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Synthesis of benzamide derivatives and their evaluation as antiprion agents

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ABSTRACT

A new set of 5-(2-(pyrrolidin-1-yl)acetamido)-*N*-butyl-2-(substituted)benzamide and 5-(2-(piperidin-1-yl)acetamido)-*N*-butyl-2-(substituted) benzamide derivatives were synthesized in which as structural features the 2-(1-pyrrolidinyl)- or 2-(1-piperidyl)acetylamino group or a diphenylether moiety are associated to a benzamide scaffold. Their binding affinity for human PrP^{C} and inhibition of its conversion into PrP^{Sc} were determined in vitro; moreover, the antiprion activity was assayed by inhibition of PrP^{Sc} accumulation in scrapie-infected mouse neuroblastoma cells (ScN2a) and scrapie mouse brain (SMB) cells. The results clearly indicate the benzamide derivatives as attractive lead compounds for the development of potential therapeutic agents against prion disease.

1. Introduction

Prion diseases are fatal neurodegenerative diseases afflicting both humans and animals. The most known disease in humans is the Creutzfeldt–Jakob disease (CJD); other examples include Gerstmann–Straussler–Scheincker syndrome (GSS), fatal familial insomnia, and kuru in humans, scrapie in sheep and goat, bovine spongiform encephalopathy in cattle, and chronic wasting disease in deer and elk.[1,2] The crucial molecular event in the pathogenesis of all prion diseases is the post-translational refolding of normal cellular prion protein PrP^{C} into the pathological-associated isoform PrP^{Sc}. This β-sheet rich form of the protein readily generates insoluble aggregates, the deposition of which is thought to be central to pathogenesis and disease progression.[3] This conversion can occur spontaneously as a result of inherited mutations in the PrP^{C} gene. Moreover, the available evidences suggest that PrP^{Sc} acts both as a template for this conversion and as a neurotoxic agent causing neuronal dysfunction and cell death.[1,4] Based on this knowledge, one of the most promising therapeutic approaches for prion diseases appears to be interference with PrP^{Sc} amplification and accumulation. In fact evidence deriving from cell culture and in vivo studies suggests that once formation of PrP^{Sc} is inhibited, clearance of PrP^{Sc} can take place. However, despite the identification of a number of compounds that are capable of inhibiting PrP^{Sc} accumulation and cleaning PrP^{Sc} from infected cell lines, little therapeutic efficacy in vivo has been achieved so far and no viable treatments of these devastating disorders are currently available.[5]

Several classes of chemical compounds are already known to possess antiprion properties and among these the acridines[6,7] (e.g., quinacrine, and structurally related tricyclic antidepressants), dimeric[8] and chimeric[9] analogues of statins,[10] 2,4-diphenylthiazole and 2,4-diphenyloxazole amides,[11] pyrazolones,[12,13] indole-3-glyoxamides,[14] pyridyl hydrazones[15] and GN8,[16] a molecule characterized by a diphenylmethane scaffold and two 2-(1-pyrrolidinyl)acetylamino groups at the 4,4′-positions of diphenylmethane. In addition, larger molecules of the polyanionic (suramin, pentosan polysulfate) or polycationic chemotype (dendritic polyamines, cationic polysaccharides[17]) have been reported to exhibit antiprion activity in cells, although it seems unlikely that such species could be therapeutically useful. In fact, only a pyridyl hydrazone derivative and GN8 (Fig. 1) have been reported to significantly extend survival in animals.[15,16] A possible explanation for this poor track record is that the majority of small molecules, investigated to date, was originally designed for other purposes (e.g., malaria, hyperlipidemia) and not optimized for either antiprion effects or for crossing the blood–brain barrier (BBB).[18]

In our laboratories, there has been an ongoing effort to develop new small molecules that bind to human PrP^{C} and stabilize it against the conversion into PrP^{Sc}, with the aim of identifying novel

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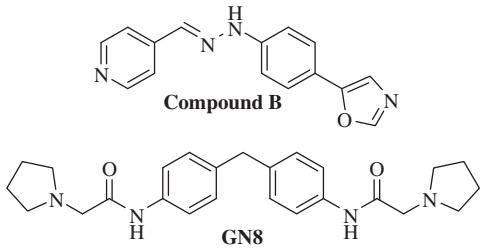


Figure 1. Structures of the small molecules compound **8** and GN8 with significant antiprion properties in vivo.

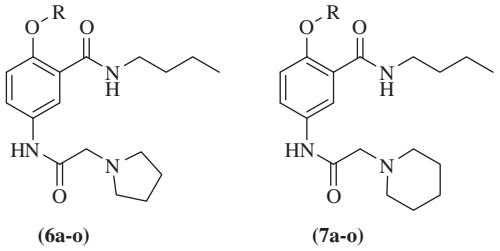


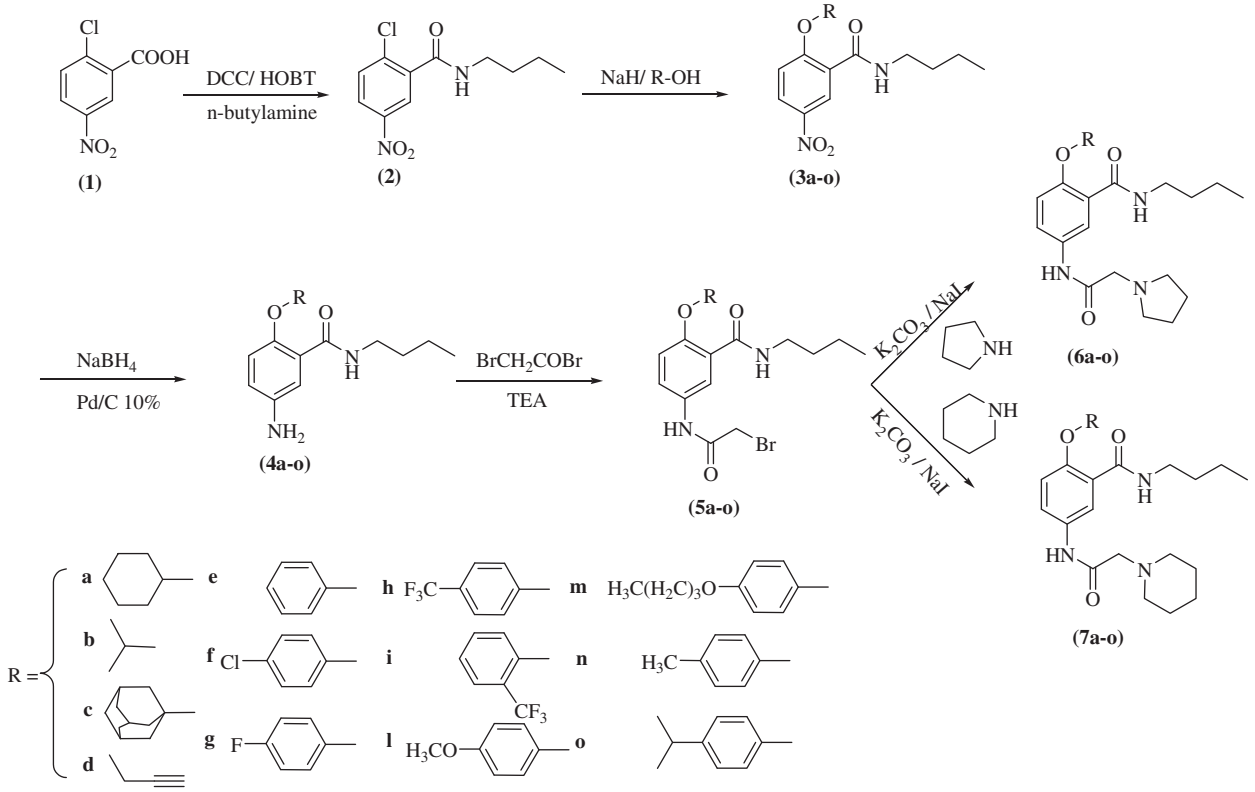
Figure 2. General structures of benzamide derivatives.

pharmacological tools characterized by more efficient antiprion activity and that could improve also our knowledge about the mechanism of formation and clearance of PrP^{Sc}. In this context a new set of 5-(2-(pyrrolidin-1-yl)acetamido)-*N*-butyl-2-(substituted)benzamide (**6a–o**) and 5-(2-(piperidin-1-yl)acetamido)-*N*-butyl-2-(substituted)benzamide (**7a–o**) derivatives (Fig. 2) was synthesized. These derivatives contain a 2-(1-pyrrolidinyl)acetylamino group or a diphenylether moiety as in the GN8 structure and in some GN8 derivatives, associated to a benzamide scaffold that was already investigated in the synthesis of substituted benzamides as anti-inflammatory agents.[19] All synthesized compounds were screened in vitro for their binding affinity to human PrP^C ($hPrP^C$), truncated human PrP^C ($t\text{-}hPrP^C$), and murine PrP^C ($rPrP^C$). For this purpose a high-throughput screening assay was applied which is based on scanning intensely fluorescent targets (SIFT) with an inverted dual-color confocal microscope setup. This technique was chosen as it is well suited for in vitro screening of a library of synthetic compounds, in order to identify inhibitors of the aggregation processes accompanying prion diseases.[20] Pharmacological data allowed to identify compounds capable of inhibiting PrP^{Sc} accumulation in scrapie-infected mouse neuroblastoma cells (ScN2a) and scrapie mouse brain (SMB) cells. Taken together, the results suggest that benzamide derivatives represent a promising new class of lead compounds useful for further optimization in order to obtain novel pharmacological tools for prion diseases.

2. Results and discussion

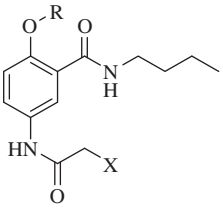
2.1. Chemistry

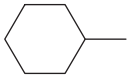
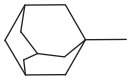
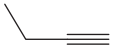
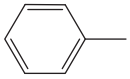
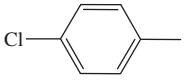
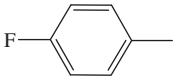
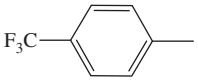
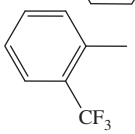
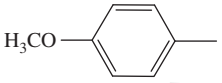
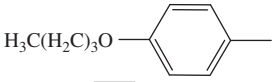
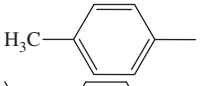
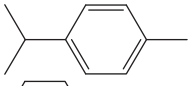
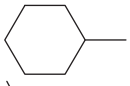
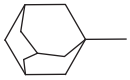
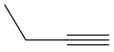
The synthetic strategy employed for the preparation of the target compounds is summarized in Scheme 1. The general procedure is based on condensation of commercially available 2-chloro-5-nitrobenzoic acid (**1**) with *n*-butylamine in anhydrous DMF in the presence of DCC/HOBt, to produce *N*-butyl-2-chloro-5-nitro-benzamide (**2**). Subsequent dissolution of **2** in anhydrous dioxane and treatment with a solution of the appropriate alcohol and NaH in anhydrous dioxane afforded smoothly the corresponding intermediates **3a**–**o**. Reduction of the nitro group with sodium borohydride led to intermediates **4a**–**o** which were acylated with bromoacetyl



Scheme 1. Synthesis of compounds **6a–o** and **7a–o**.

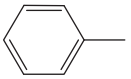
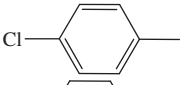
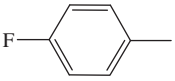
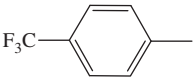
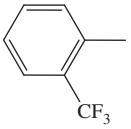
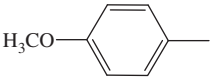
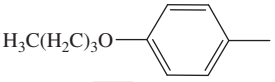
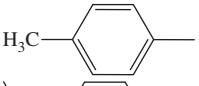
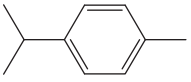
Table 1
Antiprion activities in the three screening steps of compounds **6a–o** and **7a–o**



Compd	Substituents		SIFT EC_{50} (μM)	SMB cells (% control)		ScN2a cells (% control)		EC_{50} (μM)	
	R	X		20 μM compound	2 μM compound	20 μM compound	2 μM compound	SMB cells	ScN2a cells
6a			200	38	67	32	38	7.81	3.14
6b			Inactive	48	59	24	42	10.77	2.76
6c			80	42	77	16	34	12.43	2.00
6d			Inactive	65	74	32	43	99.22	3.52
6e			200	82	92	41	48	>1000	5.52
6f			55	46	68	16	25	12.32	1.70
6g			200	92	57	39	45	>1000	4.70
6h			40	41	50	12	26	5.81	1.61
6i			25	54	61	15	16	16.49	1.42
6l			198	75	74	31	36	866.34	2.92
6m			30	58	82	19	52	53.65	2.98
6n			70	55	61	13	32	18.95	1.83
6o			18	48	73	20	32	16.27	2.08
7a			80	65	76	22	37	108.22	2.39
7b			Inactive	72	58	29	78	160.60	6.86
7c			50	36	54	12	27	5.22	1.65
7d			Inactive	66	70	37	76	97.25	9.28

(*continued on next page*)

Table 1 (*continued*)

Compd	Substituents		SIFT EC_{50} (μM)	SMB cells (% control)		ScN2a cells (% control)		EC_{50} (μM)	
	R	X		20 μM compound	2 μM compound	20 μM compound	2 μM compound	SMB cells	ScN2a cells
7e			200	75	65	30	50	501.35	3.86
7f			20	49	68	21	35	15.04	2.25
7g			70	81	61	32	35	>1000	2.93
7h			70	53	80	17	35	30.04	2.08
7i			70	55	75	12	39	30.55	2.04
7l			150	82	83	28	49	>1000	3.56
7m			50	52	76	21	61	24.27	3.73
7n			40	86	86	26	36	>1000	2.57
7o			15	46	72	13	43	13.80	2.22

All the compounds were validated in SIFT dilution series and whenever possible, EC_{50} values were determined.

bromide in the presence of triethylamine to generate **5a–o**. Subsequent condensation of **5a–o** with pyrrolidine or piperidine, performed in CH_3CN in the presence of K_2CO_3 and NaI under reflux, provided the final compounds **6a–o** and **7a–o**. The final products were purified by silica gel chromatography and crystallization from appropriate solvents. All new compounds gave satisfactory elemental analyses and were characterized by ^1H NMR and mass spectrometry. ^1H NMR and MS data for all final compounds were consistent with the proposed structures.

2.2. In vitro antiprion activity

All the synthesized compounds were screened with the SIFT assay for antiprion activity. As reported by Bertsch et al.[20] this antiprion assay represents a technique to determine the inhibitory effect of compounds on the association of PrP^C and PrP^{Sc}. The compounds were checked in a dilution series (0.1–100 μM) for dose-dependent inhibition of PrP^C/PrP^{Sc} association. Most of the compounds afforded dose response curves that confirmed their concentration-dependent inhibitory activity. Half-maximal inhibition of binding of rPrP to PrP^{Sc} was observed at 50% effective concentration (EC_{50}) values in the range of 15–200 μM compared to the effect of 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA), a cationic lipid, that previously used at concentration of 17 μM, has been found to inhibit PrP_{Sc} formation in scrapie-infected mouse cells (Table 1).[20] Besides the excellent inhibitory activity of compounds **6o** (EC_{50} = 18 μM) and **7o** (EC_{50} = 15 μM), other interesting activities were found for the compounds **6h**, **6i**, **6m**, **7f**, and **7n** with EC_{50} values in the range of 20–40 μM while compounds **6c**, **6f**, **6n**, **7a**, **7c, 7g, 7h, 7i** and **7m** were less active with EC_{50} values ranging from 50 μM to 80 μM.

Concerning the influence of the substituent of the benzamide moiety, the 4-isopropylphenoxy (**6o** and **7o**), 2-trifluoromethylphenoxy (**6i**) and *p*-chlorophenoxy group (**7f**) conferred the highest inhibitory activity on PrP^C/PrP^{Sc} association. The presence of a 4-trifluoromethylphenoxy or 4-butoxyphenoxy group on the benzamide moiety, i.e. **6h** and **6m**, respectively led also to compounds of high inhibitory activity. Non aromatic moieties as well as phenoxy, 4-fluorophenoxy, and 4-methoxyphenoxy groups afforded compounds with a dramatic decrease in inhibitory activity compared to other substituents at the position 2 of the benzamide moiety.

2.3. Cell based antiprion activity

Scrapie-infected mouse neuroblastoma cells (ScN2a) and scrapie mouse brain (SMB) cells as established cell culture models of prion propagation were selected to evaluate the antiprion activity compounds **6a–o** and **7a–o** (Figs. 3 and 4). To quantify the reduction of PrP^{Sc} obtained after the treatment with the new small molecules, the relative amount of PrP^{Sc}, present in control cells, was set at 100% and reduction was calculated from three independent experiments. In the ScN2a cell culture model numerous compounds significantly interfered with the accumulation of PrP^{Sc} without showing any overt sign of cytotoxicity at 2 μM and 20 μM concentrations. PrP^{Sc} levels were reduced in the ScN2a cells by 22–84% at 2 μM and by 59–88% at 20 μM concentration (Fig. 5).

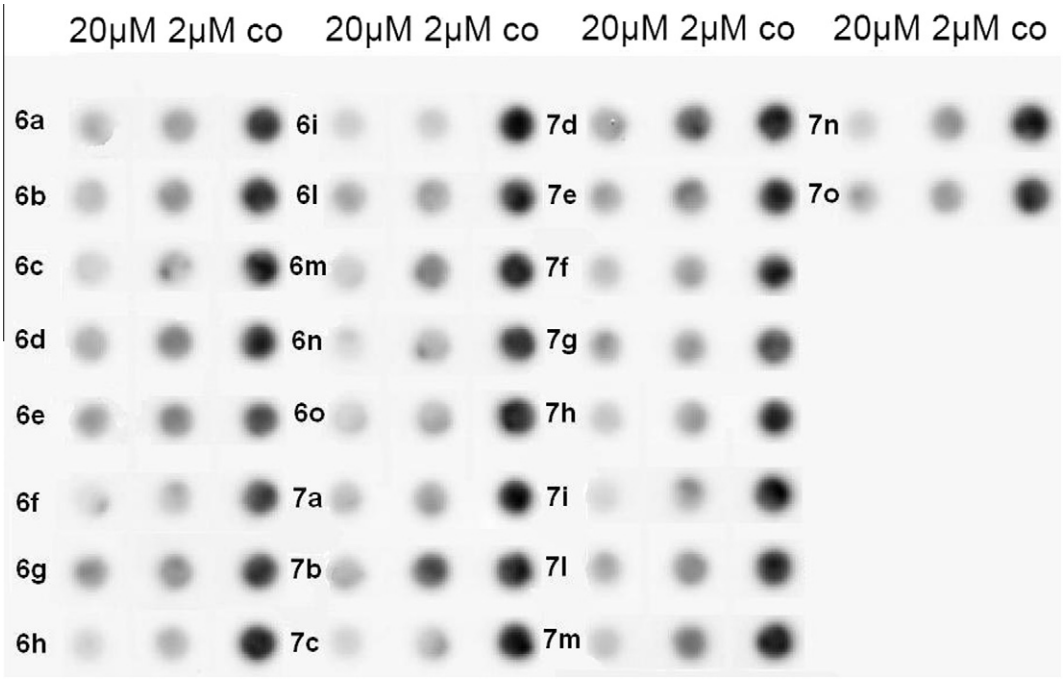


Figure 3. Inhibition of PrP^{sc} formation in mouse neuroblastoma (ScN_2a) cells treated for 3 days with the synthetic compounds at 20 μM or 2 μM concentration compared to infected control cells (K).

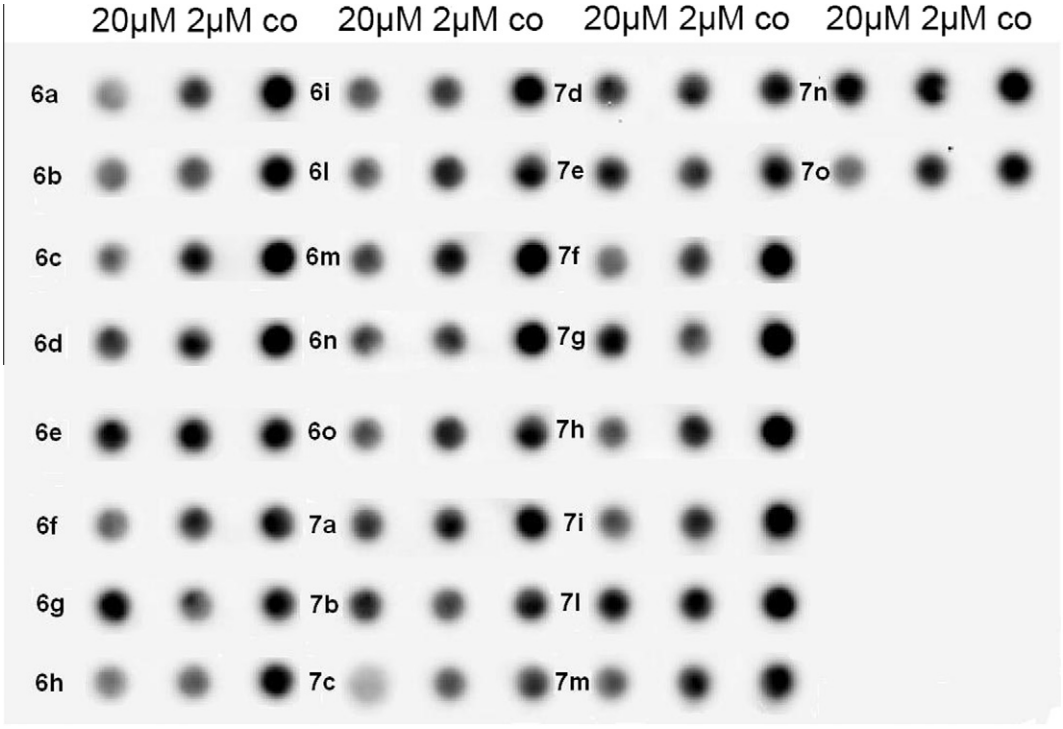


Figure 4. Inhibition of PrP^{sc} formation in scrapie mouse brain (SMB) cells treated for 3 days with the synthetic compounds at 20 μM or 2 μM concentration compared to infected control cells (K).

Beside the remarkable inhibitory activity of compounds **6h** (EC_{50} = 1.61 μM), **7c** (EC_{50} = 1.65 μM) and **7i** (EC_{50} = 2.04 μM) with 12% residual PrP^{Sc} level at 20 μM concentration, other interesting inhibitory effects with residual PrP^{sc} levels in the range of 13–17% were observed at this concentration for compounds **6n**, **6o**, **6i**, **6c**, **6f** and **7h** while the remaining compounds were less active with residual PrP^{Sc} levels ranging from 19% to 41%. At 2 μM concentration compounds **6f** (EC_{50} = 1.70 μM), **6h** (EC_{50} = 1.61 μM), **6i** (EC_{50} = 1.42 μM) and **7c** (EC_{50} = 1.65 μM) retained significant inhibitory activity (Figs. 3 and 5).

Concerning the results obtained in the SMB cell culture model, as a general trend, the compounds showed a weaker inhibitory effect

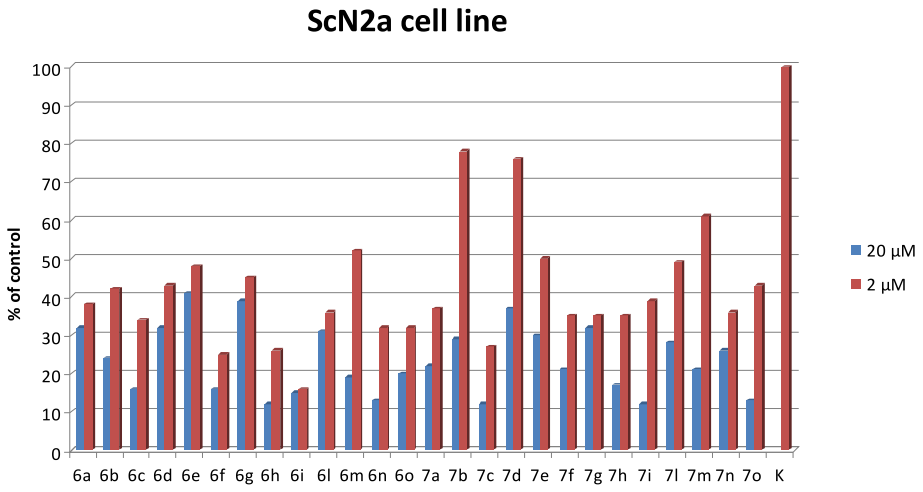


Figure 5. Antiprion activities of **6a–o** and **7a–o** at 20 μM and 2 μM on ScN2a cells.

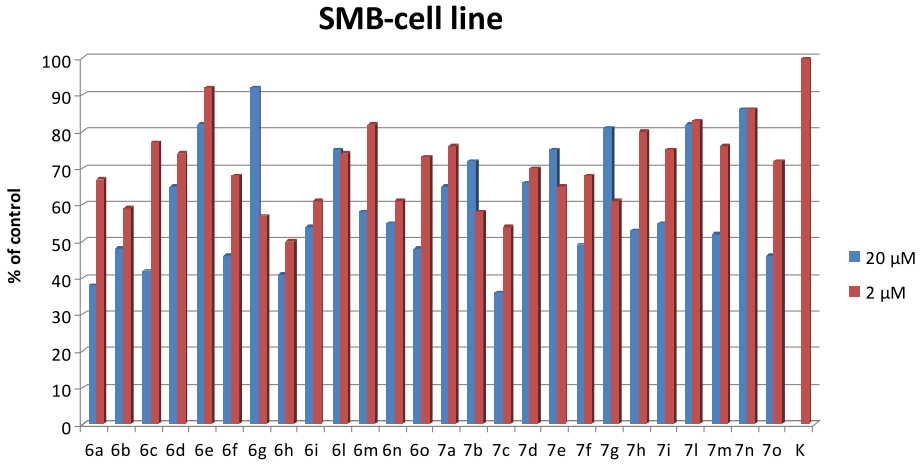


Figure 6. Antiprion activities of **6a–o** and **7a–o** at 20 μM and 2 μM on SMB-cells.

than that recorded on the ScN2a cells (Figs. 4 and 6). A reduction of PrP^{Sc} levels in the range of 50–14% and 64–14% was observed at concentrations of 2 μM and 20 μM, respectively. In this cell system the best inhibitory activity against PrP^{Sc} propagation was observed with compounds **6a** (residual PrP^{Sc} level of 67% at 2 μM and 38% at 20 μM; EC_{50} = 7.81 μM), **6h** (residual PrP^{Sc} level of 50% at 2 μM and 41% at 20 μM; EC_{50} = 5.81 μM), **7c** (residual PrP^{Sc} level of 54% at 2 μM and 36% at 20 μM; EC_{50} = 5.22 μM) and **7f** (residual PrP^{Sc} level of 68% at 2 μM and 49% at 20 μM; EC_{50} = 15.04 μM).

The results obtained with neuronal (ScN2a) and scrapie mouse brain (SMB) cells suggest an interesting selectivity towards ScN2a cells. Moreover, the collected data would suggest that the new scaffold benzamide may play a critical role in determining antiprion activity and allow for some preliminary structure–activity relationship considerations. As already demonstrated[21] in a series of GN8 derivatives, the combination of hydrophobic properties with the presence of a structural feature like a diphenylether moiety, recognizable also inside the structure of some of the most active benzamide derivatives, seems to play a crucial role in the ligand–protein interaction and in the inhibitory activity of the synthesized compounds. The antiprion profile of these new derivatives, bearing only one acylamino group linked to a basic cyclic group (pyrrolidine or piperidine) is apparently not affected by the size of the basic cycle. In fact, both series of compounds showed similar potencies, whereas the aliphatic or aromatic nature of the residue R led to different results both in SIFT assay and in biological systems (ScN2a and SMB cell lines). Therefore alkyl and cycloalkyl residues as R substituents led to inactive or less potent compounds, while, as already demonstrated in a series of aminothiazole derivatives,[18] electronic effects of the substituents appear to be of smaller importance; analogues with electron rich (e.g. compounds **6f**, **6h, 6i** etc.) or electron deficient (e.g. compound **6n**, **6o, 7o** etc.) groups showed similar activities. Rather the steric hindrance, represented for example by an isopropyl (**6o**) or a trifluoromethyl (**6h**) group linked to the *para* positon of phenoxybenzamide moiety, could be useful to determine a favorable inhibitory activity on the PrP^{C}/PrP^{Sc} association. The good correlation of the results from the SIFT assay with the antiprion activity found in cell cultures confirms that the cell activities are indeed due to interference of the small molecules with PrP^{c}/PrP^{Sc} interactions.

3. Conclusions

In a screening of a new series of small molecules with benzamide as core structure by the SIFT antiprion assay and successive evaluation of their antiprion activity in an in vitro cell-based assay the derivatives **6h**, **6i**, **7c** and **7i** were found to exhibit interesting antiprion properties. Moreover, the good correlation of the SIFT

assay and cell culture data confirms the working assumption that antiprion activities may derive from interference of these small molecules with conversion of PrP^{c} into PrP^{Sc}.[20] However, further studies are required to confirm this conclusion and to identify by in vivo studies compounds with favorable physiochemical properties for crossing the blood–brain barrier (BBB). For this purpose the benzamide core structure may be a promising new lead for antiprion agents.

4. Experimental section

4.1. Chemistry

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument. Unless otherwise stated, all spectra were recorded in $CDCl_3$. Chemical shifts are reported in ppm using Me_4Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), br s (broad singlet), d (doublet), t (triplet), qd (quadruplet), qt (quintuplet), hep (heptet), m (multiplet). Mass spectra of the final products were recorded on API 2000 Applied Biosystem mass spectrometer. Where analyses are indicated only by the symbols of the elements, results obtained are within ±0.4% of the theoretical values. All reactions were followed by TLC on Merck Silica Gel 60 F_{254} plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed on silica gel column (Kieselgel 60). Solutions were dried over Na_2SO_4 and concentrated with Büchi rotary evaporator at low pressure.

4.1.1. *N*-Butyl-2-chloro-5-nitro-benzamide (2)

To a solution of 2-chloro-5-nitrobenzoic acid **(1)** (10.00 g, 50.0 mmol) in anhydrous DMF (100 ml) was added HOBt (7.30 g, 54.0 mmol) followed by DCC (11.14 g, 54.0 mmol) at 0 °C. The resulting reaction mixture was stirred for 30 min and then *n*-butylamine hydrochloride (4.08 g, 50.0 mmol) and TEA (5.06 g, 50.0 mmol) were added. The reaction mixture was stirred at 0 °C for 2 h and overnight at room temperature. *N,N′*-Dicyclohexylurea (DCU) was filtered off and the DMF was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2, washed consecutively with 1 N NaOH and 1 N HCl. The organic phase was dried over anhydrous Na_2SO_4, filtered, concentrated and purified by crystallization from diethyl ether affording 9.49 g of pure **2** as a yellow solid, mp 134 °C.

^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 8.46 (s, 1H, ArH); 8.19 (dd, 1H, J = 7.9 Hz, ArH); 7.59 (d, 1H, J = 7.9 Hz, ArH); 6.23 (br s, 1H, CONH); 3.48 (qd, 2H, J = 6.9 Hz, CH_2NH); 1.69 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.44 (m, 2H, CH_2CH_3); 0.98 (t, 3H, J = 6.9 Hz, CH_2CH_3).

Anal. ($C_{11}H_{13}ClN_2O_3$): C, H, Cl, N.

4.1.2. *N*-Butyl-2-cyclohexyloxy-5-nitro-benzamide (3a)

A solution of sodium hydride (60% in mineral oil, 3.35 g, 84.0 mmol) in dry dioxane (150 mL) was added to cyclohexanol (7.91 g, 79.0 mmol) in an ice-water bath under a nitrogen atmosphere. After the evolution of hydrogen had ceased, the mixture was stirred at 70 °C for 2 h. Then intermediate **2** (19.6 g, 76.4 mmol) in anhydrous dioxane (100 mL) was added dropwise and the resulting reaction mixture was stirred at 70 °C for 4 h and overnight at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and washed with brine. The organic phase was dried over magnesium sulfate, filtered, concentrated and the residue purified by chromatography on silica gel column (elution with diethyl ether/hexane, 6:4 v/v) to afford **3a** (9.4 g, 75%), mp 64–65 °C.

^{1}H NMR ($CDCl_3$, δ, ppm): 9.09 (s, 1H, ArH), 8.27 (d, 1H, J = 7.9 Hz, ArH), 7.88 (br s, 1H, CONH), 7.05 (dd, 1H, J = 7.9 Hz, ArH), 4.62 (m, 1H, cyclohexyl), 3.49 (qd, 2H, J = 6.9 Hz,CH_2NH), 2.10–1.85 (m, 8H, cyclohexyl), 1.67 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH), 1.58 (m, 2H, cyclohexyl), 1.48 (m, 2H, CH_2CH_3,), 0.98 (t, 3H, J = 6.9 Hz, CH_2CH_3).

Anal. **($C_{17}H_{24}N_2O_4$):** C, H, N.

Using the procedure described for preparing **3a** the following compounds were prepared: *N*-butyl-2-isopropoxy-5-nitrobenzamide **(3b)**, *N*-butyl-2-adamantanoxy-5-nitrobenzamide **(3c)**, *N*-butyl-2-propargyloxy-5-nitrobenzamide **(3d)**, *N*-butyl-2-phenoxy-5-nitrobenzamide **(3e)**, *N*-butyl-2-*p*-chlorophenoxy-5-nitrobenzamide **(3f)**, *N*-butyl-2-*p*-fluorophenoxy-5-nitrobenzamide **(3g)**, *N*-butyl-2-*p*-trifluoromethylphenoxy-5-nitrobenzamide **(3h)**, *N*-butyl-2-*o*-trifluoromethylphenoxy-5-nitrobenzamide **(3i)**, *N*-butyl-2-*p*-methoxyphenoxy-5-nitrobenzamide **(3l)**, *N*-butyl-2-*p*-butoxyphenoxy-5-nitrobenzamide **(3m)**, *N*-butyl-2-*p*-tolyloxy-5-nitrobenzamide **(3n)** and *N*-butyl-2-*p*-isopropylphenoxy-5-nitrobenzamide **(3o)** in yields ranging between 70% and 80%. The ^{1}H NMR spectra of the intermediates **3b–o** were consistent with the assigned structures.

4.1.3. 5-Amino-*N*-butyl-2-cyclohexyloxybenzamide (4a)

To a suspension of Pd/C (0.15 g) in 15 mL water was added a suspension (20 mL) of $NaBH_4$ (1.0 g, 26.4 mmol) in water. The resulting reaction mixture was stirred at room temperature under nitrogen atmosphere for 10 min. Then **3a** (2.89 g, 8.1 mmol) in 100 mL methanol was added dropwise and the mixture was stirred for 30 min. The reaction mixture was filtered through a Celite bed. The solution was acidified with 1 N HCl to remove the excess of $NaBH_4$. The acidified solution was adjusted to alkaline pH with 2 N NaOH solution and the resulting suspension was extracted with diethyl ether. The organic phase was dried over magnesium sulfate, filtered, and evaporated to dryness to yield intermediate **4a** as a white solid (2.70 g, 87%); ^{1}H NMR data are reported as hydrochloride salt.

^{1}H NMR ($CDCl_3$, δ, ppm): 8.24 (br s, 1H, CONH), 7.53 (s, 1H, ArH), 6.81 (d, 1H, J = 7.9 Hz, ArH), 6.73 (dd, 1H, J = 7.9 Hz, ArH), 4.62 (m, 1H, cyclohexyl), 3.98 (br s, 2H, NH_2), 3.49 (qd, 2H, J = 6.9 Hz, CH_2NH), 2.10–1.85 (m, 8H, cyclohexyl), 1.67 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH), 1.58 (m, 2H, cyclohexyl), 1.48 (m, 2H, CH_2CH_3,), 0.98 (t, 3H, J = 6.9 Hz, CH_2CH_3).

Anal. ($C_{17}H_{26}N_2O_2$): C, H, N.

Using the procedure described for preparing **4a** the following compounds were prepared: 5-amino-*N*-butyl-2-isopropoxybenzamide **(4b)**, 5-amino-*N*-butyl-2-adamantaniloxybenzamide **(4c)**, 5-amino-*N*-butyl-2-propargyloxybenzamide **(4d)**, 5-amino-*N*-butyl-2-phenoxybenzamide **(4e)**, 5-amino-*N*-butyl-2-*p*-chlorophenoxybenzamide **(4f)**, 5-amino-*N*-butyl-2-*p*-fluorophenoxybenzamide **(4g)**, 5-amino-*N*-butyl-2-*p*-trifluoromethylphenoxybenzamide **(4h)**, 5-amino-*N*-butyl-2-*o*-trifluoromethylphenoxybenzamide **(4i)**, 5-amino-N-butyl-2-*p*-methoxyphenoxybenzamide **(4l)**, 5-amino-*N*-butyl-2-*p*-butoxyphenoxybenzamide **(4m)**, 5-amino-*N*-butyl-2-*p*-tolyloxybenzamide **(4n)** and 5-amino-*N*-butyl-2-*p*-isopropylphenoxybenzamide **(4o)** in yields ranging between 70% and 80%. The ^{1}H NMR spectra of intermediates **4b–o** are consistent with the proposed structures.

4.1.4. 5-(2-Bromoacetamido)-*N*-butyl-2-(cyclohexyloxy)benzamide (5a)

To a precooled solution (−10 °C) of 5-amino-*N*-butyl-2-cyclohexyloxybenzamide **(4a)** (1.20 g, 4.16 mmol) and triethylamine (0.50 g, 5.0 mmol) in CH_2Cl_2 (90 mL) and water (8 mL) a solution of bromoacetyl bromide (1.68 g, 8.34 mmol) in CH_2Cl_2 (20 mL) was added dropwise under stirring. Then the mixture was allowed to warm up to room temperature within 3 h. The phases were separated, the aqueous phase was washed with CH_2Cl_2, and the organic

phase was reextracted with water. The organic phases were combined and the solvent was evaporated to a small volume. Upon addition of hexane and cooling, the precipitate was filtered off, washed with hexane and recrystallized from CH_2Cl_2/hexane; yield: 1.55 g (91%), light yellow needles.

^{1}H NMR ($CDCl_3$, δ, ppm): 8.89 (s, 1H, ArH), 8.02 (br s, 1H, NHCO), 7.90 (d, 1H, *J* = 7.9 Hz, ArH), 7.88 (br s, 1H, CONH), 6.90 (dd, 1H, *J* = 7.9 Hz, ArH), 4.62 (m, 1H, cyclohexyl), 4.20 (s, 2H, CH_2Br), 3.49 (qd, 2H, *J* = 6.9 Hz,CH_2NH), 2.10–1.85 (m, 8H, cyclohexyl), 1.67 (qt , 2H, *J* = 6.9 Hz, CH_2CH_2NH), 1.58 (m, 2H, cyclohexyl), 1.48 (m, 2H, CH_2CH_3), 0.98 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

Anal. ($C_{19}H_{27}BrN_2O_3$): C, H, N.

Using the procedure described for preparing **5a** the following compounds were prepared: 5-(2-bromoacetamido)-2-*N*-butyl-isopropoxybenzamide **(5b)**, 5-(2-bromoacetamido)-2-*N*-butyl-adamantanoxybenzamide **(5c)**, 5-(2-bromoacetamido)-2-*N*-butyl-propargyloxybenzamide **(5d)**, 5-(2-bromoacetamido)-2-*N*-butyl-phenoxybenzamide **(5e)**, 5-(2-bromoacetamido)-2-*N*-butyl-*p*-chlorophenoxybenzamide **(5f)**, 5-(2-bromoacetamido)-2-*N*-butyl-*p*-fluorophenoxybenzamide **(5g)**, 5-(2-bromoacetamido)-2-*N*-butyl-*p*-trifluoromethylphenoxybenzamide **(5h)**, 5-(2-bromoacetamido)-2-*N*-butyl-*o*-trifluoromethylphenoxybenzamide **(5i)**, 5-(2-bromoacetamido)-2-*N*-butyl-*p*-methoxyphenoxybenzamide **(5l)**, 5-(2-bromoacetamido)-2-*N*-butyl-*p*-butoxyphenoxybenzamide **(5m)**, 5-(2-bromoacetamido)-2-*N*-butyl-*p*-tolyloxybenzamide **(5n)** and 5-(2-bromoacetamido)-2-*N*-butyl-*p*-isopropylphenoxybenzamide **(5o)** in yields ranging between 35% and 98%. The ^{1}H NMR spectra of intermediates **5b–o** are consistent with the proposed structures.

4.1.5. General procedure for the preparation of 5-(2-(pyrrolidin-1-yl)acetamido)-*N*-butyl-2-(substituted)benzamide (6a–o) and 5-(2-(piperidin-1-yl)acetamido)-*N*-butyl-2-(substituted)benzamide (7a–o) derivatives

A mixture of 5-(2-bromoacetamido)-*N*-butyl-2-(substituted)benzamide **(5a–o)** (3.14 mmol) and NaI (0.70 g; 4.72 mmol) in acetonitrile was stirred under reflux for 30 min. Then the appropriate pyrrolidine or piperidine derivative (0.22 g; 3.14 mmol and 0.27 g; 3.14 mmol respectively) and anhydrous K_2CO_3 (0.65 g; 4.72 mmol) were added. The reaction mixture was stirred under reflux for 4 h. After cooling, the mixture was filtered, evaporated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was evaporated. The crude products were purified by silica gel column chromatography using diethyl ether/methanol 9:1 (v/v) as eluent. The resulting products were recrystallized from diethyl ether.

4.1.5.1. 5-(2-(Pyrrolidin-1-yl)acetamido)-N-butyl-2-(cyclohexyloxy)benzamide (6a).

Yield: 85%; mp 83–84 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.17 (br s, 1H, CONH); 8.25 (m, 2H, ArH); 7.82 (br s, 1H, NHCO); 6.97 (d, 1H, *J* = 7.9 Hz, ArH); 4.72 (m, 1H, cyclohexyl); 3.48 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.25 (s, 2H, $COCH_2$); 2.67 (br s, 4H, Pyrr); 2.04 (m, 8H, cyclohexyl); 1.85 (br s, 4H, Pyrr); 1.64 (m, 2H, cyclohexyl); 1.55 (m, 2H, CH_2CH_2NH); 1.45 (m, 2H, CH_2CH_3); 0.98 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 402.7 $[M+H]^+$; 424.4 $[M+Na]^+$.

Anal. ($C_{23}H_{35}N_3O_3$): C, H, N.

4.1.5.2. 5-(2-(Pyrrolidin-1-yl)acetamido)-N-butyl-2-isopropoxybenzamide (6b).

Yield: 87%; mp 56–59 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.18 (br s, 1H, CONH); 8.26 (dd, 1H, *J* = 8.8 Hz, ArH); 8.17 (br s, 1H, NHCO); 7.83 (s, 1H, ArH); 6.96 (d, 1H, *J* = 8.8 Hz, ArH); 4.73 (hep, 1H, *J* = 5.8 Hz, CH-Isopropyl); 3.48 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.26 (s, 2H, $COCH_2$); 2.68 (br s, 4H, Pyrr); 1.85 (br s, 4H, Pyrr); 1.62 (qt, 2H, *J* = 6.9 Hz, CH_2CH_2NH); 1.48 (m, 2H, CH_2CH_3); 1.46 (d, *J* = 5.8 Hz, 6H, 2 CH_3-Isopropyl); 0.98 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 362.3 $[M+H]^+$; 384.3 $[M+Na]^+$; 400.2 $[M+K]^+$.

Anal. ($C_{20}H_{31}N_3O_3$): C, H, N.

4.1.5.3. 5-(2-(Pyrrolidin-1-yl)acetamido)-N-butyl-2-(adamantanyloxy)benzamide (6c).

Yield: 81%; mp 126–128 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.23 (br s, 1H, CONH); 8.20 (dd, 1H, *J* = 8.8 Hz, ArH); 8.05 (br s, 1H, NHCO); 7.78 (s, 1H, ArH); 7.06 (d, 1H, *J* = 8.8 Hz, ArH); 3.47 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.25 (s, 2H, $COCH_2$); 2.67 (br s, 4H, Pyrr); 1.91 (m, 6H, Adamantane); 1.84 (br s, 4H, Pyrr); 1.66–1.55 (m, 9H, Adamantane); 1.47 (qt, 2H, *J* = 6.9 Hz, CH_2CH_2NH); 1.34 (m, 2H, CH_2CH_3); 0.97 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 454.2 $[M+H]^+$; 476.4 $[M+Na]^+$.

Anal. ($C_{27}H_{39}N_3O_3$): C, H, N.

4.1.5.4. 5-(2-(Pyrrolidin-1-yl)acetamido)-N-butyl-2-(prop-2-ynyloxy)benzamide (6d).

Yield: 76%; mp 157–162 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.26 (br s, 1H, CONH); 8.28 (dd, 1H, *J* = 8.8 Hz, ArH); 7.86 (s, 1H, ArH); 7.83 (br s, 1H, NHCO); 7.02 (d, 1H, *J* = 8.8 Hz, ArH); 4.79 (s, 1H, CH-Propargyl); 3.48 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.32 (s, 2H, $COCH_2$); 2.72 (br s, 4H, Pyrr); 2.59 (s, 2H, CH_2-Propargyl); 1.87 (br s, 4H, Pyrr); 1.62 (qt, 2H, *J* = 6.9 Hz, CH_2CH_2NH); 1.46 (m, 2H, CH_2CH_3); 0.98 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 358.3 $[M+H]^+$; 380.4 $[M+Na]^+$; 396.3 $[M+K]^+$.

Anal. ($C_{20}H_{27}N_3O_3$): C, H, N.

4.1.5.5. 5-(2-(Pyrrolidin-1-yl)acetamido)-N-butyl-2-phenoxybenzamide (6e).

Yield: 40%; mp 111–112 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.30 (br s, 1H, CONH); 8.24 (dd, 1H, *J* = 8.8 Hz, ArH); 7.88 (s, 1H, ArH); 7.69 (br s, 1H, NHCO); 7.38 (t, 2H, *J* = 7.6 Hz, ArH); 7.18 (t, 1H, *J* = 7.6 Hz, ArH); 7.01 (d, 2H, *J* = 7.6 Hz, ArH); 6.88 (d, 1H, *J* = 8.8 Hz, ArH); 3.44 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.27 (s, 2H, $COCH_2$); 2.69 (br s, 4H, Pyrr); 1.86 (br s, 4H, Pyrr); 1.54 (qt, 2H, *J* = *J* = 6.9 Hz, CH_2CH_2NH); 1.34 (m, 2H, CH_2CH_3); 0.88 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 396,1 $[M+H]^+$.

Anal. ($C_{23}H_{29}N_3O_3$): C, H, N.

4.1.5.6. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(4-chlorophenoxy)-N-butylbenzamide (6f).

Yield: 64%; mp 118–121 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.31 (br s, 1H, CONH); 8.24 (dd, 1H, *J* = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.50 (br s, 1H, NHCO); 7.33 (d, 2H, *J* = 8.4 Hz, ArH); 6.94 (d, 2H, *J* = 8.4 Hz, ArH); 6.87 (d, 1H, *J* = 8.8 Hz; ArH); 3.43 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.28 (s, 2H, $COCH_2$); 2.69 (br s, 4H, Pyrr); 1.87 (br s, 4H, Pyrr); 1.50 (qt, 2H, *J* = 6.9 Hz, CH_2CH_2NH); 1.31 (m, 2H, CH_2CH_3); 0.89 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 430.1 $[M+H]^+$; 452.4 $[M+Na]^+$.

Anal. ($C_{23}H_{28}ClN_3O_3$): C, H, N.

4.1.5.7. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(4-fluorophenoxy)-N-butylbenzamide (6g).

Yield: 85%; mp 66–69 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.28 (br s, 1H, CONH); 8.22(dd, 1H, *J* = 8.8 Hz, ArH); 7.88 (s, 1H, ArH); 7.61 (br s, 1H, NHCO); 7.08 (t, 2H, *J* = 8.4 Hz, ArH); 6.99 (m, 2H, *J* = 8.4 Hz, ArH); 6.83 (d, 1H, *J* = 8.8 Hz ArH); 3.45 (qd, 2H, *J* = 6.9 Hz, *J* = 5.8 Hz, CH_2NH); 3.27 (s, 2H, $COCH_2$); 2.69 (br s, 4H, Pyrr); 1.86 (br s, 4H, Pyrr); 1.54 (qt, 2H, *J* = 6.9 Hz, CH_2CH_2NH); 1.34 (m, 2H, CH_2CH_3); 0.90 (t, 3H, *J* = 6.9 Hz, CH_2CH_3).

ESI-MS: 414.1 $[M+H]^+$.

Anal. ($C_{23}H_{28}FN_3O_3$): C, H, N.

4.1.5.8. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(4-(trifluoromethyl)phenoxy)-N-butylbenzamide (6h). Yield: 43%; mp 110–112 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.33 (br s, 1H, CONH); 8.28 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.61 (d, 2H, J = 8.4 Hz, ArH); 7.29 (br s, 1H, NHCO); 7.05 (d, 2H, J = 8.4 Hz, ArH); 6.95 (d, 1H, J = 8.8 Hz, ArH); 3.40 (qd, 2H, J = 6.9 Hz, J = 5.8 Hz, CH_2NH); 3.28 (s, 2H, $COCH_2$); 2.69 (br s, 4H, Pyrr); 1.87 (br s, 4H, Pyrr); 1.47 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.28 (m, 2H, CH_2CH_3); 0.85 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 464.0 $[M+H]^+$; 486.1 $[M+Na]^+$; 502.2 $[M+K]^+$.

Anal. ($C_{24}H_{28}F_3N_3O_3$): C, H, N.

4.1.5.9. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(2-(trifluoromethyl)phenoxy)-N-butylbenzamide (6i). Yield: 92%; mp 119–120 °C; ^{1}H NMR (400 MHz, $CDCl_3$ δ: 9.33 (br s, 1H, CONH); 8.29 (d, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.71 (d, 1H, J = 7.3 Hz, ArH); 7,48 (t, 1H, J = 7.3 Hz, ArH), 7.42 (br s, 1H, NHCO); 7.23 (t, 1H, J = 7.3 Hz, ArNH); 6.92 (d, 1H, J = 7.3 Hz, ArH); 6.86 (d, 1H, J = 8.8 Hz, ArH); 3.38 (qd, 2H, J = 6.9 Hz, J = 5.8 Hz, CH_2NH); 3.28 (s, 2H, $COCH_2$); 2.69 (br s, 4H, Pyrr); 1.87 (br s, 4H, Pyrr); 1.47 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.27 (m, 2H, CH_2CH_3); 0.85 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 464.2 $[M+H]^+$; 486.3 $[M+Na]^+$.

Anal. ($C_{24}H_{28}F_3N_3O_3$): C, H, N.

4.1.5.10. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(4-methoxyphenoxy)-N-butylbenzamide (6l). Yield: 97%; mp 64–66 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.26 (br s, 1H, CONH); 8.18 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.83 (br s, 1H, NHCO); 6.98 (d, 2H, J = 8.7 Hz, ArH); 6.91 (d, 2H, J = 8.7 Hz, ArH); 6.78 (d, 1H, J = 8.8 Hz, ArH); 3.81 (s, 3H, OCH_3); 3.46 (qd, 2H, J = 6.9 Hz, J = 5.8 Hz, CH_2NH); 3.26 (s, 2H, $COCH_2$); 2.68 (br s, 4H, Pyrr); 1.86 (br s, 4H, Pyrr); 1.55 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.36 (m, 2H, CH_2CH_3); 0.91 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 426.2 $[M+H]^+$.

Anal. ($C_{24}H_{31}N_3O_4$): C, H, N.

4.1.5.11. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(4-butoxyphenoxy)-N-butylbenzamide (6m). Yield: 69%; mp 65–67 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.24 (br s, 1H, CONH); 8.17(dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.83 (br s, 1H, NHCO); 6.96 (d, 2H, J = 8.7 Hz, ArH); 6.90 (d, 2H, J = 8.7 Hz, ArH); 6.79 (d, 1H, J = 8.8 Hz ArH); 3.96 (t, 2H, J = 6.6 Hz, OCH_2); 3.46 (qd, 2H, J = 6.9 Hz, J = 5.8 Hz, CH_2NH); 3.26 (s, 2H, $COCH_2$); 2.68 (br s, 4H, Pyrr); 1.86 (br s, 4H, Pyrr); 1.80 (qt, 2H, J = 6.6 Hz, OCH_2CH_2); 1.57 (m, 4H, $2CH_2$, $OCH_2CH_2CH_2$, CH_2CH_2NH); 1.36 (m, 2H, CH_2CH_3); 0.99 (t, 3H, J = 6.6 Hz, $O(CH_2)_3CH_3$,); 0.91 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 468.6 $[M+H]^+$.

Anal. ($C_{27}H_{37}N_3O_4$): C, H, N.

4.1.5.12. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(p-tolyloxy)-N-butylbenzamide (6n). Yield: 71%; mp 82–84 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.26 (br s, 1H, CONH); 8.21(dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.75 (br s, 1H, NHCO); 7.17 (d, 2H, J = 8.4 Hz, ArH); 6.91 (d, 2H, J = 8.4 Hz, ArH); 6.84 (d, 1H, J = 8.8 Hz, ArH); 3.44 (qd, 2H, J = 6.9 Hz, J = 5.8 Hz, CH_2NH); 3.26 (s, 2H, $COCH_2$); 2.68 (br s, 4H, Pyrr); 2.34 (s, 3H, $ArCH_3$); 1.86 (br s, 4H, Pyrr); 1.54 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.34 (m, 2H, CH_2CH_3); 0.89 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 410.2 $[M+H]^+$.

Anal. ($C_{24}H_{31}N_3O_3$): C, H, N.

4.1.5.13. 5-(2-(Pyrrolidin-1-yl)acetamido)-2-(4-isopropylphenoxy)-N-butylbenzamide (6o). Yield 78%; mp 68–69 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.27 (br s, 1H, CONH); 8.21(dd, 1H, J = 8.8 Hz, ArH); 7.88 (s, 1H, ArH); 7.76 (br s, 1H, NHCO); 7.22 (d, 2H, J = 8.4 Hz, ArH); 6.94 (d, 2H, J = 8.4 Hz, ArH); 6.86 (d, 1H, J = 8.8 Hz, ArH); 3.44 (qd, 2H, J = 6.9 Hz, J = 5.8 Hz, CH_2NH); 3.26 (s, 2H, $COCH_2$); 2.93 (hep, 1H, J = 6.9 Hz, CH-Isopropyl); 2.69 (br s, 4H, Pyrr); 1.86 (br s, 4H, Pyrr); 1.54 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.31 (m, 2H, CH_2CH_3); 1.26 (d, 6H, J = 6.9 Hz, $2CH_3$-Isopropyl); 0.89 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 438.2 $[M+H]^+$.

Anal. ($C_{26}H_{35}N_3O_3$): C, H, N.

4.1.5.14. 5-(2-(Piperidin-1-yl)acetamido)-N-butyl-2-(cyclohexyloxy)benzamide (7a). Yield: 74%; mp 96–98 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.27 (br s, 1H, CONH); 8.23 (dd, 1H, J = 8.8 Hz, ArH); 8.05 (br s, 1H, NHCO); 7.82 (s, 1H, ArH); 6.97 (d, 1H, J = 8.8 Hz, ArH); 4.44 (m, 1H, Cyclohexyl); 3.48 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.04 (s, 2H, $COCH_2$); 2.51 (br s, 4H, Pip); 2.06 (m, 8H, Cyclohexyl); 1.78 (m, 2H, Cyclohexyl); 1.65 (m, 6H, Pip); 1.44 (m, 4H, $-CH_2CH_2-$); 0.94 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 416.4 $[M+H]^+$.

Anal. ($C_{24}H_{37}N_3O_3$): C, H, N.

4.1.5.15. 5-(2-(Piperidin-1-yl)acetamido)-N-butyl-2-isopropoxybenzamide (7b). Yield: 97%, mp 90–92 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.27 (br s, 1H, CONH); 8.25 (dd, 1H, J = 8.8 Hz, ArH); 8.17 (br s, 1H, NHCO); 7.82 (s, 1H, ArH); 6.96 (d, 1H, J = 8.8 Hz, ArH); 4.71 (hep, 1H, J = 5.8 Hz, CH-Isopropyl); 3.48 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.04 (s, 2H, $COCH_2$); 2.51 (br s, 4H, Pip); 1.68–1.57 (m, 6H, Pip, 2H, CH_2CH_2NH); 1.46 (m, 2H, CH_2CH_3); 1.41 (d, 6H, J = 5.8 Hz, $2CH_3$-Isopropyl); 0.98 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 376.4 $[M+H]^+$; 398.4 $[M+Na]^+$; 414.3 $[M+K]^+$.

Anal. ($C_{21}H_{33}N_3O_3$): C, H, N.

4.1.5.16. 5-(2-(Piperidin-1-yl)acetamido)-N-butyl-2-(adamantanyloxy)benzamide (7c). Yield: 84%; mp 99–100 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.33 (br s, 1H, CONH); 8.18 (dd, 1H, J = 8.8 Hz, ArH); 8.03 (br s, 1H, NHCO); 7.70 (s, 1H, ArH); 7.06 (d, 1H, J = 8.8 Hz, ArH); 3.47 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.04 (s, 2H, $COCH_2$); 2.51 (br s, 4H, Pip); 2.18 (m, 6H, Adamantane); 1.91 (m, 6H, Pip); 1.64–1.55 (m, 2H, CH_2.Butyl, 9H, Adamantane); 1.45 (m, 2H, CH_2CH_3); 0.97 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 468.5 $[M+H]^+$.

Anal. ($C_{28}H_{41}N_3O_3$): C, H, N.

4.1.5.17. 5-(2-(Piperidin-1-yl)acetamido)-N-butyl-2-(prop-2-ynyloxy)benzamide (7d). Yield: 71%; mp 96–99 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.31 (br s, 1H, CONH); 8.28 (dd, 1H, J = 8.8 Hz, ArH); 8.03 (br s, 1H, NHCO); 7.83 (s, 1H, ArH); 7.02 (d, 1H, J = 8.8 Hz, ArH); 4,79 (s, 1H, CH-Propargyl); 3.49 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.04 (s, 2H, $COCH_2$); 2.59 (s, 2H, CH_2-Propargyl); 2.51 (br s, 4H, Pip); 1.63 (m, 6H, Pip; 2H, CH_2CH_2NH); 1.46 (m, 2H, CH_2CH_3); 0.96 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 372.3 $[M+H]^+$; 394.5 $[M+Na]^+$; 410.4 $[M+K]^+$.

Anal. ($C_{21}H_{29}N_3O_3$): C, H, N.

4.1.5.18. 5-(2-(Piperidin-1-yl)acetamido)-N-butyl-2-phenoxybenzamide (7e). Yield 89%; mp 99–101 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.30 (br s, 1H, CONH); 8.22 (dd, 1H, J = 8.8 Hz, ArH); 7.88 (s, 1H, ArH); 7.66 (br s, 1H, NHCO); 7.38 (t, 2H, J = 7.6 Hz, ArH); 7.18 (t, 1H, J = 7.6 Hz, ArH); 7.00 (d, 2H, J = 7.6 Hz, ArH); 6.88 (d, 1H, J = 8.8 Hz, ArH); 3.44 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.06 (s, 2H, $COCH_2$); 2.53 (br s, 4H, Pip); 1.66 (m, 6H, Pip); 1.51 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.32 (m, 2H, CH_2CH_3); 0.88 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 410.5 $[M+H]^+$; 432.3 $[M+Na]^+$.

Anal. ($C_{24}H_{31}N_3O_3$): C, H, N.

4.1.5.19. 5-(2-(Piperidin-1-yl)acetamido)-2-(4-chlorophenoxy)-N-butylbenzamide (7f). Yield: 78%; mp 122–123 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.40 (br s, 1H, CONH); 8.22 (dd, 1H, J = 8.8 Hz, ArH); 7.86 (s, 1H, ArH); 7.47 (br s, 1H, NHCO); 7.32 (d, 2H, J = 8.7 Hz, ArH); 6.93 (d, 2H, J = 8.7 Hz, ArH); 6.87 (d, 1H, J = 8.8 Hz, ArH); 3.43 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.06 (s, 2H, $COCH_2$); 2.53 (br s, 4H, Pip); 1.66 (m, 6H, Pip); 1.52 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.32 (m, 2H, CH_2CH_3); 0.89 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 444.3 $[M+H]^+$; 466.5 $[M+Na]^+$.

Anal. ($C_{24}H_{30}ClN_3O_3$): C, H, N.

4.1.5.20. 5-(2-(Piperidin-1-yl)acetamido)-2-(4-fluorophenoxy)-N-butylbenzamide (7g). Yield: 90%; mp 125–126 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.38 (br s, 1H, CONH); 8.20 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.60 (br s, 1H, NHCO); 7.05 (d, 2H, J = 8.7 Hz, ArH); 6.98 (d, 2H, J = 8.7 Hz, ArH); 6.83 (d, 1H, J = 8.8 Hz, ArH); 3.45 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.05 (s, 2H, $COCH_2$); 2.52 (br s, 4H, Pip); 1.67 (m, 6H, Pip); 1.53 (m, 2H, CH_2CH_2NH); 1.34 (m, 2H, CH_2CH_3); 0.90 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 428.4 $[M+H]^+$; 450.5 $[M+Na]^+$; 466.4 $[M+K]^+$.

Anal. ($C_{24}H_{30}FN_3O_3$): C, H, N.

4.1.5.21. 5-(2-(Piperidin-1-yl)acetamido)-2-(4-(trifluoromethyl)phenoxy)-N-butylbenzamide (7h). Yield: 34%; mp 62–64 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.45 (br s, 1H, CONH); 8.24 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.61 (d, 2H, J = 8.0 Hz, ArH); 7.39 (br s, 1H, NHCO); 7.04 (d, 2H, J = 8.0 Hz, ArH); 6.96 (d, 1H, J = 8.8 Hz, ArH); 3.40 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.07 (s, 2H, $COCH_2$); 2.54 (br s, 4H, Pip); 1.67 (m, 6H, Pip); 1.48 (m, 2H, CH_2CH_2NH); 1.27 (m, 2H, CH_2CH_3); 0.86 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 478.5 $[M+H]^+$; 500.6 $[M+Na]^+$.

Anal. ($C_{25}H_{30}F_3N_3O_3$): C, H, N.

4.1.5.22. 5-(2-(Piperidin-1-yl)acetamido)-2-(2-(trifluoromethyl)phenoxy)-N-butylbenzamide (7i). Yield: 54%; mp 86–88 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.44 (br s, 1H, CONH); 8.27 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.72 (d, 1H, J = 7.6 Hz, ArH), 7,49 (t, 1H, J = 8.0 Hz, ArH), 7.41 (br s,1H, NHCO); 7.24 (t, 1H, J = 7.6 Hz, ArH); 6.92 (d, 1H, J = 9.1 Hz, ArH); 6.84 (d, 1H, J = 8.8 Hz, ArH); 3.41 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.07 (s, 2H, $COCH_2$); 2.54 (br s, 4H, Pip); 1.68 (m, 6H, Pip); 1.44 (m, 2H, CH_2CH_2NH); 1.27 (m, 2H, CH_2CH_3); 0.86 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 478.5 $[M+H]^+$; 500.5 $[M+Na]^+$; 516.6 $[M+K]^+$.

Anal. ($C_{25}H_{30}F_3N_3O_3$): C, H, N.

4.1.5.23. 5-(2-(Piperidin-1-yl)acetamido)-2-(4-methoxyphenoxy)-N-butylbenzamide (7l). Yield 97%; mp 48–50 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.35 (br s, 1H, CONH); 8.15 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.81 (br s, 1H, NHCO); 6.97 (d, 2H, J = 8.8 Hz, ArH); 6.90 (d, 2H, J = 8.8 Hz, ArH); 6.78 (d, 1H, J = 8.8 Hz, ArH); 3.80 (s, 3H, OCH_3); 3.46 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.04 (s, 2H, $COCH_2$); 2.52 (br s, 4H, Pip); 1.70–1.63 (m, 6H, Pip); 1.55 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.36 (m, 2H, CH_2CH_3); 0.90 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 440.4 $[M+H]^+$; 462.4 $[M+Na]^+$.

Anal. ($C_{25}H_{33}N_3O_4$): C, H, N.

4.1.5.24. 5-(2-(Piperidin-1-yl)acetamido)-2-(4-butoxyphenoxy)-N-butylbenzamide (7m). Yield: 86%; mp 52–54 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.35 (br s, 1H, CONH); 8.14 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.83 (br s, 1H, NHCO); 6.96 (d, 2H, J = 8.4 Hz, ArH); 6.90 (d, 2H, J = 8.4 Hz, ArH); 6.79 (d, 1H, J = 8.8 Hz, ArH); 3.96 (t, 2H, J = 6.6 Hz OCH_2); 3.46 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.05 (s, 2H, $COCH_2$); 2.53 (br s, 4H, Pip); 1.80 (m, 2H, J = 6.6 Hz OCH_2CH_2); 1.64 (m, 6H, Pip); 1.57 (m, 4H, $2CH_2$, $OCH_2CH_2CH_2$, CH_2CH_2NH); 1.36 (m, 2H, CH_2CH_3); 0.99 (t, 3H, J = 6.9 Hz, $O(CH_2)_3CH_3$,); 0.91 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 482.7 $[M+H]^+$; 504.6 $[M+Na]^+$.

Anal. ($C_{28}H_{39}N_3O_4$): C, H, N.

4.1.5.25. 5-(2-(Piperidin-1-yl)acetamido)-2-(p-tolyloxy)-N-butylbenzamide (7n). Yield: 88%; mp 79–82 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.37 (br s, 1H, CONH); 8.19 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, ArH); 7.75 (br s, 1H, NHCO); 7.15 (d, 2H, J = 8.4 Hz, ArH); 6.91 (d, 2H, J = 8.4 Hz, ArH); 6.84 (d, 1H, J = 8.8 Hz, ArH); 3.45 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.05 (s, 2H, $COCH_2$); 2.52 (br s, 4H, Pip); 2.34 (s, 3H, $ArCH_3$); 1.66 (m, 6H, Pip); 1.54 (qt, 2H, J = 6.9 Hz, CH_2CH_2NH); 1.36 (m, 2H, CH_2CH_3); 0.89 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 424.4 $[M+H]^+$; 446.3 $[M+Na]^+$; 462.2 $[M+K]^+$.

Anal. ($C_{25}H_{33}N_3O_3$): C, H, N.

4.1.5.26. 5-(2-(Piperidin-1-yl)acetamido)-2-(4-isopropylphenoxy)-N-butylbenzamide (7o). Yield: 92%; mp 75–77 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.37 (br s, 1H, CONH); 8.20 (dd, 1H, J = 8.8 Hz, ArH); 7.87 (s, 1H, J = 7.9 Hz, ArH); 7.75 (br s, 1H, NHCO); 7.22 (d, 2H, J = 8.4 Hz, ArH); 6.94 (d, 2H, J = 8.4 Hz, ArH); 6.86 (d, 1H, J = 8.8 Hz, ArH); 3.44 (qd, 2H, J = 6.9 Hz, J = 6.2 Hz, CH_2NH); 3.05 (s, 2H, $COCH_2$); 2.94 (hep, 1H, J = 6.9 Hz, CH-Isopropyl); 2.53 (br s, 4H, Pip); 1.66 (m, 6H, Pip); 1.52 (m, 2H, , CH_2CH_2NH); 1.33 (m, 2H, CH_2CH_3); 1.27 (d, 6H, J = 6.9 Hz, $2CH_3$-Isopropyl); 0.89 (t, 3H, J = 6.9 Hz, CH_2CH_3).

ESI-MS: 452.7 $[M+H]^+$.

Anal. ($C_{27}H_{37}N_3O_3$): C, H, N.

4.2. Pharmacology

4.2.1. Production of recombinant mouse PrP 23–231

Recombinant PrP 23–231 was produced and purified essentially as previously reported,[19] except that for bacterial expression, BL21(DE3) RIL *Escherichia coli* cells (Novagen) were transformed with plasmid pET17b-MmPrP23–231WT31 for mouse PrP23–231.

4.2.2. Preparation of PrP^{Sc}

PrP^{Sc} was prepared from brain of CJD patients as described by Bertsch et al.[19] Aliquots of the final pellet resuspended in 1× phosphate-buffered saline (PBS) plus 0.1% sarcosyl solution were diluted fivefold into 20 mM potassium phosphate buffer at pH 6.0, 0.1% Nonidet P-40 and sonicated in a water bath sonicator for 60 s. After centrifugation at 1.000 rpm for 1 min, the supernatant was diluted 100-fold in potassium phosphate buffer, pH 6, 0.1% Nonidet P40 for the assay.

4.2.3. Fluorescent labelling of antibodies and recombinant PrP

L42 monoclonal antibody (r-biopharm, Darmstadt, Germany) was labelled with Alexa Fluor 647 (Alexa-647; Invitrogen, Eugene) according to the manufactureŕs manual. Mouse rPrP was labelled with the Alexa Fluor 488 (Alexa-488; Invitrogen, Eugene) in 20 mM potassium phosphate buffer, pH 6, 0.1% Nonidet P40, 40 mM sodium bicarbonate buffer, pH 8.3. Unbound fluorophores were separated by gelfiltration on PD10 columns (GE Healthcare, Freiburg, Germany) equilibrated with 20 mM potassium phosphate buffer, pH 6, 0.1% Nonidet P40. Quality control of labeling reaction and ratio was performed by fluorescence correlation spectroscopy (FCS) measurements on an Insight Reader (Evotec Technologies, Hamburg, Germany). The labeling ratio was approximately 1.3 fluorophores per rPrP molecule.

4.2.4. Assay for PrP^{C}-PrP^{Sc} association

For the assay, the synthetized compounds **6a–o** and **7a–o** (approximately 10 mM in DMSO) were first diluted 10-fold into DMSO. This dilution was again diluted 10-fold into potassium phosphate buffer, pH 6, 0.1% Nonidet P40. A mixture of labelled mouse rPrP and labeled L42 monoclonal antibody was prepared in 20 mM potassium phosphate buffer, pH 6, 0.1% Nonidet P40 so that the labeled molecules were approximately equally abundant at 2–6 nM. In a 20 μL assay volume 8 μL of the rPrP/antibody mixture, 2 μL compound and 10 μL of the diluted PrP^{Sc} preparation were mixed. The samples were loaded onto 96-well plates with cover-glass bottom (Evotec-Technologies, Hamburg, Germany) and measured on an Insight Reader.

4.2.5. Single-particle measurement and analysis

FIDA (fluorescence intensity distribution analysis) measurements were performed at excitation energies of 200 μW for the 488 nm laser and 300 μW for the 633 nm laser. Scanning parameters were set to 100 μm scan path length, 50 Hz beamscanner frequency, and 2000 μm positioning table movement. The measurement time was 10 s. Fluorescence from the two fluorophores was recorded separately with single photon detectors and photons were summed over time intervals of constant length (bins) using a bin length of 40 μs. The number of red and green fluorescent photon counts was measured and analysed in a two-dimensional intensity distribution histogram, as previously described .[22]

The fluorescence intensity data was evaluated using a 2D-SIFT software module (Evotec-Technologies, Hamburg, Germany). Cut-off values for bin intensities for each measurement series were adjusted manually according to the control measurements.

4.2.6. Inhibition of PrP^{Sc} accumulation in a cell based dot blot model

Mouse neuroblastoma (ScN_2a) and scrapie mouse brain (SMB) cells, infected with the Rocky Mountain Laboratory (RML) scrapie strain, were seeded in a concentration of 20.000 cells per well in 100 μl of medium at a Costar 3599 flat-bottom 96-well plate (Corning Inc., Corning, N.Y.) prior to compound addition. Test compounds were added to the cell medium in a final dilution of 20.0 μM and 2.0 μM, respectively. After an incubation period of three days at 37 °C (ScN_2a cells) or at 35 °C (SMB cells) in a CO_2 incubator the cultures were lysed and analyzed for PrP^{Sc}/PrP^{res} (protease resistant prion protein) formation. After cell medium removal, 100 μl of lysis puffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl; 0.5% (w/v) DOC; 0,5% (v/v) Triton X-100) was added to each well for 5 min at room temperature. Using a dot blot apparatus (Sigma–Aldrich) cell lysate was transferred under vacuum to an activated polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Milipore) and fixed to the membrane by incubation for 1 h at 37 °C. The membrane was incubated in lysis puffer and treated with Proteinase K solution (final dilution 25 μg/ml) for 90 min at 37 °C. Subsequently, the membrane was washed twice with pure water, the denaturation solution (3 M guanidinium thiocyanate, 0.1 M Tris HCl pH 8) was added for 10 min at room temperature and membranes were washed five times with pure water. After the denaturation step the membrane was blocked with 5% (w/v) non-fat milk-0.1% (v/v) tween-20 (Sigma) in phosphate buffered saline (PBS-T) for 60 min. An appropriate dilution of polyclonal antibody rabbit 10[23] in blocking solution was incubated with the membrane for 60 min. After PBS-T rinsing, the membrane was incubated in a 0.2 μg/ml solution of Horseradish Peroxidase (HRP) conjugated with anti-rabbit IgG antibody (Promega) in PBST-milk for 1 h at room temperature. After additional PBS-T rinsing the bound antibodies were detected using a chemiluminescence reagent system (ECL, Amersham) and were visualized directly in an image analysis system (Versa Doc, Quantity One, Bio-Rad, Munich, Germany). Inhibition was calculated as relative signal volume compared to the untreated control (100%).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.026.

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