Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 5974–5981
Bioorganic & Medicinal Chemistry

Benzanilides with spasmolytic activity: Chemistry, pharmacology, and SAR

Gerda Brunhofer,[a] Norbert Handler,[a] Klaus Leisser,[a] Christian R. Studenik[b] and Thomas Erker[a,*]

[a]*Department of Pharmaceutical Chemistry, University of Vienna, A-1090 Vienna, Althanstrasse 14, Austria*
[b]*Department of Pharmacology and Toxicology, University of Vienna, A-1090 Vienna, Althanstrasse 14, Austria*

Received 18 January 2008; revised 16 April 2008; accepted 23 April 2008
Available online 26 April 2008

Abstract—The following study describes the synthesis of new benzanilide derivatives and their pharmacological investigation on smooth muscle preparations of guinea pigs. All compounds were synthesized in good yields and showed a spasmolytic activity without significant effect on vascular smooth muscles and heart muscle preparations. Moreover, further pharmacological investigations as well as in silico studies were performed to elucidate the mechanism of action. Compound 3 showed the most potent spasmolytic activity with an IC_{50} of 3.25 μM.

1. Introduction

Gastrointestinal hypermotility and abdominal pain are symptoms of the irritable bowel syndrome (IBS) and other functional bowel disorders.[1] Unfortunately, the basis of these gastrointestinal motility disorders which induce among others abnormally stimulated motility is still not understood. It is our attempt to identify a new class of compounds exhibiting spasmolytic activity and influencing the gastrointestinal hypermotility and overaction of the intestinal tract without having strong side-effects like the standard anti-diarrhoeal agent loperamide, which influences significantly the central nervous system. According to the international literature, stilbenoids show a highly active principle. Gigantol, lusianthridin, and batatasin III are stilbenoids which showed a higher than or comparable to the spasmolytic activity of papaverine at the rat ileum.[2,3] Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a phytoalexin mainly found in the skin of grapes and in red wine. This natural product displays a wide range of pharmacological-effects like for example cardiovascular protective and cancer chemopreventive properties.[4] Navarette-Vazquez and colleagues synthesized benzimidazole derivatives as stilbene isosteres and proved their spasmolytic activity by using the rat ileum test.[5] In a previous study we investigated the spasmolytic activity of resveratrol that shows an IC_{50} of 46.4 μM.[6] Therefore, starting point of this study was also the modulation of the stilbene scaffold. The new synthesized substances exhibit an increase of the spasmolytic activity. We replaced the ethylene linker by a carboxamide group getting a benzanilide structure also widely known as a very active principle. We tested the effect of our novel molecules on the terminal ileum of the guinea pig. Further pharmacological in vitro experiments were undertaken concerning the underlying modes of action as well as the effect of the substances on the vasculature. In the following study we present new substances with spasmolytic activity, which could, indeed, be beneficial in the treatment of gastrointestinal disorders.

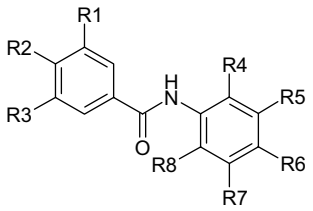
2. Chemistry

A series of new benzanilide derivatives were synthesized by using a standard chemical methodology. The reaction between the appropriate benzoylchloride and the corresponding substituted aniline yielded the desired benzanilide derivative. The reaction was carried out at room temperature and afforded all compounds in a good yield (Table 1).

Keywords: Benzanilide; SAR; Spasmolytic activity; Smooth muscle preparations.
* Corresponding author. Tel.: +43 1 4277 55003; fax: +43 1 4277 9551; e-mail: thomas.erker@univie.ac.at

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.04.057

Table 1. Structures of the synthesized benzanilide derivatives **1**–**10**



Compound	R1	R2	R3	R4	R5	R6	R7	R8
1	–H	–F	–H	–F	–H	–F	–H	–F
2	–F	–H	–F	–H	$-OCH_3$	$-OCH_3$	$-OCH_3$	–H
3	–F	–H	–F	–H	–H	$-SCH_3$	–H	–H
4	–F	–H	–F	–H	–H	$-N(CH_3)_2$	–H	–H
5	–H	$-OCF_3$	–H	–H	$-OCH_3$	–H	$-OCH_3$	–H
6	–F	–H	–F	–F	–H	–F	–H	–H
7	–F	–H	–F	–F	–H	–F	–H	–F
8	–F	–H	–F	–H	–H	–F	–H	–H
9	–H	–F	–H	–H	–F	–H	–F	–H
10	$-NO_2$	–H	$-NO_2$	–F	–H	–F	–H	–F

3. Pharmacological results and discussion

The inotropic and chronotropic effects of the compounds were studied on isolated heart muscle preparations of guinea pigs in a concentration range between 0.1 and 100 μM. None of the derivatives showed a significant change in inotropy or chronotropy up to a concentration of 100 μM (data not shown). The relaxing effect of the compounds was also studied on aortic rings and terminal ilea. The preparations were stimulated with 90 mM KCl (aortic rings) and 60 mM KCl (terminal ilea). There was no significant effect of all compounds on vascular smooth muscles up to a concentration of 100 μM. In terminal ilea all compounds concentration-dependently relaxed the KCl-induced contraction force (f_c). At the highest concentration studied (100 μM for compounds **1**, **2**, **4**, **10**, and 30 μM for compound **3**) **1** reduced f_c to 28.35 ± 5.67% (from 11.46 ± 1.33 to 3.76 ± 1.30 mN, $n = 6$, $P < 0.001$), **2** to 22.87 ± 5.67% (from 17.05 ± 2.36 to 4.15 ± 1.36 mN, $n = 4$, $P < 0.001$), **3** to 2.80 ± 1.24% (from 12.32 ± 1.34 to 2.80 ± 1.24 mN, $n = 7$, $P < 0.001$), **4** to 23.93 ± 4.11% (from 11.06 ± 1.27 to 3.17 ± 0.85 mN, $n = 6$, $P < 0.001$), **5** to 11.99 ± 9.27% (from 17.25 ± 2.45 to 0.84 ± 1.18 mN, $n = 5$, $P < 0.001$), **6** to 51.13 ± 0.69% (from 13.58 ± 3.05 to 6.96 ± 1.56 mN, $n = 5$, $P < 0.01$), **7** to 9.00 ± 5.22% (from 15.26 ± 2.80 to 3.14 ± 1.32 mN, $n = 4$, $P < 0.001$), **8** to 30.55 ± 1.20% (from 8.56 ± 0.61 to 2.61 ± 0.19 mN, $n = 5$, $P < 0.001$), **9** to 1.66 ± 5.86% (from 16.26 ± 2.49 to 0.79 ± 1.16 mN, $n = 5$, $P < 0.001$), and **10** to 25.66 ± 5.64% (from 14.72 ± 2.40 to 3.04 ± 0.98 mN, $n = 6$, $P < 0.001$). Resveratrol showed a reduction of f_c to 35.23 ± 2.00% ($n = 5$, $P < 0.001$).[6] The decrease in percent for **1**, **6**, **7**, **8**, and **9** is shown in Figure 1 and for **2**, **3**, **4**, **5**, and **10** is shown in Figure 2. The evaluation of all the synthesized compounds is listed in Table 2 (IC_{50} values and potency index related to resveratrol).

Spasmolytic drugs have severe side-effects such as QT-prolongation and cardiac arrhythmias.[7–10] Our results demonstrate a selective spasmolytic activity of the presented compounds on terminal ilea. All compounds (**1** to **10**) produced a significant spasmolytic effect in a concentration-dependent manner without a significant action on vascular smooth muscle and heart muscle preparations. The most potent compound was compound **3** (IC_{50} of 3.25 μM), which was 7.39 times more potent than resveratrol, followed by **5** with an IC_{50} of 7.5 μM and **9** with an IC_{50} of 8.50 μM, which were 3.20 and 2.82 times more active compared to resveratrol. Compounds **1**, **2**, **4**, **7**, **8**, and **10** were less effective. Compound **6** showed the weakest spasmolytic effect (IC_{50} over 100 μM). Due to the presented pharmacological results we are able to do some initial structure–activity relationships. Two fluoro substituents in both *meta* positions of the acidic side of the amide linker demonstrated a good dilating activity. In particular, compound **3** was

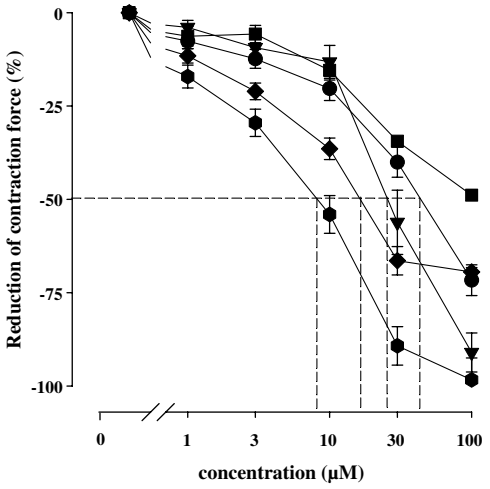


Figure 1. Concentration-dependent effect of **1** (●), **6** (■), **7** (▼), **8** (♦), and **9** (⬢) on terminal ilea contracted by 60 mM KCl. The decrease in percent of contraction force is semi logarithmically plotted on the ordinate against the concentration of the compounds on the abscissa. Symbols represent the arithmetic means ± SEM from 4 to 6 experiments.

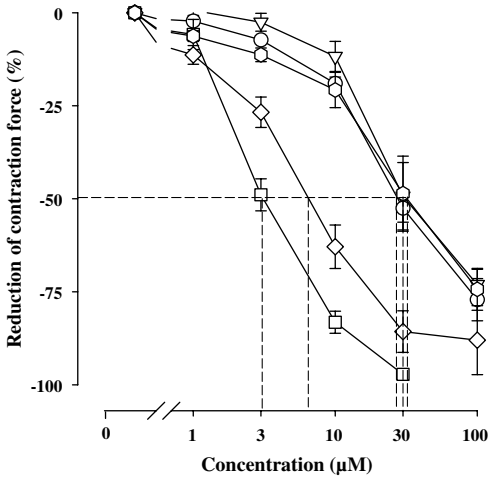


Figure 2. Concentration-dependent effect of **2** (○), **3** (□), **4** (▽), **5** (◇), and **10** (⬡) on terminal ilea contracted by 60 mM KCl. The decrease in percent of contraction force is semi logarithmically plotted on the ordinate against the concentration of the compounds on the abscissa. Symbols represent the arithmetic means ± SEM from 4 to 6 experiments.

Table 2. IC_{50}-values for the spasmolytic activity of the synthesized benzanilide derivatives **1**–**10** (μM) and their potency index related to resveratrol

Compound	IC_{50} (μM)	Potency index
1	42.00	0.57
2	27.50	0.87
3	3.25	7.39
4	30.00	0.80
5	7.50	3.20
6	>100	>0.24
7	26.00	0.92
8	21.00	1.14
9	8.50	2.82
10	32.50	0.74
Resveratrol	24.00	1

the most potent substance in this series bearing a thiomethyl substituent in *para* position on the anilino moiety (IC_{50} 3.25 μM). Exchange of the thiomethyl substituent of the basic side of compound **3** against 3,4,5-trimethoxy (compound **2**), 4-dimethylamino (compound **4**), 2,4,6-trifluoro (compound **7**), or 4-fluoro substituents (compound **8**) resulted in a decrease of the spasmolytic activity (IC_{50} 21.00–30.00 μM). But on the other hand, 2,4-difluoro substituents at the aniline moiety and two fluoro substituents in both *m*-positions of the acidic side of the amide linker (compound **6**) caused a detrimental impact on the spasmolytic activity (IC_{50} > 100 μM). Moreover, a trifluoromethoxy group as well as a fluoro atom in the *para* position of the acidic side together with dimethoxy or difluoro substituents in both *meta* positions of the aniline moiety (compounds **5** and **9**) showed also a good spasmolytic efficacy (IC_{50} 7.50 and 8.50 μM). 2,4,6-Trifluoro substituents at the basic side of the amide linker demonstrated a moderately spasmolytic activity, which is not strictly depending on the substituents of the acidic side (compound **7**: 3,5-difluoro, IC_{50} 26.00 μM; compound **10**: 3,5-dinitro, IC_{50} 32.50 μM; compound **1**: 4-fluoro, IC_{50} 42.00 μM).

The results from this study demonstrate that new benzanilide derivatives presented herein act concentration dependently as selective spasmolytic agents. There are different mechanisms that are responsible for this effect. To identify a possible involvement of cholinergic, serotoninergic and histaminergic mechanisms we studied the effect of the substances on contractions caused by acetylcholine, serotonin, and histamine, but there were no significant inhibitory responses observable. Since it has been demonstrated that an NO synthase is present in the mesenteric plexus we investigated a possible involvement of NO. However, inhibition of the NO synthase by nitro-L-arginine did not show any change of the spasmolytic activity of the substances (data not shown).[11,12] Therefore, we assume that large conductance calcium-activated potassium channels known as BK channels or maxi-K which are expressed in various tissues are responsible for the selective spasmolytic activity of the presented compounds. BK activating substances modulate muscular and neuronal hyperexcitability and although maxi-K channels are even found in the vascular system it is described that small molecules are able to affect selectively BK channels present in the intestinal tract.[13–15] Hence, further experiments need to be done to clarify the exact mechanism of action underlying the selective spasmolytic activity of these highly interesting compounds.

4. Structure–activity relationship

In order to better understand the spasmolytic activity of compounds **1** to **10** we tried to correlate the IC_{50} value to energetic, electrostatic, and structural parameters derived from ab initio calculations.

Thioamide analogs of the compounds **1** to **10** have also been synthesized (results to be published). These show a significant increase in spasmolytic activity and also exhibit, due to the larger van der Waals radius of the sulfur atom, a larger dihedral angle of the two benzene rings.

We therefore started from the assumption, that the spasmolytic action of compounds **1** to **10** is related to the conformation of the two benzene rings and the conformation of the benzamide C–N bond. We tried to model the energetic barrier of rotation around the C–N bond. The amide bond was held fixed at intervals of 5° (compounds **1**, **2**, **3**, and **4**), respectively, 10° (compounds **5**, **6**, **7**, **8**, **9**, and **10**) and let the rest of the molecule relax. The energy of the optimized structures was sampled. The graphical representation of the results can be seen in Figure 3.

We used the energies of the corresponding three energy maxima and minima, the angles at these maxima and minima and the Mulliken charges of the amide nitrogen, the carbonyl carbon and the sum of the Mulliken charges of the two benzene rings as independent variables of the subsequent regression analysis. Table 3 shows the resulting dihedral angles of the C–N bond at the calculated respective minima and maxima.

Because of the large number of independent variables a backward elimination algorithm was used including all

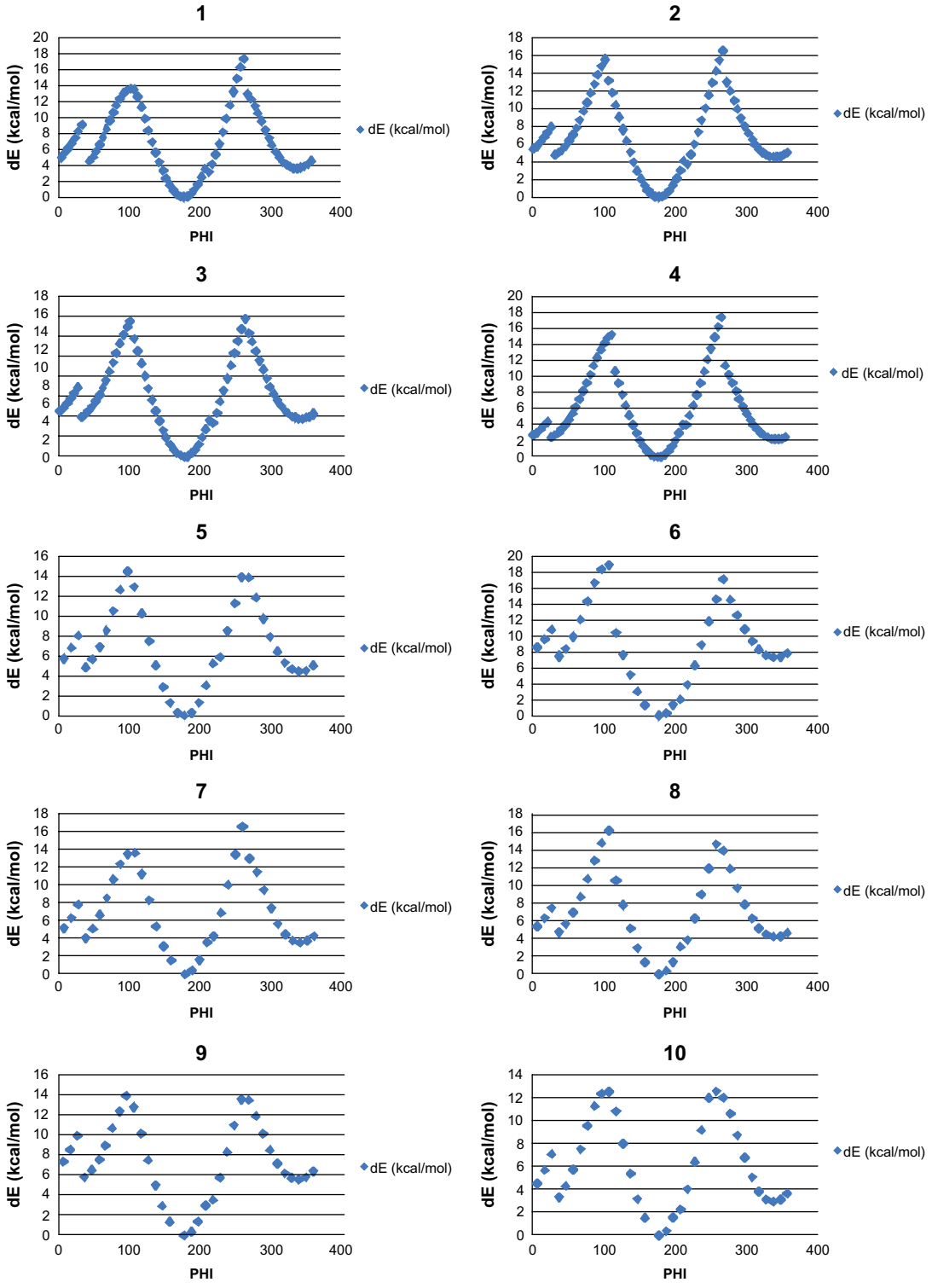


Figure 3. Potential energies of compounds **1** to **10** obtained through rotation of the C–N bond. The amide bond was held fix at intervals of 5° (compounds **1**, **2**, **3**, and **4**), respectively, 10° (compounds **5**, **6**, **7**, **8**, **9**, and **10**).

independent variables and excluding variables stepwise starting with the variable with the smallest partial correlation and excluding variables with α F-value $\geqq$0.100. Three variables were identified as significant. The final model was derived using the significant variables only (Table 4). In order to get an impression of the predictivity of the model, we performed a leave-one-out cross-validation of the final model. The q^2 value of 0.60 indicates a moderate predictivity of the model due to the small number n of observations.

The plot of the calculated values derived from the final model versus the experimental values is shown in Figure 4. Figure 5 shows the plot of the calculated values

Table 3. AMIN1, AMIN2, AMIN3, AMAX1, AMAX2, and AMAX3 are the angles at minimum 1, 2, and 3 and at maximum 1, 2, and 3, respectively

Compound	LNINVBIO	AMIN1	AMIN2	AMIN3	AMAX1	AMAX2	AMAX3
1	−3.95	42.74000	177.74001	337.73999	32.74000	102.74000	262.73999
2	−3.31	31.96000	176.96001	341.95999	26.96000	101.96000	266.95999
3	−1.18	32.07000	177.07001	342.07001	27.07000	102.07000	262.07001
4	−3.40	27.45000	177.45000	342.45001	22.45000	112.45000	267.45001
5	−2.01	36.92000	176.92000	336.92001	26.92000	96.92000	256.92001
6	−4.61	37.29000	177.28999	337.29001	27.29000	107.29000	267.29001
7	−3.26	37.80000	177.80000	337.79999	27.80000	107.80000	257.79999
8	−3.04	32.27000	177.27000	337.26999	27.27000	107.27000	257.26999
9	−2.14	36.55000	176.55000	336.54999	26.55000	96.55000	256.54999
10	−3.33	37.03000	177.03000	337.03000	27.03000	107.03000	257.03000

LNINVBIO is the natural logarithm of the inverse biological activity.

Table 4. Final model using three significant variables

Significant variables only $n = 10$; $r^2 = 0.868$; $r^2_{cv} = 0.601$				
	Coefficient	*t*	SD	*p*
Intercept	−72.849	−3.293	22.19	0.017
AMAX2	0.385	4.648	0.083	0.004
AMAX3	−0.092	−2.893	0.032	0.028
AMIN3	−0.195	−4.365	0.054	0.005

The q^2 value of 0.60 indicates a moderate predictivity of the model due to the small number *n* of observations.

derived from the cross-validated final model versus the experimental values.

5. Conclusion

We may conclude that this study demonstrates the synthesis of new compounds possessing a relaxant effect on guinea pig ileum and that especially compound **3** showed a 7.39-fold higher activity than the comparing compound resveratrol. Further studies investigating the chronotropic, inotropic, or vasodilating activity of the compounds proved the selectivity of the presented compounds which would be a great asset compared to other anti-diarrhoeal agents. We hypothesize that the spasmolytic activity of the compounds is, at least partly, due to the activation of BK-channels. Moreover, we could show a correlation between the spasmolytic activity of compounds **1** to **10** and the benzamide C–N angle. Due to the small amount of compounds under investigation the predictivity of the model is rather low.

6. Experimental

6.1. Chemistry

6.1.1. General experimental methods. All chemicals obtained from commercial suppliers were used as received and were of analytical grade. Melting points were deter-

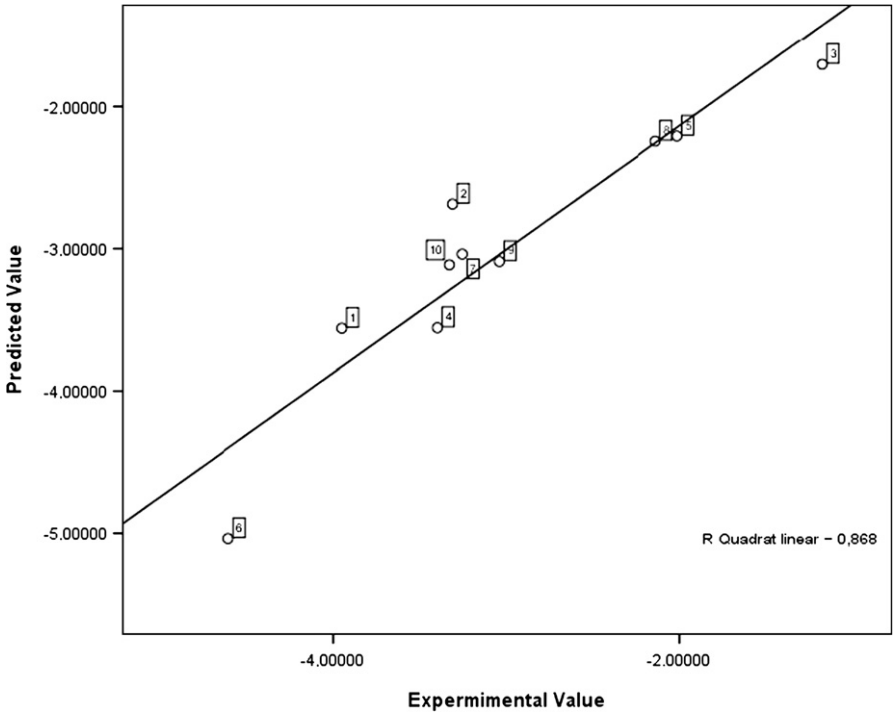


Figure 4. The calculated values predicted by the three component model are plotted against the experimental values.

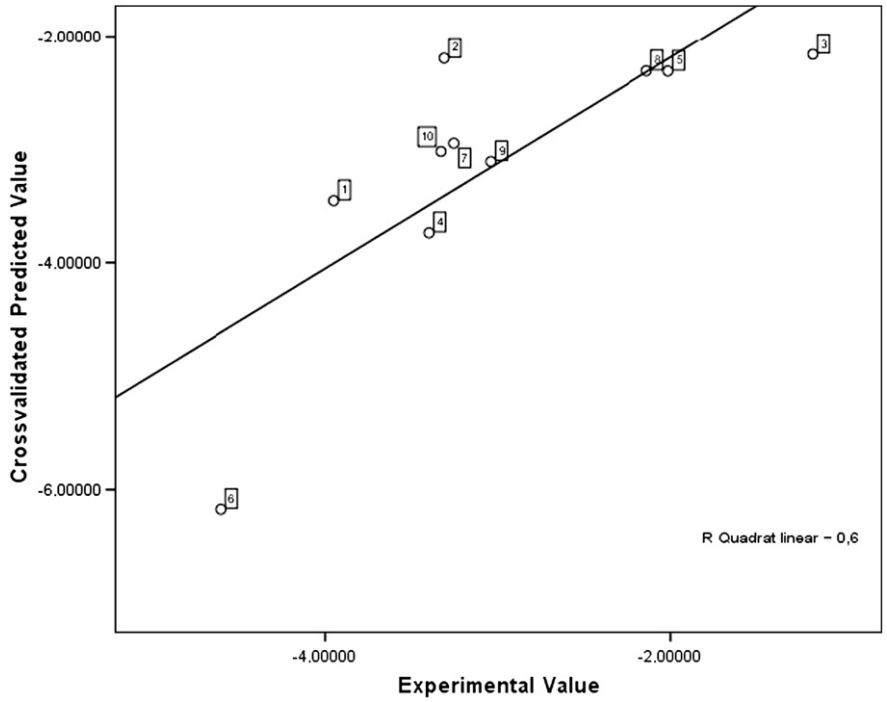


Figure 5. The calculated values predicted by the cross-validated three component model (leave-one-out cross-validation) are plotted against the experimental values.

mined on a Kofler hot stage apparatus and are uncorrected. The ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker Avance DPx200 (200 and 50 MHz). Chemical shifts are reported in δ units (ppm) relative to Me_4Si line as internal standard and J values are reported in Hertz. Mass spectra were obtained by a Hewlett Packard (GC: 5890; MS: 5970) spectrometer. Solutions in organic solvents were dried over anhydrous sodium sulfate.

6.1.2. General procedure for the synthesis of the benzanilide derivatives. To a solution of 10 mmol appropriate benzoylchloride in 10 ml anhydrous dioxane was quickly added a solution of 10 mmol substituted aniline derivative also dissolved in 10 ml anhydrous dioxane. The mixture was shaken immediately. The reaction was carried out in a 100 ml extraction funnel at room temperature. The reaction mixture was allowed to stand for a period of 10 min and then it was purged into ice water. The formed solid was filtered and purified by recrystallization.

6.1.2.1. *N*-(2,4,6-Trifluorophenyl)-4-fluorobenzamide (1). Yield: 2.28 g (85%), mp 150 °C; ^{1}H NMR (d_6-DMSO): δ 10.17 (s, 1H), 8.13–7.97 (m, 2H), 7.49–7.25 (m, 4H); ^{13}C NMR (d_6-DMSO): δ 164.5, 164.4 ($J_{C,F}$ = 247.1 Hz), 158.3 ($J_{C,F}$ = 249.6 Hz), 155.5–155.8 (m), 130.5 ($J_{C,F}$ = 9.5 Hz), 129.3 ($J_{C,F}$ = 2.9 Hz), 115.6 ($J_{C,F}$ = 22.5 Hz), 111.6–111.2 (m), 101.5–100.4 (m); MS *m*/*z* 269 [M^+, 19%], 123 [100%].

6.1.2.2. *N*-(3,4,5-Trimethoxyphenyl)-3,5-difluorobenzamide (2). Yield: 2.85 g (88%), mp 186 °C; ^{1}H NMR (d_6-DMSO): δ 10.25 (s, 1H,), 7.78–7.61 (m, 2H), 7.60–7.45 (m, 1H), 7.21 (s, 2H), 3.78 (s, 6H), 3.65 (s, 3H); ^{13}C NMR (d_6-DMSO): δ 162.5, 162.3 ($J_{C,F}$ = 247.0 Hz), 162.1 ($J_{C,F}$ = 247.0 Hz), 152.8, 138.3 (t, $J_{C,F}$ = 8.1 Hz), 134.7, 111.0 ($J_{C,F}$ = 26.0 Hz), 107.1, 98.1, 60.1, 55.7; MS *m*/*z* 323 [M^+, 27%], 141 [100%].

6.1.2.3. *N*-(4-Methylthiophenyl)-3,5-difluorobenzamide (3). Yield: 2.57 (92%), mp 175 °;. ^{1}H NMR (d_6-DMSO): δ 10.36 (s, 1H), 7.77–7.61 (m, 4H), 7.58–7.45 (m, 1H), 7.33–7.21 (m, 2H), 2.47 (s, 3H); ^{13}C NMR (d_6-DMSO): δ 162.7, 162.3 ($J_{C,F}$ = 247.0 Hz), 162.1 ($J_{C,F}$ = 247.0 Hz), 138.3, 136.0, 133.0, 126.7, 121.1, 111.1 ($J_{C,F}$ = 26.0 Hz), 107.0 (t, $J_{C,F}$ = 26.0 Hz), 15.3; MS *m*/*z* 279 [M^+, 55%], 141 [100%].

6.1.2.4. *N*-(4-Dimethylaminophenyl)-3,5-difluorobenzamide (4). Yield: 1.38 g (50%), mp 161 °C; ^{1}H NMR ($CDCl_3$): δ 7.85 (br s, 1H), 7.46–7.30 (m, 4H), 7.01–6.86 (m, 1H), 6.68 ($J_{A,B}$ = 8.0 Hz, 2H), 2.93 (s, 6H); ^{13}C NMR ($CDCl_3$): δ Amid-C not detectable, 163.0 ($J_{C,F}$ = 251.6 Hz), 162.8 ($J_{C,F}$ = 251.6 Hz), 148.4, 126.7, 122.4, 112.8, 110.3 (d, $J_{C,F}$ = 26.3 Hz), 106.7 (t, $J_{C,F}$ = 25.2 Hz), 40.7; MS *m*/*z* 276 [M^+, 35%], 135 [100%].

6.1.2.5. *N*-(3,5-Dimethoxyphenyl)-(4-trifluoromethoxy)benzamide (5). Yield: 2.34 g (68%), mp 111–113 °C; ^{1}H NMR ($CDCl_3$): δ 7.95–7.82 (m, 3H), 7.29 (d, $J_{A,B}$ = 8.0 Hz, 2H), 6.87 (d, J = 2.3 Hz, 2H), 6.28 (t, J = 2.3 Hz, 1H), 3.79 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 164.5, 161.1, 139.3, 133.3, 128.9, 120.7 ($J_{C,F}$ = 1.1 Hz), 98.4, 97.1, 55.3; MS *m*/*z* 341 [M^+, 29%], 189 [100%].

6.1.2.6. *N*-(2,4-Difluorophenyl)-3,5-difluorobenzamide (6). Yield: 2.05 g (76%), mp 143 °C; ^{1}H NMR ($CDCl_3$): δ 8.65 (s, 1H), 8.16–7.97 (m, 1H), 7.53–7.39 (m, 2H), 7.07–6.84 (m, 3H); ^{13}C NMR ($CDCl_3$): δ 162.9 ($J_{C,F}$ = 250.6 Hz), 162.7 ($J_{C,F}$ = 250.6 Hz), 161.7, 124.9–124.7

(m), 111.4–110.4 (m), 107.2 (t, $J_{C,F}$ = 25.2 Hz), 104.0 and 103.5 (dd, $J_{C,F}$ = 26.5 Hz and 23.4 Hz); MS *m*/*z* 269 [M^+, 15%], 141 [100%].

6.1.2.7. *N*-(2,4,6-Trifluorophenyl)-3,5-difluorobenzamide (7). Yield: 2.10 g (74%), mp 171 °C; ^{1}H NMR ($CDCl_3$): δ 9.12 (s, 1H), 7.71–7.52 (m, 2H), 7.08–6.93 (m, 1H), 6.91–6.64 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 162.6 ($J_{C,F}$ = 249.8 Hz), 160.3, 111.4 and 111.1 (dd, $J_{C,F}$ = 16.3 Hz and 10.2 Hz), 107.2 (t, $J_{C,F}$ = 25.2 Hz), 100.4 (dt, $J_{C,F}$ = 25.6 Hz and 3.3 Hz); MS *m*/*z* 287 [M^+, 8%], 141 [100%].

6.1.2.8. *N*-(4-Fluorophenyl)-3,5-difluorobenzamide (8). Yield: 2.33 g (93%), mp 157 °C; ^{1}H NMR ($CDCl_3$): δ 9.52 (s, 1H), 7.83–7.63 (m, 2H), 7.62–7.41 (m, 2H), 7.11–6.89 (m, 3H); ^{13}C NMR ($CDCl_3$): δ 163.4, 162.7 ($J_{C,F}$ = 250.1 Hz), 162.5 ($J_{C,F}$ = 250.1 Hz), 159.3 ($J_{C,F}$ = 243.8 Hz), 138.3 ($J_{C,F}$ = 8.1 Hz), 134.1 ($J_{C,F}$ = 2.7 Hz), 122.5 ($J_{C,F}$ = 8.1 Hz), 115.2 ($J_{C,F}$ = 22.6 Hz), 110.8 ($J_{C,F}$ = 10.0 Hz), 106.6 (t, $J_{C,F}$ = 25.2 Hz); MS *m*/*z* 251 [M^+, 32%], 141 [100%].

6.1.2.9. *N*-(3,5-Difluorophenyl)-4-fluorobenzamide (9). Yield: 2.25 g (90%), mp 155 °C; ^{1}H NMR ($CDCl_3$): δ 7.95 (s, 1H), 7.90–7.80 (m, 2H), 7.30–7.09 (m, 4H), 6.65–6.54 (tt, $J_{H,H}$ = 8.8 Hz, $J_{H,F}$ = 2.3 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 165.1 ($J_{C,F}$ = 253.6 Hz), 164.7, 163.3 ($J_{C,F}$ = 247.0 Hz), 163.0 ($J_{C,F}$ = 247.0 Hz), 140.4 ($J_{C,F}$ = 13.4 Hz), 130.4, 129.5 ($J_{C,F}$ = 9.2 Hz), 116.0 ($J_{C,F}$ = 22.2 Hz), 103.2 ($J_{C,F}$ = 29.5 Hz), 99.9 (t, $J_{C,F}$ = 25.7 Hz); MS *m*/*z* 251 [M+, 15%], 123 [100%].

6.1.2.10. *N*-(2,4,6-Trifluorophenyl)-3,5-dinitrobenzamide (10). Yield: 2.39 g (70%), mp 232–236 °C; ^{1}H NMR ($CDCl_3$): δ 10.94 (s, 1H), 9.12 (s, 2H), 9.11–8.97 (m, 1H), 7.56–7.41 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 161.6, 148.4, 135.2, 128.1, 121.7, 101.2; MS *m*/*z* 341 [M^+, 21%], 195 [100%].

6.1.3. Purity of compounds 1–10. The purity of the compounds **1**–**10** was confirmed by elemental analysis and was within ±0.4% of the theoretical values calculated for the structural formulas.

6.2. Pharmacological studies

Male and female guinea pigs (Him DH) (340–480 g) were obtained from the Department of Laboratory Zoology and Genetics (Medical University, Himberg, Austria) and housed in an air-conditioned room with a 12-h photo period at a temperature of 22–24 °C and at a relative humidity of 50–60%. The sex of the experimental animal has no significant impact on the results of these studies and therefore, both sexes were used. The guinea pigs were killed with a blow on the neck. After excision of the heart, papillary muscles were dissected from the right ventricle for contractility measurements. Purkinje fibers were carefully removed to prevent spontaneous activity. Only muscles with a diameter of less than 0.87 mm were used in order to have a sufficient oxygen supply. Chronotropic activity was tested on guinea pig isolated right atria. The isolated preparations were stored at room temperature in a Krebs–Henseleit solution with the following composition (in mM) NaCl 114.9, KCl 4.73, $CaCl_2$ 3.2, $MgSO_4$ 1.18, $NaHCO_3$, 24.9, KH_2PO_4 1.18, glucose 10; pH 7.2–7.4. Experiments were performed at a temperature of 35 ± 1 °C. The bathing solutions were continuously bubbled by a mixture of 95% O_2 and 5% CO_2 to guarantee sufficient oxygen supply and appropriate pH as well as circulation of nutrient solution with and without test substance.

Isometric contraction force of electrically stimulated papillary muscles and spontaneous activity in right atria was measured by the method described by Reiter.[16] A force transducer and amplifier (Transbridge™, 4-Channel Transducer Amplifier, World Precision Instruments, Sarasota, FL, USA) were used for measurements of isometric contractions. Resting tension of 3.92 mN for papillary muscles and 10.37 mN for right atria was kept constant throughout the experiments. Papillary muscles were electrically driven with an Anapulse Stimulator Model 301-T and an Isolation Unit Model 305-1 (WPI, Hamden, CT, USA) at a frequency of 1 Hz and pulse duration of 3 ms. Amplitude of stimulation pulse was adjusted 10% above threshold level. After a control period of 30 minutes the compounds **1–10** were added to the bathing solution cumulatively, until a steady state was reached. The aorta and the ileum were dissected as well. The aorta was stored at room temperature in gassed (95% O_2 and 5% CO_2), modified Krebs–Henseleit solution with the following composition (in mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 5.8, KH_2PO_4 1.4, $NaHCO_3$ 11.9, and glucose 10. The aorta was cleaned of loosely adhering fat and connective tissue and cut into rings of 2 mm width. Aortic rings were precontracted with 90 mM KCl.

The terminal portion of the ileum was removed and the 10 cm nearest to the caecum was discarded. The intestine was placed in a nutrient solution. The intestine was cleaned by flushing with nutrient solution and cut into pieces of 1 to 2 cm length. The experiments were performed at a temperature of 37 ± 1°C. The smooth muscle preparations were placed in a continuously oxygenated (95% O_2 and 5% CO_2) bath of 28 ml nutrient solution with the following composition (in mM): NaCl 114.9, KCl 4.73, $CaCl_2$ 3.2, $MgSO_4$ 1.18, $NaHCO_3$ 24.9, KH_2PO_4 1.18, glucose 10; pH 7.2–7.4. One end was connected to a tissue holder and the other to a force transducer (Transbridge™, 4-Channel Transducer Amplifier, World Precision Instruments, Sarasota, FL, USA). The preparations were precontracted by 60 mM KCl and a resting tension of 4.9 mN was kept constant throughout the experiments. After a control period of 45 min the different concentrations of the compounds were added to the bathing solution cumulatively, until a steady state was reached.

Signals were recorded with a chart recorder (BD 112 Dual Channel, Kipp & Zonen) and evaluated. For statistical analyses the arithmetic means and standard error of the mean (SEM) of *n* experiments were calculated. Statistical significance of the results was evaluated by the Student's *t*-test for paired observations.

Due to insolubility of the test compounds in aqueous nutrient solution, stock solutions of the compounds **1–10** were dissolved in dimethylsulfoxide (DMSO) every day and were further diluted with modified Krebs–Henseleit solution to the required concentrations. To exclude the DMSO effect, a series of experiments with DMSO only were performed at the same experimental conditions. The DMSO effect was subtracted from the results of the compounds.

6.3. Computational methods

Molecular modeling was done using program MOE[17] on a Sun Ultra 40 workstation running Linux. Ab initio calculations were done with GAMESS [18] on the same machine and operating system.

Structures were derived using MOE and the MMFF94x force field and a stochastic search algorithm as implemented in MOE. The structure with the lowest energy was taken as the starting point for the subsequent grid search algorithm in GAMESS.

Statistical calculations were carried out with the multiple linear regression module implemented in SPSS 14.0.[19] The regression models were estimated by their correlation coefficients r^2 and the cross-validated correlation coefficients q^2 (leave-one-out cross-validation).

References and notes

1. Mertz, H. R. *Gastroenterol. Clin. North Am.* **2003**, *32*, 463.
2. Estrada, S.; Rojas, A.; Mathison, Y.; Israel, A.; Mata, R. *Planta Med.* **1999**, *65*, 109.
3. Hernández-Romero, Y.; Rojas, J. I.; Castillo, R.; Rojas, A.; Mata, R. *J. Nat. Prod.* **2004**, *67*, 160.
4. Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnswoth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218.
5. Navarette-Vazquez, G.; Moreno-Diaz, H.; Aguierre-Crespo, F.; Leon-Rivera, I.; Villalobos-Molina, R.; Munoz-Muniz, O.; Estrada–Soto, S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4169.
6. Handler, N.; Brunhofer, G.; Studenik, C.; Leisser, K.; Jaeger, W.; Parth, S.; Erker, T. *Bioorg. Med. Chem.* **2007**, *15*, 6109.
7. Meyers, N. L.; Rickling, R. L. *J. Int. Med. Res.* **2007**, *35*, 848.
8. Toga, T.; Kohmura, Y.; Kawatsu, R. *J. Pharmacol. Sci.* **2007**, *105*, 207.
9. Mohammad, S.; Zhou, Z.; Gong, Q.; January, C. T. *Am. J. Physiol.* **1997**, *273*, H2534.
10. Paakkari, I. *Toxicol. Lett.* **2002**, *127*, 279.
11. Li, C. G.; Rand, M. J. *Eur. J. Pharmacol.* **1990**, *191*, 303.
12. Konturek, S. K.; Konturek, P. C. *Digestion* **1995**, *56*, 1.
13. Calderone, V. *Curr. Med. Chem.* **2002**, *9*, 1385.
14. Biagi, G.; Giorgi, I.; Livi, O.; Nardi, A.; Calderone, V.; Martelli, A.; Martinotti, E.; LeRoy Salerni, O. *Eur. J. Med. Chem.* **2004**, *39*, 491.
15. Sivarao, D. V.; Newberry, K.; Langdon, S.; Lee, A. V.; Hewawasam, P.; Plym, M. J.; Signor, L.; Myers, R.; Lodge, N. J. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 840.
16. Reiter, M. *Arzneim.-Forsch.* **1967**, *17*, 1249.
17. *MOE 2007.09 (Molecular Operating Environment)*. **2007**, Chemical Computing Group: 1010 Sherbrooke Street West, Suite 910, Montreal Que., Canada H3A 2R7.
18. Schmidt, M. W.; Baldridge, K. K.; Boatz, J. A.; Elbert, S. T.; Gordon, M. S.; Jensen, J. H.; Koseki, S.; Matsunaga, N.; Nguyen, K. A.; Su, S.; Windus, T. L.; Dupius, M.; Montgomery, J. A., Jr. *J. Comput. Chem.* **1993**, *14*, 1347.
19. *SPSS, version 14*, SPSS: Chicago IL, USA.