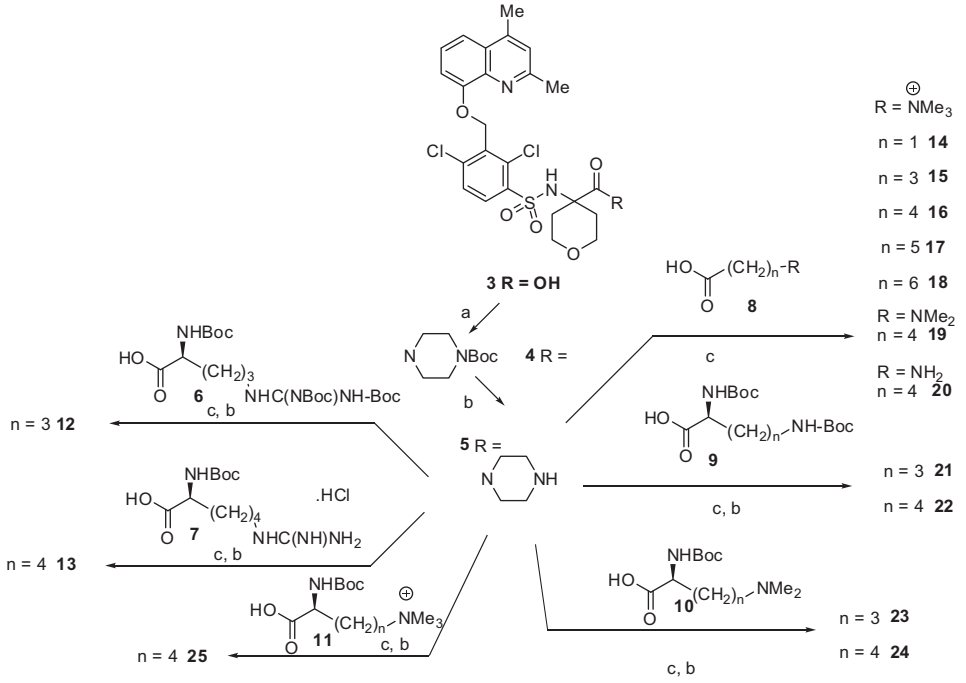
[a] pK_i for inhibition of specific binding of [^{3}H]-BK to hB_2 receptor in stably transfected CHO cells membrane preparations.
[b] pA_2 for the BK mediated accumulation of inositolphosphates in stably transfected CHOdhfr-/hB_2R cells. For details see Section 5.

The crude products were purified by preparative HPLC affording the final compounds as the corresponding trifluoroacetate salts.

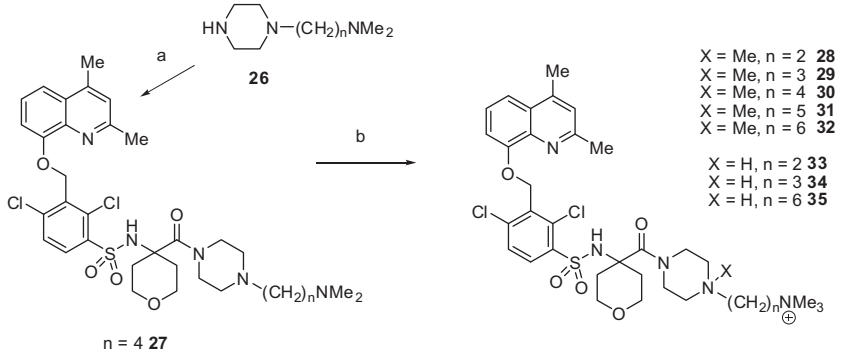
3. Results and discussion

3.1. Binding affinity

The purified compounds were evaluated for their ability to inhibit the binding of tritiated BK to the hB_2R (Tables 1–3) and showed pKi values in the sub-nanomolar range. The SARs were quite flat and the observed differences were often within the experimental error. From these and previous results, it seems that the rigid left portion of the molecule is the one manly responsible for site specificity and binding affinity, while the remainder acts by giving the molecule solubility and some additional interactions, mainly of an electrostatic nature. There is clearly a favorable cationic binding interaction at some distance from the aromatic part of the molecule, but this doesn't seem to have strict geometrical requirements. This is in agreement with data obtained from various research groups which showed that an increase in the positive charge of the antagonist/agonist increased its binding affinity for the receptor. The crucial importance of the Asp266 and Asp284 (—located at the top of transmembrane helices 6 and 7), on the binding affinity of BK and the peptide antagonist Icatibant has been demonstrated,[7] but this is not generally true for other non-peptidic antagonists, and π-cation interactions or general electrostatic effects cannot be completely ruled out. The receptor selectivity of the compounds (hB_2 vs hB_1) was assessed by measuring their ability to inhibit the binding of the tritiated B_1 selective agonist [^{3}H][desArg$_9$]Lys-BK to the hB_1R (stably expressed in CHO cells). All the compounds showed pK_i values <5–6, thus indicating their high selectivity for the hB_2R system.



Scheme 1. (a) EDC·HCl, HOAt, Boc-piperazine; (b) HCl, dioxane; (c) EDC·HCl, HOBt.



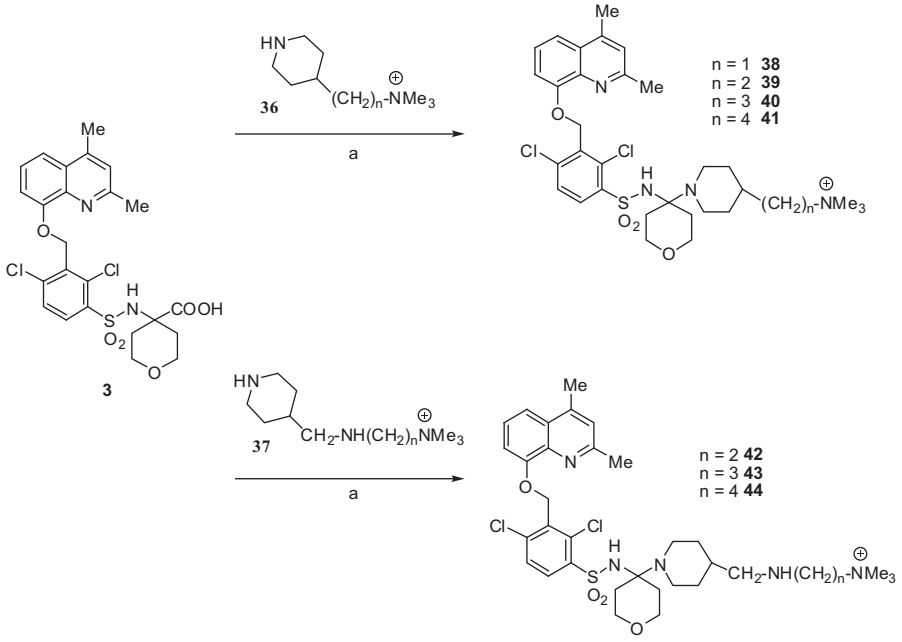
Scheme 2. (a) EDC·HCl, HOAt, **3**, DMF; (b) MeI.

3.2. Functional activity

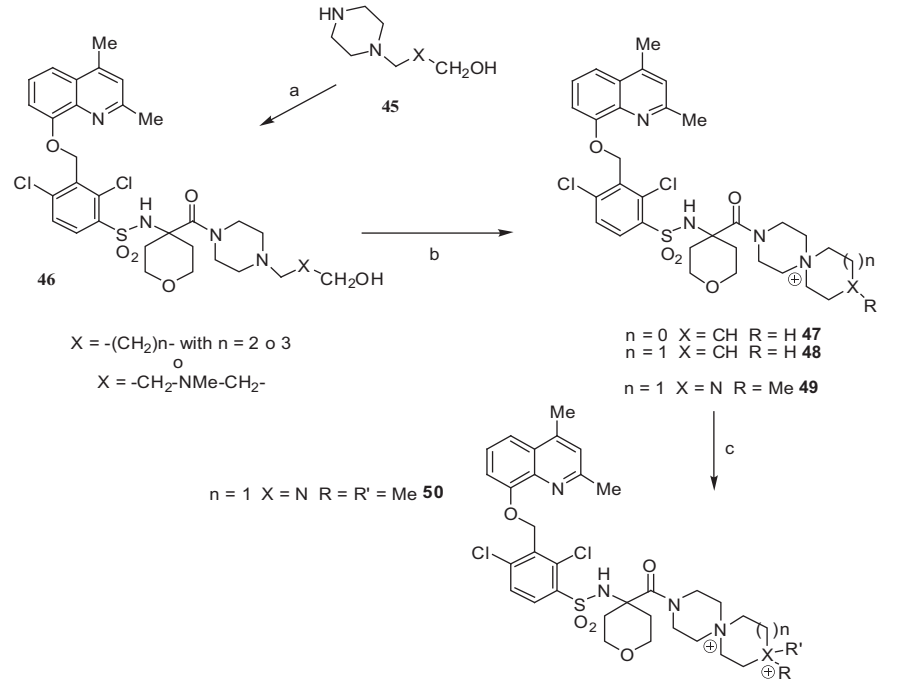
When the molecules were tested for their antagonist potency (pA_2) toward functional responses induced by BK—using the accumulation of inositolphosphates (IP) as an index of phospholipase C activation—the results were somewhat unexpected, in that the high binding affinities were not always translated into high functional activity. The antagonist efficacy of the compounds lacking an amide group on the second nitrogen of the piperazine ring was reduced by at least one log unit and a similar effect was observed for the piperidine derivatives. A possible reason for this may be a difference in the stability of the drug–target complex, also termed residence time,[8] of the various analogs with the receptor.

Functional washout experiments (involving the exposure of antagonist pre-treated tissue to fresh medium and monitoring the timewise restoration of receptor responsiveness to an agonist) provide information about antagonist dissociation characteristics. Thus, with the aim of comparing the residence times of compounds **41** and **48** with that of **1a**, the time-course of the reversibility patterns of the functional blockade was evaluated by exploiting washout experiments in the IP accumulation assay in CHO/hB_2R cells. Equieffective concentrations (3 μM for **41** and **48**, and 100 nM for **1a**) of the three compounds were pre-incubated (15 min) with the cells and the agonist (BK, 2 nM) was added. After one hour the reaction was stopped and the quantity of the IP formed was evaluated. In parallel, other cells underwent washing to remove the antagonist, that is, the incubation media was totally substituted every 15 min, and the residual antagonist capability was evaluated by adding BK as above. The results, expressed as the % of inhibition of time-matched control BK responses, obtained in cells pre-incubated with vehicle, are reported in Table 4. The data obtained clearly show that after three wash periods compound **1a** can still inhibit 50% of the agonist time-matched response, while the other two antagonists (**41** and **48**) had been completely washed out after two wash periods, thus indicating that they dissociate from the receptor more swiftly than compound **1a**. These differences in the reversibility of the functional blockade can support the relative differences in antagonist potency, as previously shown for Icatibant.[9]

Compound **16**, having the highest pA_2 on hB_2 receptor, and the added advantage of being an achiral molecule, was tested for



Scheme 3. (a) EDC·HCl, HOAt.



Scheme 4. (a) PyBOP, DIPEA, **3**, DMF; (b) MsCl, TEA; (c) MeI.

Table 4
Evaluation of the reversibility of B_2 receptor blockade of compounds **1a**, **41** and **48**

Wash period (15 min)	Compd **1a**	Compd **41**	Compd **48**
	% of inhibition of time-matched control BK response		
0	100	100	100
1	96 ± 3	23 ± 11	72 ± 16
2	60 ± 2	6 ± 8	9 ± 4
3	52 ± 2	0	0

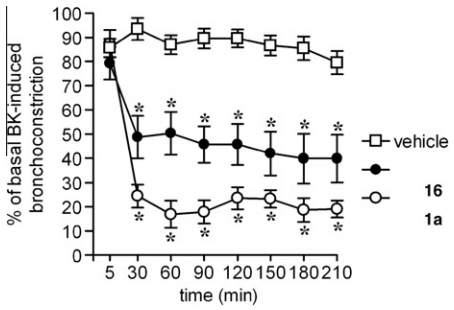


Figure 2. In vivo activity of compounds **16** and **1a** after 30 mmol/kg intratracheal administration, on bronchoconstriction induced by repeated challenges with BK (10 nmol/kg iv) in anaesthetized guinea pigs.

functional activity on the guinea pig B_2 receptor by measuring the antagonism towards the BK induced contractile responses in the isolated longitudinal smooth muscle assay. The excellent results ($pA_2 = 10.2$) encouraged us to perform a comparison of the in vivo activity of **16** with that of **1a** and this was carried out via intratracheal administration at a dose of 30 nmol/kg (Fig. 2). Unfortunately, despite having good activity in vivo compound **16** was unable to reach the inhibition levels seen with **1a**. As a consequence **1a** remained our pre-clincal candidate.

4. Conclusions

Two conclusions can be drawn from the results presented herein, together with those from our earlier work.[5] None of the initially proposed modifications to the starting lead **1a** resulted in an improvement in its overall pharmacological properties: the chiral primary ammine cannot be replaced by an amino group or a second ammonium group within the alkyl chain, nor eliminated; the elimination of the amide bond linking the polar side-chain is deleterious as is the substitution of the piperazine with a piperidine. On the other side, it seems that the portion of the molecule from the quinoline to the piperidine is the major determinant for binding recognition and that we have reached a form of plateau for the molecules represented by the general formula **2**. The piperazine terminal functionalization provides a contribution to the binding affinity, but also plays a role in solubilizing the compounds, and can be used to modulate the pharmaco-kinetic and pharmaco-dynamic properties of molecules belonging to this class. In addition, our data further highlight that the different experimental conditions used for the radioligand and functional assays reveal the different pharmacodynamic properties of closely related analogs. In fact, lower antagonist potency values may be dictated by faster dissociation from the receptor which is revealed in the functional assay, where higher concentrations (up to μM) of agonist are used to displace the antagonist, with respect to those used in the binding assay.

Further work must be carried out to elucidate the reasons for the observed differences in the functional activities of compounds which otherwise are so similar in structure and binding potency., We think that this can be seen as an interesting example of a chemical series that seems to have reached the maximum of its possibilities.

5. Experimental section

5.1. Chemistry

Commercial chemicals and solvents were of reagent grade and used without further purification.

Purity evaluation was performed via analytical HPLC using the following systems: a 600 E Waters pump coupled to a Jasco 875 UV detector, and a Merck-Hitachi D-2500 integrator, a Jasco PU-980 pump, LG-980-02 gradient unit, a Jasco UV-975 UV/vis detector, and a Merck-Hitachi D-2500 integrator; a Beckman System Gold apparatus and an Agilent 1100 analytic HPLC system. Solvents: (A) water 0.1% TFA and (B) ACN 0.1% TFA, flow 1 mL/min. System A: Column Symmetry 300 RP-18, 250 × 4.6 mm, λ = 220 nm; from 80% to 20% solvent (A) in 20 min. System B: Column Symmetry 300 RP-18, 250 × 4.6 mm, λ = 214 nm; 10 min isocratic 10% (B), then from 10%(B) to 80%(B) in 10 min. System C: Column Vydac pept. and Prot., 5 μm, 150 × 4.6 mm; λ = 220 nm; from 80% to 20% solvent (A) in 20 min.

All compounds tested had purities ⩾95%.

Preparative reverse phase HPLC was performed on a Waters 600E apparatus with a Jasco 874 UV detector or on a Waters Delta-Prep 3000 apparatus. The mobile phases were the same as for the analytical systems. Gradient elution was employed. The columns used were a SymmetryPrep™ C18, 7 μm, 19 × 300 mm, a Hibar Lichrosorb RP-18, 7 μm, 25 × 250 mm, a Vydac C18, 10 μm, 22 × 250 mm, or a Jupiter, 15 μm, 250 × 21.2 mm. Peak detection was at 220 and 254 nm. Chemical yields have not been optimized.

NMR experiments were recorded on a Varian 300 MHz spectrometer (equipped with a 5 mm inverse probe) or a Bruker Avance 400 MHz, and are referenced to residual solvent signals: $CDCl_3$ (δ 7.26) or DMSO-d_6 (δ 2.49). Chemical shifts are reported in δ units (parts per million) and are assigned as singlets (s), doublets (d), doublets of doublets (dd), triplets (t), quartet (q), quintet (quin), multiplets (m), broad signals (br), or very broad signals (v br). Coupling constants (J) are reported in hertz (Hz).

Mass spectra were recorded using a Waters Alliance 2795 HPLC system fitted with a UV-PDS 996 diode array detector, a ZMD mass spectrometer and a GL Science Inertsil ODS-3 column (50 × 3 mm, 3 μm) or a ThermoFinnigan LCQ equipped with APCI or ESI source.

5.1.1. General procedure for coupling amine 5[5] with carboxylic acids 6–11 to produce compounds 12–25

A solution of the acid (0.06 mmol) in DMF (3.0 mL) was cooled in an ice bath. EDC·HCl (0.06 mmol) and HOAt (0.06 mmol) were added and the resulting mixture was stirred for an additional hour at 0 °C. Amine **5** (0.04 mmol) and DIPEA (0.05 mmol) were added and stirring was continued at room temperature overnight. At the end of the reaction solvents were distilled off in vacuo and the residue purified by reparative HPLC. When BOC deprotection was necessary, the residue was dissolved in 4 N HCl in dioxane and stirred at room temperature until deprotection was complete. The solvents were then distilled off and the residue was purified by preparative HPLC.

5.1.2. *N*-{4-[4-((*S*)-2-Amino-5-guanidino-pentanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-2,4-dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (12)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.85 (1H, s), 8.20 (2H, br s), 8.00 (1H, d), 7.90–6.90 (10H, m), 5.65 (H, br s), 4.50 (1H, br s), 4.30–3.05 (14H, m), 2.70 (3H, s), 3.65 (3H, s), 2.00–1.40 (8H, m). MS *m/z* Calcd for $C_{34}H_{44}Cl_2N_8O_6S$: 762.1. Found: 763.2 $[M+H]^+$. HPLC purity: system B, t_R 5.29 min.

5.1.3. *N*-{4-[4-((*S*)-2-Amino-6-guanidino-hexanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-2,4-dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoro acetate salt (13)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.85 (1H, s), 8.15 (2H, br s), 8.0 (1H, d), 7.90–6.70 (11H, m), 5.65 (2H, br s), 4.70–3.00 (15H, m), 2.80 (br s, 3H), 3.7 (3H, br s), 2.00–1.25 (10H, m). MS *m/z* Calcd

for $C_{35}H_{46}Cl_2N_8O_6S$: 776.3. Found:777.2 $[M+H]^+$. HPLC purity: system B, t_R 5.38 min.

5.1.4. [2-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-2-oxo-ethyl]-trimethylammonium trifluoroacetate salt (14)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.45 (1H, s), 8.05 (1H, d), 7.80 (2H, m), 7.60 (1H, m), 7.45 (2H, m), 5.70 (2H, br s), 4.50 (2H, s), 4.10 (3H, m), 3.80 (4H, m), 3.60 (2H, m), 3.50 (2H, m), 3.40 (3H, m), 3.30 (9H, s), 2.75 (3H, s), 3.70 (9H, s), 2.00 (2H, m), 1.75 (2H, m). MS *m/z* Calcd for $C_{33}H_{42}Cl_2N_5O_6S$: 706.2. Found: 706.4 $[M]^+$. HPLC purity: system B, t_R 5.71 min.

5.1.5. [4-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-4-oxo-butyl]-trimethyl-ammonium trifluoroacetate salt (15)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.65 (1H, s), 8.05 (1H, d), 7.80 (4H, m), 7.60 (2H, m), 7.50 (2H, m), 5.70 (2H, br s), 3.80 (3H, m), 3.60 (4H, m), 3.45 (3H, m), 3.30 (4H, m), 3.05 (9H, s), 7.75 (3H, s), 2.70 (3H, s), 2.50 (2H, m), 2.00 (4H, m), 1.70 (2H, m). MS *m/z* Calcd for $C_{35}H_{46}Cl_2N_5O_6S$: 734.2. Found: 734.2 $[M]^+$. HPLC purity: system B, t_R 5.69 min.

5.1.6. [5-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-5-oxo-pentyl]-trimethyl-ammonium trifluoroacetate salt (16)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.85 (1H, s), 8.15 (1H, d), 7.70 (5H, m), 5.70 (2H, br s), 4.45–3.40 (7H, m), 3.40–3.20 (7H, m), 3.10 (9H, s), 2.80 (6H, br s), 2.50 (2H, m), 1.95 (2H, m), 1.70 (2H, m), 1.55 (2H, m). MS *m/z* Calcd for $C_{36}H_{48}Cl_2N_5O_6S$: 748.3. Found: 748.2 $[M]^+$. HPLC purity: system B, t_R 5.65 min.

5.1.7. [6-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-6-oxo-hexyl]-trimethyl-ammonium trifluoroacetate salt (17)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.80 (1H, s), 8.05 (1H, d), 7.80 (5H, m), 5.70 (2H, br s), 3.80 (7H, vbr m), 3.35 (7H, m), 3.00 (9H, s), 2.80 (6H, br s), 2.40 (2H, m), 1.95 (2H, m), 1.70 (2H, m), 1.50 (2H, m), 1.30 (2H, m). MS *m/z* Calcd for $C_{37}H_{50}Cl_2N_5O_6S$: 762.3. Found: 762.3 $[M]^+$. HPLC purity: system B, t_R 5.77 min.

5.1.8. [7-(4-{4-[2,4-Dichloro-3-(2,4-dimetyl-[7-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-7-oxo-heptyl]-trimethyl-ammonium trifluoroacetate salt (18)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.80 (1H, s), 8.00 (1H, d), 7.80 (1H, d), 7.00 (4H, m), 5.70 (2H, br s), 3.80 (3H, m), 3.70 (4H, m), 3.50 (3H, m), 3.30 (4H, m), 3.00 (9H, s), 2.80 (6H, br s), 2.40 (2H, m), 1.90 (2H, m), 1.70 (4H, m), 1.55 (2H, m), 1.30 (4H, m). MS *m/z* Calcd for $C_{38}H_{52}Cl_2N_5O_6S$: 776.3. Found: 776.3 $[M]^+$. HPLC purity: system B, t_R 5.77 min.

5.1.9. 2,4-Dichloro-N-{4-[4-(5-dimethylamino-pentanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (19)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.10(1H, br s), 8.45 (1H, s), 8.00(1H, d), 7.75 (2H, m), 7.50 (1H, m), 7.35 (2H, m), 5.70 (2H, s), 3.75 (4H, m), 3.60 (4H, m), 3.40 (4H, m), 2.80 (6H, s), 2.75 (3H, s), 2.70 (3H, s), 2.45 (2H, m), 2.00 (2H, m), 1.70 (4H, m), 1.35 (2H, m). MS *m/z* Calcd for $C_{35}H_{45}Cl_2N_5O_6S$: 733.2. Found: 734.2 $[M+H]^+$. HPLC purity: system B, t_R 5.64 min.

5.1.10. *N*-{4-[4-(5-Amino-pentanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-2,4-dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (20)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.50 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.60 (1H, m), 7.45 (1H, m), 5.70 (2H, m), 3.70 (8H, br m), 3.45 (4H, m), 3.35 (2H, m), 2.85 (2H, m), 2.70 (3H, s), 3.65 (3H, s), 2.40 (2H, m), 2.00 (2H, m), 1.70 (2H, m), 1.60 (4H, m). MS *m/z* Calcd for $C_{33}H_{41}Cl_2N_5O_6S$: 705.2. Found: 706.2 $[M+H]^+$. HPLC purity: system B, t_R 5.60 min.

5.1.11. 2,4-Dichloro-N-{4-[4-((*S*)-2,5-diamino-pentanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (21)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.55 (1H, s), 8.1 (3H, br s), 8.00 (1H, s), 7.75 (3H, br s), 7.70 (2H, m), 7.50 (1H, m), 7.35 (2H, m), 5.70 (2H, s), 4.50 (1H, s), 3.80–3.35 (12H, vbrm), 2.85 (2H, br s), 2.70 (3H, s), 2.65 (3H, s), 2.00(2H, m),1.70 (6H, m). MS *m/z* Calcd for $C_{33}H_{42}Cl_2N_6O_6S$: 720.23. Found: 721.2 $[M+H]^+$. HPLC purity: system B, t_R 5.20 min.

5.1.12. [(*S*)-5-Amino-6-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-y)-6-oxo-hexyl]-trimethyl-ammonium trifluoroacetate salt (22)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.50 (1H, s), 8.05 (3H, br s), 8.00 (1H, d), 7.75 (2H, m), 7.70 (3H, m), 7.50 (1H, m), 7.35 (2H, m), 5.70 (2H,s), 4.40 (2H, br s), 3.95–3.30 (12H, m), 2.85(2H, m), 2.75 (3H, s), 3.70 (3H, s), 2.00 (2H, m), 2.75 (4H, m), 1.60 (2H, m), 1.45 (2H, m). MS *m/z* Calcd for $C_{34}H_{44}Cl_2N_6O_6S$: 734.2. Found: 735.6 $[M+H]^+$. HPLC purity: system B, t_R 5.20 min.

5.1.13. *N*-{4-[4-((*S*)-2-Amino-5-dimethylamino-pentanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-2,4-dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (23)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.70 (1H, br s), 8.60 (1H, s), 8.20 (3H, br s), 8.00 (1H, d), 7.80 (2H, m), 7.60 (1H, m), 7.50 (2H, m), 5.70 (2H, s), 3.90–3.50 (8H, m), 3.45 (2H, m), 3.30 (2H, m), 3.10 (2H,m), 2.80 (6H, s), 2.70 (3H, s), 2.65 (3H, s), 1.95 (2H, m), 1.70 (6H, m). MS *m/z* Calcd for $C_{35}H_{46}Cl_2N_6O_6S$: 748.3. Found: 749.4 $[M+H]^+$. HPLC purity: system B, t_R 5.42 min.

5.1.14. *N*-{4-[4-((*S*)-2-Amino-6-dimethylamino-hexanoyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-2,4-dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (24)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.70 (1H, br s), 8.85 (1H, s), 8.20 (3H, br s), 8.00 (1H, d), 7.90-7.50 (5H, m), 5.70 (2H, br s), 4.50 (1H, br s), 4.00–3.15 (18H, m), 3.00 (2H, m), 2.75 (6H, br s), 1.90 (2H, m), 1.60 (6H, m), 1.30 (2H, m). MS *m/z* Calcd for $C_{36}H_{48}Cl_2N_6O_6S$: 762.3. Found: 763.3 $[M+H]^+$. HPLC purity: system B, t_R 5.28 min.

5.1.15. (*S*)-5-Amino-6-(4-(4-(2,4-dichloro-3-((2,4-dimethyl quinolin-8-yloxy)methyl)phenylsulfonamido)tetrahydro-2*H*-pyran-4-carbonyl)piperazin-1-yl)-*N*,*N*,*N*-trimethyl-6-oxohexan-1-aminium trifluoroacetate salt (25)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.80 (1H, s), 8.20 (3H, s), 8.00 (1H, d), br7.80 (2H, m), 7.60 (1H, m), 7.50 (2H, m), 5.70 (2H, br s), 4.45 (1H, m), 4.00–3.20 (14H, m), 3.00 (9H, s), 2.70 (3H, s), 2.65

(3H, s), 1.90 (2H, m), 1.70 (6H, m), 1.35 (2H, m). MS *m/z* Calcd for $C_{37}H_{51}Cl_2N_6O_6S$: 777.3. Found: 777.4 $[M]^+$. HPLC purity: system A, t_R 8.84 min.

5.1.16. General method for the coupling of acid 3 with amines 26 and subsequent methylation to obtain compounds 27–35

PyBOP (0.06 mmol) and DIPEA (0.09 mmol) were added to a solution of acid **3** (0.06 mmol) in DMF (1 mL). Amine **26** (0.06 mmol) and DIPEA (0.09 mmol) in DMF (1 mL) were added to the reaction and stirring was continued at room temperature. At the end of the reaction the solvents were distilled off in vacuo. EtOAc was added to the residue, the organic solution was washed three times with a saturated $NaHCO_3$ solution. The organic solvent was removed under reduced pressure to give the crude product which was used in the successive methylation step.

5.1.17. 2,4-Dichloro-*N*-{4-[4-(4-dimethylamino-butyl)-piperazine-1-carbonyl]-tetrahydro-pyran-4-yl}-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonamide trifluoroacetate salt (27)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.50 (1H, br s), 8.70 (1H, s), 8.00 (1H, d), 7.80 (2H, m), 7.70 (1H, m), 7.45(2H, m), 5.70 (2H, m), 3.45 (4H, m), 3.35 (4H, m), 3.10 (4H, m), 2.80 (6H, s), 2.70 (3H, s), 2.65 (3H, s), 1.95 (2H, m), 1.70(6H, m). MS *m/z* Calcd for $C_{34}H_{45}Cl_2N_5O_5S$ 705.35. Found: 706.7 $[M+H]^+$. HPLC purity: system A, t_R 7.90 min.

5.1.18. 4-(4-(2,4-Dichloro-3-((2,4-dimethylquinolin-8-yloxy)methyl)phenylsulfonamido)tetrahydro-2*H*-pyran-4-carbonyl)-1-methyl-1-(2-(trimethylammonio)ethyl)piperazin-1-ium trifluoroacetate salt (28)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.55 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.55 (1H, m), 7.40 (2H, m), 5.70 (2H, m), 4.35–3.75 (8H, m), 3.60 (4H, m), 3.50 (2H, m), 3.30 (2H, m), 3.25 (3H, s), 3.20 (9H, s), 2.70 (3H, s), 2.65 (3H, s), 2.00 (2H, m), 1.75 (2H, m). MS *m/z* Calcd for $C_{34}H_{47}Cl_2N_5O_5S$: 707.3. Found: 324.4. HPLC purity: system C, t_R 6.12 min.

5.1.19. 4-(4-(2,4-Dichloro-3-((2,4-dimethylquinolin-8-yloxy)methyl)phenylsulfonamido)tetrahydro-2*H*-pyran-4-carbonyl)-1-methyl-1-(3-(trimethylammonio)propyl)piperazin-1-ium trifluoroacetate salt (29)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.55 (1H, s), 8.00 (1H, d), 7.80 (1H, d), 7.75 (1H, d), 7.50 (1H, dd), 7.40 (1H, d), 7.35 (1H, s), 5.70 (2H, s), 4.25 (2H, m), 4.00 (2H, m), 3.70–3.20 (12H, m), 3.25 (3H, s), 3.15 (9H, s), 2.70 (3H, s), 3.65 (3H, s), 2.25 (2H, m), 1.95 (2H, m), 1.70 (2H, m). MS *m/z* Calcd for $C_{35}H_{49}Cl_2N_5O_5S$: 722.8. Found: 720.2,722.2 $[M-H^-]^+$. HPLC purity: system C, t_R 6.24 min

5.1.20. (4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-1-methyl-piperazinium-1-yl)-butyl]-trimethyl-ammonium trifluoroacetate salt (30)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.60 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.50 (1H, m), 7.40 (2H, m), 5.70 (2H, s), 4.30 (2H, m), 3.90 (2H, m), 3.70–3.30 (12H, m), 3.20 (3H, s), 3.10 (9H, s), 2.70 (3H, s), 2.65 (3H, s), 2.00 (2H, m), 1.80 (4H, m), 1.70 (2H, m). MS *m/z* Calcd for $C_{36}H_{51}Cl_2N_5O_5S$: 735.3. Found: 338.5. HPLC purity: system C, t_R 6.26 min.

5.1.21. 4-(4-(2,4-Dichloro-3-((2,4-dimethylquinolin-8-yloxy)methyl)phenylsulfonamido)tetrahydro-2*H*-pyran-4-carbonyl)-1-methyl-1-(5-(trimethylammonio)pentyl)piperazin-1-ium trifluoroacetate salt (31)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.70 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.60 (1H, m), 7.45 (2H, m), 5.70 (2H, m), 4.30 (2H, m), 3.90 (2H, m), 3.60–3.20 (12H, m), 3.20 (3H, s), 3.05 (9H, s), 1.95 (2H, m), 1.70 (6H, m), 1.40(2H, m). MS *m/z* Calcd for $C_{37}H_{53}Cl_2N_5O_5S$: 749.31. Found: 862.2 $[M+TFA]^+$, 748.1 $[M-H]^+$. HPLC purity: system A, t_R 8.98 min.

5.1.22. 4-(4-(2,4-Dichloro-3-((2,4-dimethylquinolin-8-yloxy)methyl)phenylsulfonamido)tetrahydro-2*H*-pyran-4-carbonyl)-1-methyl-1-(6-(trimethylammonio)hexyl)piperazin-1-ium trifluoroacetate salt (32)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.70 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.60 (1H, m), 7.40 (2H, m), 5.70 (2H, s), 4.30 (2H, m), 3.90(2H, m), 3.60–3.25 (12H, m), 3.20 (3H, s), 3.20 (9H, s), 2.70 (3H, s), 2.65 (3H, s), 2.00 (2H, m), 1.70 (6H, m), 1.35 (4H, m). MS *m/z* Calcd for $C_{38}H_{55}Cl_2N_5O_5S$: 763.3. Found: 762.6 $[M-H]^+$. HPLC purity: system C, t_R 6.48 min.

5.1.23. [2-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-ethyl]-trimethyl-ammonium trifluoroacetate salt (33)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.40 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.65 (1H, m), 7.45 (2H, m), 5.70 (2H, s), 3.70–3.25 (10H, m), 3.20 (9H, s), 2.90 (2H, m), 2.70 (3H, s), 2.65 (3H, s), 2.60 (4H, m), 1.95 (2H, m), 1.70 (2H, m). MS *m/z* Calcd for $C_{33}H_{44}Cl_2N_5O_5S$: 693.7. Found: 692.5, 694.5 $[M]^+$. HPLC purity: system A, t_R 8.86 min.

5.1.24. [3-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-propyl]-trimethyl-ammonium trifluoroacetate salt (34)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.85 (1H, s), 8.00 (1H, d), 7.85–7.30 (5H, m), 5.65 (2H, br s), 4.80–3.20 (16H, m), 3.19 (9H, s), 2.70 (3H, s), 2.65 (3H, s), 2.15 (2H, m), 1.80 (2H, m), 1.65 (2H, m). MS *m/z* Calcd for $C_{34}H_{46}Cl_2N_5O_5S$: 707.7. Found: 706.1, 708.1 $[M]^+$. HPLC purity: system A, t_R 8.67 min.

5.1.25. [6-(4-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperazin-1-yl)-hexyl]-trimethyl-ammonium trifluoroacetate salt (35)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.50 (1H, s), 8.00 (1H, d), 7.70 (2H, m), 7.50 (1H, m), 7.35 (2H, m), 5.70 (2H, m), 3.50–3.00 (16H, m), 2.70 (3H, s), 2.65 (3H, s), 2.00 (2H, m), 1.70 (6H, m), 1.35 (4H, m). MS *m/z* Calcd for $C_{37}H_{52}Cl_2N_5O_5S$: 748.3. Found: 748.6 $[M]^+$. HPLC purity: system C, t_R 6.60 min

5.1.26. General method for coupling acid 3 with amines 36 and 37 to obtain compounds 38–44

A solution of acid **3** (0.11 mmol), HOAt (0.14 mmol) and EDC·HCl (0.17 mmol) in DMF (2 mL) was stirred in an ice-bath for 30 min. Then a solution of the amine (0.073 mmol) in DMF (2.0 mL) was added, followed, if necessary, by dropwise addition of DIPEA (0.075 mmol). Stirring was continued at 0 °C for an additional 30 min, then at room temperature overnight. At the end of the reaction the solvents were removed in vacuo and the residue was purified by preparative HPLC.

5.1.27. (1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-ylmethyl)-trimethyl-ammonium trifluoroacetate salt (38)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.75 (1H, s), 8.00 (1H, d), 7.80–7.30 (5H, m), 5.65 (2H, br s), 4.45 (2H, m), 3.90 (2H, m), 3.40 (2H, m), 3.25 (2H, m), 3.10 (4H, m), 3.10 (9H, s), 2.75 (6H, br s), 1.85

(6H, m), 1.70 (2H, m), 1.40(2H, m). MS *m/z* Calcd for $C_{33}H_{43}Cl_2N_4O_5S$: 678.7. Found: 677.2, 679.3 $[M]^+$. HPLC purity: system A, t_R 9.68 min.

5.1.28. [2-(1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-yl)-ethyl]-trimethyl-ammonium trilfuoroacetate salt (39)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.75 (1H, m), 8.05 (1H, d), 7.90–7.50 (5H, m), 5.65 (2H, br s), 4.50 (2H, m), 3.60–3.15 (8H, m), 3.05 (9H, s), 2.70 (6H, br s), 1.90 (2H, m), 1.80–1.45 (7H, m), 1.25 (2H, m). MS *m/z* Calcd for $C_{34}H_{45}Cl_2N_4O_5S$: 692.7. Found: 691.2, 693.3 $[M]^+$. HPLC purity: system A, t_R 9.86 min.

5.1.29. [3-(1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-yl)-propyl]-trimethyl-ammonium trilfuoroacetate salt (40)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.40 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.50 (1H, m), 7.40 (2H, m), 5.70 (2H, s), 4.50 (2H, m), 3.50–3.20 (6H, m), 3.05 (9H, s), 2.80 (2H, m), 2.70 (3H, s), 2.65 (3H, s), 1.95 (2H, m), 1.75 (6H, m), 1.55 (1H, m), 1.20 (4H, m). MS *m/z* Calcd for $C_{35}H_{47}Cl_2N_4O_5S$: 706.7. Found: 705.2, 707.3 $[M]^+$. HPLC purity: system A, t_R 10.27 min.

5.1.30. [4-(1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-yl)-butyl]-trimethyl-ammonium trilfuoroacetate salt (41)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.40 (1H, s), 8.00 (1H, d), 7.80 (2H, m), 7.60 (1H, m), 7.45 (2H, m), 5.70 (2H, s), 4.50 (2H, m), 3.35 (6H, m), 3.10 (9H, s), 2.85 (2H, m), 2.75 (3H, s), 2.70 (3H, s), 1.95 (2H, m), 1.70 (6H, m), 1.55 (1H, m), 1.35 (4H, m), 1.15 (2H, m). MS *m/z* Calcd for $C_{36}H_{49}Cl_2N_4O_5S$: 720.8. Found: 719.2, 722.3 $[M]^+$. HPLC purity: system C, t_R 7.94 min.

5.1.31. {2-[(1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-ylmethyl)-amino]-ety}-trimethyl-ammonium trifluoroacetate salt (42)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.00 (1H, br s), 8.65 (1H, s), 8.00 (1H,d), 7.80 (2H, m), 7.55 (1H, m), 7.45 (2H, m), 5.65 (2H, s), 4.55 (2H, m), 4.00–3.30 (10H, m), 3.20 (9H, s), 2.95 (2H, m), 2.70 (3H, s), 2.65 (3H, s), 2.05–1.65 (5H, m), 1.20 (4H, m). MS *m/z* Calcd for $C_{35}H_{48}Cl_2N_5O_5S$: 721.7. Found: 720.3, 722.3 $[M]^+$. HPLC purity: system C, t_R 6.50 min.

5.1.32. {3-[(1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-ylmethyl)-amino]-propyl}-trimethyl-ammonium trifluoroacetate salt (43)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.67 (1H, br s), 8.65 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.55 (1H, m), 7.45 (2H, m), 5.70 (2H, s), 4.50 (2H, m), 4.30–3.10 (10H, m), 3.00 (9H, s), 3.05–2.80 (2H, m), 2.70 (3H, s), 2.65 (3H, s), 2.30–1.65 (7H, m), 1.20 (4H, m). MS *m/z* Calcd for $C_{36}H_{50}Cl_2N_5O_5S$: 735.8. Found: 736.2 $[M]^+$. HPLC purity: system C, t_R 6.50 min.

5.1.33. {4-[(1-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxymethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-piperidin-4-ylmethyl)-amino]-butyl}-trimethyl-ammonium trifluoroacetate salt (44)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.65 (1H, s), 8.50 (1H, br s), 8.00 (1H, d), 7.50 (2H, m), 7.55 (1H, m), 7.45 (2H, m), 5.70 (2H, s), 4.55 (2H, m), 4.00–3.20 (6H, m), 3.10 (9H, s), 3.05–2.80 (6H, m), 2.70 (3H, s), 2.65 (3H, s), 2.00–1.60 (9H, m), 1.25 (4H, m). MS *m/z* Calcd for $C_{37}H_{52}Cl_2N_5O_5S$: 749.8. Found: 692.2, 694.2 $[M-NMe_3+H]^+$. HPLC purity: system C, t_R 6.46 min.

5.1.34. 8-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxy methyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbo nyl}-8-aza-5-azonia-spiro[4.5]decane trifluoroacetate salt (47)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.55 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.55 (1H, m), 7.35 (2H, m), 6.75 (2H, s), 4.00 (4H, m), 3.70 (4H, m), 3.55 (4H, m), 3.45 (2H, m), 3.30 (2H, m), 2.75 (3H, s), 2.65 (3H, s), 2.20 (4H, m), 2.00 (2H, m), 1.80 (2H, m). MS *m/z* Calcd for $C_{32}H_{39}Cl_2N_4O_5S$: 662.6. Found: 661.1, 663.1 $[M]^+$. HPLC purity: system A, t_R 9.58 min

5.1.35. 3-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxym ethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-3-aza-6-azonia-spiro[5.5]undecane trifluoroacetate salt (48)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.70 (1H, s), 8.00 (1H, d), 7.75 (2H, m), 7.55 (1H, m), 7.45 (2H, m), 5.70 (2H, s), 4.05 (4H, m), 4.60–3.40 (10H, m), 3.30 (2H, m), 2.75 (3H, s), 3.70 (3H, s), 1.95 (2H, m), 1.85 (4H, m), 1.65 (4H, m). MS *m/z* Calcd for $C_{33}H_{41}Cl_2N_4O_5S$: 676.7. Found: 675.5, 677.6 $[M]^+$. HPLC purity: system A, t_R 9.89 min.

5.1.36. 3-{4-[2,4-Dichloro-3-(2,4-dimethyl-quinolin-8-yloxym ethyl)-benzenesulfonylamino]-tetrahydro-pyran-4-carbonyl}-9-methyl-3,9-diaza-6-azonia-spiro[5.5]undecane trifluoroace tate salt (49)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.85 (1H, s), 8.10–7.75 (6H, m), 5.75 (2H, s), 4.00–3.75 (10H, m), 3.40–3.05 (10H, m), 2.90 (9H, br s), 1.90–1.10 (4H, m). MS *m/z* Calcd for $C_{33}H_{42}Cl_2N_5O_5S$: 691.7. Found: 690.5, 692.5 $[M]^+$. HPLC purity: system C, t_R 6.29 min.

5.1.37. 9-(4-(2,4-Dichloro-3-((2,4-dimethylquinolin-8-yloxy) methyl)phenylsulfonamido)tetrahydro-2*H*-pyran-4-carbonyl)-3,3-dimethyl-9-aza-3,6-diazoniaspiro[5.5]undecane trifluoro acetate salt (50)

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.70 (1H, s), 8.30 (1H, br s), 8.00 (1H, d), 7.80 (1H, d), 7.70 (1H, d), 7.50 (1H, dd), 7.40 (1H, d), 7.30 (1H, s), 5.70 (2H, s), 4.15 (6H, m), 3.95 (4H, m), 3.85 (4H, m), 3.55–3.00 (12H, m), 2.70(3H, s), 2.65 (3H, s), 1.90 (2H, m), 1.65 (2H). MS *m/z* Calcd for $C_{34}H_{45}Cl_2N_5O_5S$: 706.2. Found: 633.2, 635.2 $[M-NMe_4]^{+\cdot}$. HPLC purity: system C, t_R 6.22 min.

6. Biology

6.1. Receptor binding assays

Binding assays were performed using membranes of CHO cells expressing the hB_2R, as previously described.[10] The buffer used for the binding experiments was *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (10 mM, pH 7.4) containing 1,10-phenanthroline (1 mM), bacitracin (140 μg/mL), and bovine serum albumin (1 g/L). Binding assays were performed at room temperature in a final volume of 0.5 mL, and an incubation time of 60 min was used. The [^{3}H]-BK concentration was comparable with its calculated K_d value (0.1–0.2 nM), and the membrane concentration was 100–150 μg/mL. Competing ligands were tested under a wide range of concentrations (1 pM–10 μM). Non-specific binding was defined as the amount of labeled ligand bound in the presence of 1 μM BK. The final concentration of DMSO in the binding assay was 1% and did not affect the binding parameters. Reactions were stopped by filtration with UniFilter-96 plates GF/B (Packard Instrument Company), pre-soaked for at least 2 h in polyethylenimine 0.6%, and using a MicroMate 96 Cell Harvester (Packard Instrument

Company). The tubes and filters were then washed 5 times with 0.5 mL aliquots of Tris buffer (50 mM, pH 7.4, 4 °C). The filters were dried and soaked in Microscint 40 (50 μL/well, Packard Instrument Company), and the bound radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). Binding parameters were evaluated using GraphPad Prism 4.0 (GraphPad, San Diego, CA) to determine the ligand concentration inhibiting the radioligand binding by 50% (IC_{50}). The −log of K_i values (pK_i) were calculated from the Cheng–Prusoff equation $K_i = IC_{50}/(1 + [\text{radioligand}]/K_d)$.

6.2. Measurement of inositol monophosphate accumulation

The assay was performed according to the method of Berridge.[10] CHO cells stably transfected with the hB_2R were plated in 24-well plates (1.8×10^5 cells/well) and incubated at 37 °C overnight. Cells were labeled in the presence of a labeling medium (Ham'sF-12/MEMα = 1/1, 1% dialyzed FCS, 2 mM glutamine and 50 IU/mL Penicillin/Streptomycin) by adding 1 μCi/well inositol,myo-[1,2-^{3}H(N)] for 24 h. The labeled culture medium was aspirated and the cells were pre-incubated in the absence (control) or presence of an opportune nanomolar concentration of the chosen compound for 15 min and then incubated in the absence or the presence of 1×10^{-11} up to 1×10^{-5} M BK for 40 min at 37 °C in IP1 modified buffer (135 mM PBS, 20 mM Na/Hepes pH 7.4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 11.1 mM Glucose, 25 mM LiCl, 0.05% BSA, 1 mM 1,10-phenantroline and 140 μg/mL bacitracin). At the end of the incubation period, 1 mL of ice-cold methanol/0.1 N HCl (2:1 v/v) was added to liberate the inositol phosphate formed. The aqueous phase was applied to an anion exchange column (AG 1-X8 Bio-Rad, Hercules, CA, USA) and the inositol monophosphate eluted with 0.2 M ammonium formate/0.1 M formic acid. In the IP1 fraction, the radioactivity was determined by liquid scintillation spectrometry. Agonist concentration-response curves in the absence and presence of antagonists were fitted by sigmoidal nonlinear regression (GraphPad Prism 4.0) to determine the concentration producing 50% (EC_{50}) of the agonist control maximal response (E_{max}).The affinity of competitive antagonism was expressed in terms of pA_2 calculated from the equation: $pA_2 = \log [CR - 1] - \log [\text{antagonist concentration}]$, where CR is the concentration-ratio of equieffective concentrations of agonist (EC_{50}) obtained in the presence and in the absence of antagonist.

6.3. In vivo experiments

In vivo experiments were performed in male Dunkin Hartley guinea-pigs weighing 350–400 g (Charles River, Italy) in accordance with the regulations of the European Union and the local ethical committee.

Evaluation of bronchoconstriction and hypotension induced by iv bradykinin and the antagonist effects of the compounds were performed according to the methods reported in Ref. 11

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.036.

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