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Article

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N^{ω}-carbamoylation of the argininamide moiety: an avenue to insurmountable NPY Y_1 receptor antagonists and a radiolabeled selective high affinity molecular tool ([^{3}H]UR-MK299) with extended residence time

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KEYWORDS

neuropeptide Y; NPY Y_1 receptor antagonist; radioligand; target residence time; argininamide; neuropeptide FF; carbamoylguanidine

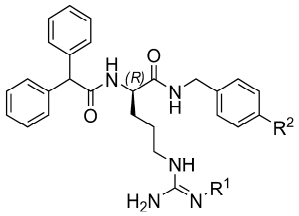
ABSTRACT Analogs of the argininamide-type NPY Y_1 receptor (Y_1R) antagonist BIBP3226, bearing carbamoyl moieties at the guanidine group, revealed subnanomolar K_i values and caused depression of the maximal response to NPY (calcium assay) by up to 90% in a concentration- and time-dependent manner, suggesting insurmountable antagonism. To gain insight into the mechanism of binding of the synthesized compounds, a tritiated antagonist, (*R*)-N^{α}-diphenylacetyl-N^{ω}-[2-([2,3-^{3}H]propionylamino)ethyl]aminocarbonyl-(4-hydroxybenzyl)arginin-amide ([^{3}H]UR-MK299, [^{3}H]**38**), was prepared. [^{3}H]**38** revealed a dissociation constant in the picomolar range (K_d 0.044 nM, SK-N-MC cells) and very high Y_1R selectivity. Apart from superior affinity, a considerably lower target off-rate ($t_{1/2}$ 95 min) was characteristic of [^{3}H]**38** compared to the higher homolog containing a tetramethylene instead of an ethylene spacer ($t_{1/2}$ 3 min, K_d 2.0 nM,). Y_1R binding of [^{3}H]**38** was fully reversible and fully displaceable by nonpeptide antagonists and the agonist pNPY. Therefore, the insurmountable antagonism observed in the functional assay has to be attributed to the extended target-residence time, a phenomenon of relevance in drug research beyond the NPY receptor field.

Introduction

The 36 amino acid peptide neuropeptide Y (NPY) is involved in the regulation of various physiological functions and was reported to be implicated in diseases such as obesity, depression and addiction to alcohol.[1-3] NPY binds to receptors of the NPY family, which, in humans, comprises four functionally expressed receptor subtypes (Y_1R, Y_2R, Y_4R and Y_5R). Due to (over)expression in malignant tumors, the Y_1R has been suggested as a target for cancer diagnosis.[4-8] Complementary to radiolabeled peptidic Y_1R agonists, highly selective radiolabeled nonpeptidic antagonists are valuable pharmacological and potential diagnostic tools, but

commercially not available. Apart from high affinity and selectivity, slow target off-rate and high in vivo stability of the radiotracer are considered favorable.

The tritiated analog of the (*R*)-argininamide **1a** (BIBP3226)[9] (Figure 1) was the first described radiolabeled Y_1R antagonist, exhibiting a K_d value of 2.1 nM.[10] Bioisosteric replacement of the guanidine group in **1a** by acyl- or carbamoylguanidine moieties afforded the radiolabeled Y_1R antagonists [^{3}H]UR-MK114 ([^{3}H]**2**)[11] and [^{3}H]UR-MK136 ([^{3}H]**4**)[12] (Figure 1) with affinities comparable to **1a**. Aiming at Y_1R radioligands with higher affinity, potential radioligands derived from the argininamide **1b** (BIBO3304)[13] (Figure 1) were recently described.[14] However, derivatization of **1b** resulted in a pronounced decrease in Y_1R affinity, leading to ligands with binding constants in the single-digit nanomolar range. In continuation of our work on argininamide-type Y_1R antagonists we focused on N^{ω}-carbamoylated argininamides, as the carbamoylguanidine moiety was recently identified as a stable bioisostere of the acylguanidine group,[12,15-16] conferring higher affinity than the guanidine group in the parent compound **1a**, as previously reported for **5-7**[17-18] (Figure 1).



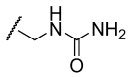
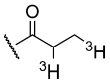
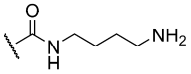
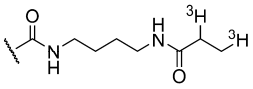
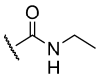
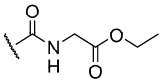
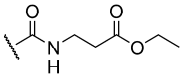
compound	R^1	R^2	K_i or K_d [nM]
BIBP3226 (**1a**)	H	OH	2.1[a] 1.3[b]
BIBO3304 (**1b**)	H		0.25[b]
[^{3}H]UR-MK114 ([^{3}H]**2**)		OH	1.2[c]
3		OH	4.3[b]
[^{3}H]UR-MK136 ([^{3}H]**4**)		OH	2.0[d]
5		OH	0.06[e]
6		OH	0.06[e] 0.3[f]
7		OH	0.3[f]

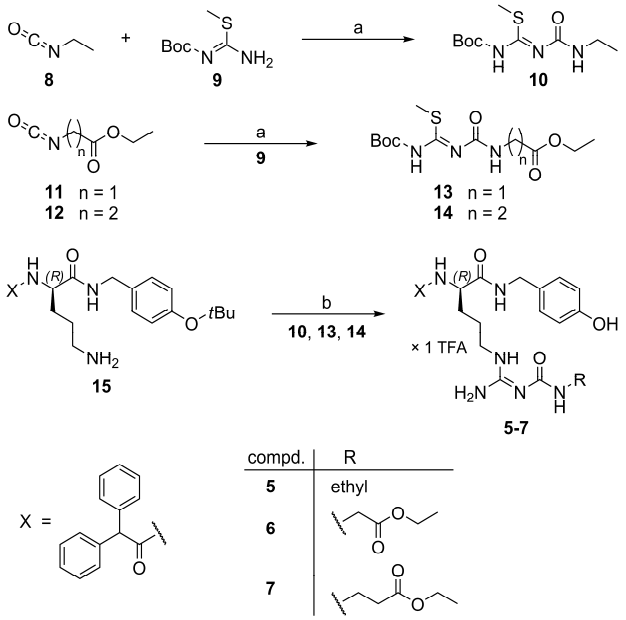
Figure 1. Structures and Y_1R affinities (determined at SK-N-MC neuroblastoma or HEL cells) of **1a**, **1b**, the argininamide-type radioligands [^{3}H]**2** and [^{3}H]**4**, and the N^{ω}-carbamoylated argininamides **3** and **5-7**. [a]K_d value reported for [^{3}H]**1a** by Entzeroth *et al.*[10] [b]K_i value determined with [^{3}H]**2** as radioligand.[15] [c]K_d value described by Keller *et al.*[11] [d]K_d value described by Keller *et al.*[12] [e,f]K_i value determined with [^{3}H]propionyl-pNPY as radioligand, [e]Brennauer *et al.*,[17] [f]Schneider *et al.*[18]

Very recent detailed functional studies with **5-7** (synthesis not reported so far; *cf.* Supporting Information) revealed insurmountable antagonism of these nonpeptide Y_1R ligands. To elucidate this phenomenon we synthesized and characterized new analogs of **5-7** including compounds with bulkier carbamoyl substituents as well as the radiolabeled version of an optimized antagonist.

Results and Discussion

Chemistry. The synthesis of argininamides **5-7**, which were prepared from amine **15** and the *S*-methylisothiourea derivatives **10**,[19] **13** and **14**, is outlined in Scheme 1. The guanidinylating reagents **10**, **13** and **14** were obtained by treatment of **9** with the commercially available isocyanates **8**, **11** or **12**. Guanidinylation of amine **15** with **10**, **13** and **14** in the presence of mercury(II) chloride gave Boc/*t*Bu-protected intermediates, which were treated with trifluoroacetic acid (TFA) to afford argininamides **5-7** (Scheme 1).

Scheme 1. Synthesis of the N^{ω}-carbamoylated argininamide-type Y_1R antagonists **5-7**.[a]

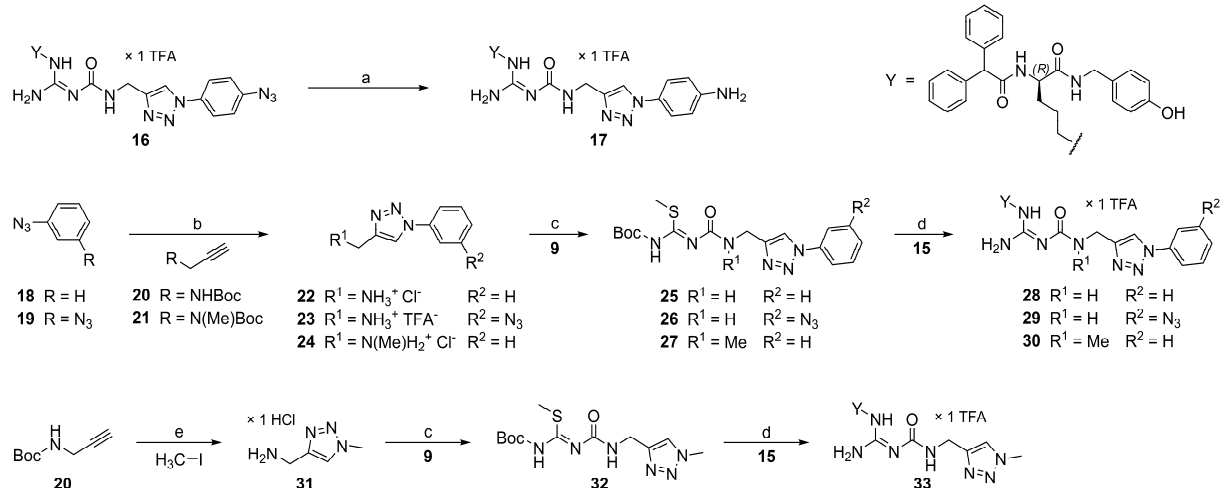


[a]Reagents and conditions: (a) DIPEA, CH_2Cl_2, 86-99%; (b) (1) $HgCl_2$, DIPEA, CH_2Cl_2; (2) TFA, CH_2Cl_2, H_2O, 62-75%.

Scheme 2 shows the synthesis of argininamides **17**, **28-30** and **33**, which contain a triazole moiety in the substituent attached to the guanidine group. Palladium-catalyzed hydrogenation of

the previously described argininamide **16**[20] afforded aniline derivative **17**. The synthesis of argininamides **28-30** started from azides **18** and **19**, which were subjected to a copper(I)-catalyzed cycloaddition reaction with alkyne **20** or **21** to give the triazole derivatives **22**,[21] **23** and **24** (Scheme 2). Compounds **22-24** were converted to the guanidinylating reagents **25-27** by treatment with triphosgene and subsequent reaction with thiourea derivative **9**. Guanidinylation of amine **15** with **25-27** followed by Boc/*t*Bu-deprotection with TFA afforded compounds **28-30**.

Scheme 2. Synthesis of the triazole-containing argininamides **17**, **28-30** and **33**.[a]



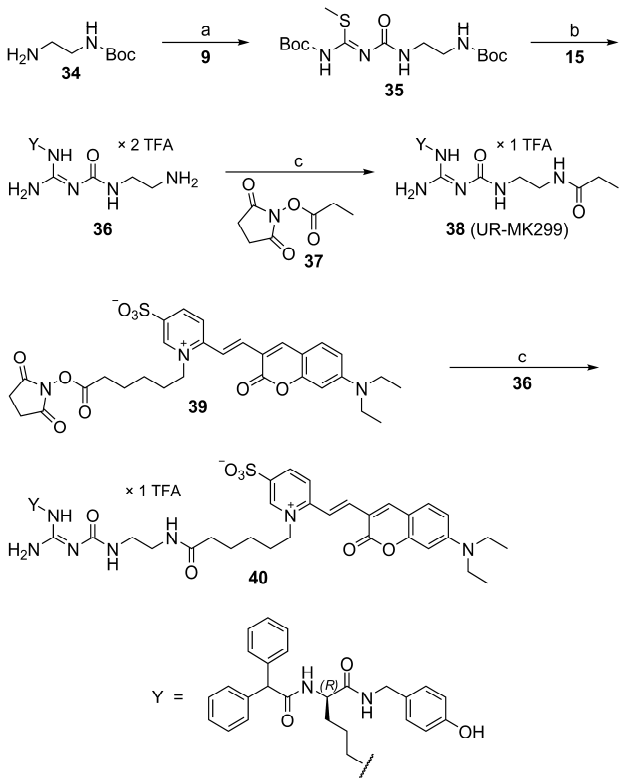
[a]Reagents and conditions: (a) 10% Pd/C, hydrogen, MeOH, 99%; (b) (1) ascorbic acid, $CuSO_4$, MeOH, H_2O; (2) MeOH/conc. aq HCl 3:1 (v/v) (**22**, **24**) or CH_2Cl_2/TFA 2:1 (v/v) (**23**), 38-86%; (c) triphosgene, DIPEA, CH_2Cl_2 (**25-27**) or acetonitrile (**32**), 25-79%; (d) (1) $HgCl_2$, triethylamine, DMF; (2) TFA, CH_2Cl_2, 21-72%; (e) (1) iodomethane, NaN_3, tetrabutylammonium tetrafluoroborate, diethyl ether, H_2O; (2) alkyne **20**, ascorbic acid, $CuSO_4$, MeOH, diethyl ether, H_2O; (3) MeOH/conc. aq HCl 3:1 (v/v), 72%.

Methyl azide, required for the preparation of compound **31**, was prepared from methyl iodide and sodium azide in a water/diethyl ether mixture using tetrabutylammonium tetrafluoroborate as

phase transfer catalyst. To circumvent the isolation of the explosive methyl azide, alkyne **20** and aqueous solutions of ascorbic acid and copper(II) sulfate were added directly to the separated diethyl ether phase, containing the azide, to obtain the respective cycloaddition product, which was chromatographed and treated with hydrochloric acid to give the unprotected amine **31**[22]. Treatment of **31** with triphosgene and subsequent addition of compound **9** gave the guanidinylating reagent **32**, which was used to convert amine **15** to argininamide **33** (Scheme 2).

For the synthesis of propionamide **38** (UR-MK299) and the fluorescently labeled argininamide **40**, thiourea derivative **9** and amine **34** were converted to guanidinylating reagent **35**,[23] using a procedure reported for the higher homolog of **35** (Scheme 3).[15] Treatment of amine **15** with **35** in the presence of mercury(II) chloride and subsequent deprotection yielded amine **36**, which was propionylated to give **38**, or treated with Fluorescence Red Mega 480 succinimidyl ester (**39**) to afford compound **40** (Scheme 3).

Scheme 3. Synthesis of the Y_1R antagonist **38** and the fluorescently labeled argininamide **40**.[a]



[a]Reagents and conditions: (a) triphosgene, DIPEA, CH_2Cl_2, 66%; (b) (1) $HgCl_2$, DIPEA, CH_2Cl_2; (2) TFA, CH_2Cl_2, H_2O, 65%; (c) DIPEA, DMF, **38**: 83%, **40**: 69%.

Functional studies at the human NPY Y_1 receptor. The carbamoylated argininamide-type Y_1R antagonists **5-7** (Figure 1), exhibiting high Y_1R affinity (K_i < 1 nM, *cf*. Figure 1 and Table 1), were investigated in a Fura-2 Ca^{2+} assay on human erythroleukemia (HEL) cells using pNPY as agonist. The effect of pNPY, elicited at a concentration of 10 nM, was inhibited by increasing concentrations of the antagonist. Interestingly, the antagonistic effect turned out to be time-dependent, increasing with the incubation period of the cells in the presence of the antagonist prior to the addition of the agonist pNPY (Figure 2 B-D). The maximum shift of the curves was reached after 5 min (compound **5**, Figure 2B) and 15-20 min (**6** and **7**, Figure 2C and 2D), respectively. By contrast, this phenomenon was not observed in case of the parent compound **1a** (Figure 2A), indicating that the time-dependent effect has to be attributed to the carbamoyl substituents attached to the guanidine group in **5-7**.

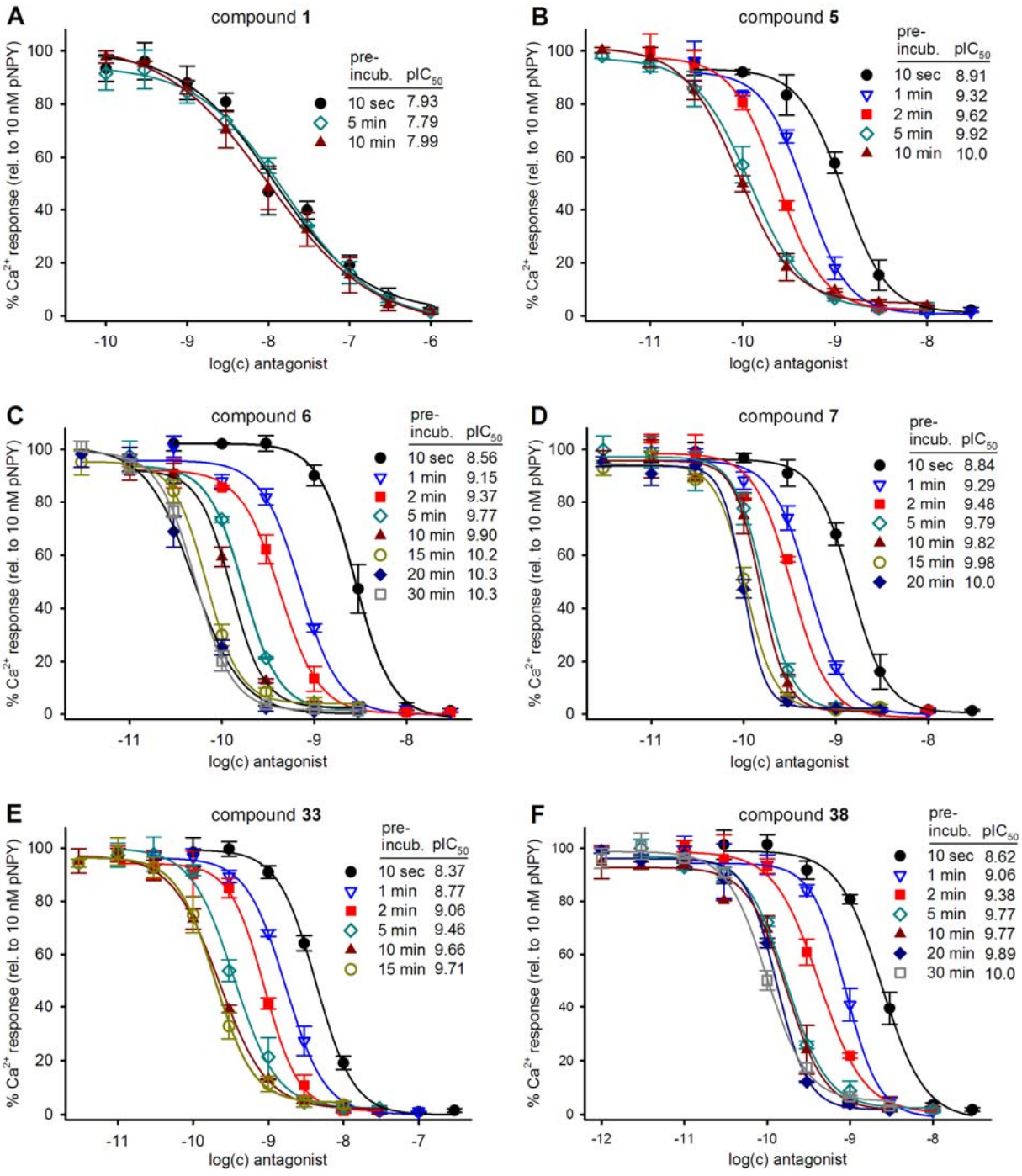


Figure 2. Concentration- and time-dependent inhibition of the pNPY (10 nM) induced Ca^{2+} response in HEL cells by the argininamides **1a** (A), **5** (B), **6** (C), **7** (D), **33** (E), and **38** (F). Mean values ± SEM from at least three independent experiments (performed in singlet).

As shown for compound **6** (Figure 3A) and **16** (Supporting Information Figure S5) as examples, the concentration-response curve of pNPY was rightward-shifted and depressed upon pre-incubation of the cells with increasing concentrations of the antagonists for 15 minutes. The maximal depression of the concentration-response curve was confirmed by a different experimental approach:[24] adding pNPY at a 'supramaximal effective concentration'[24] of 300 nM to cells pre-incubated with compound **6** at increasing concentrations for 15 minutes resulted in a biphasic curve, indicating the extent of insurmountable antagonism (Figure 3B). Partially insurmountable antagonism, although less pronounced, was also observed for compounds **5** and **38** (Figure 3B). In a more simplified kinetic experiment, only one ('supra-effective') concentration of antagonist, derived from the respective determined (**1a**, **5-7**, **33** and **38**, Figure 2) or published[11-12] IC_{50} value (**2**, **4**), was applied to inhibit the response elicited by 300 nM of pNPY (Figure 3C). The plateaus of the curves, reached in a time-dependent manner, correspond to the maximum depression caused by the antagonist, as exemplified by compound **6** at a concentration of 1 nM (Figure 3 A,B). Whereas **5-7**, **33** and **38** caused a strong depression of the maximal pNPY effect by up to 90% (Figure 3), a phenomenon characteristic of insurmountable antagonists,[25-26] the argininamides **2** and **4** caused a slight depression of the maximal Ca^{2+} response, and the parent compound **1a** did not affect the pNPY response within the limits of experimental error (Figure 3C).

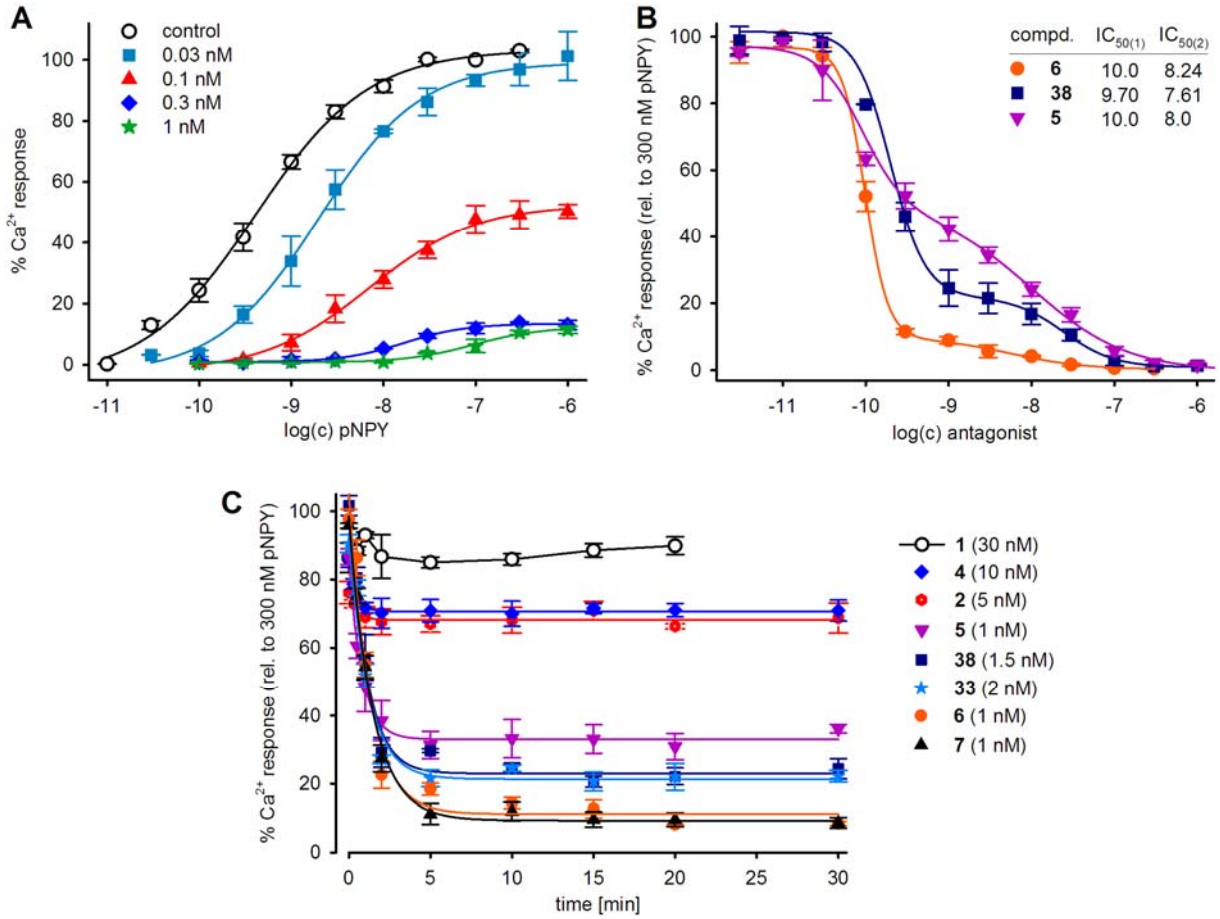


Figure 3. Investigation of the (insurmountable) antagonism of argininamides **1a**, **2**, **4**, **5-7**, **33** and **38** in a Fura-2 Ca^{2+} assay performed with Y_1R expressing HEL cells. A: Concentration-response curves of pNPY in the absence and presence of antagonist **6** (15 min pre-incubation). The presence of **6** led to a rightward shift of the curves and to a strong depression of the maximum response to pNPY. B: Concentration-dependent inhibition of the Ca^{2+} response, elicited by pNPY at a supramaximal concentration of 300 nM, by the antagonists **5**, **6** and **38** (15 min pre-incubation). The biphasic course of the curves revealed partially insurmountable antagonism.[24] C: Time-dependent depression of the pNPY (300 nM) induced Ca^{2+} response by **1a**, **2**, **4**, **5-7**, **33** and **38**. Presented are mean values ± SEM from at least two (A, B) or three (C) independent experiments (performed in singlet).

The results shown in Figure 3C might be interpreted as a hint to insurmountable antagonism but not a proof, because the Fura-2 Ca^{2+} assay is a non-equilibrium assay, which can produce depressions of the maximal agonist response due to hemi-equilibrium among receptor, agonist and antagonist.[27] However, this is improbable due to the results of the additional experiments

shown in Figure 3A,B and due to the fact that the parent compound revealed typical, that is, surmountable antagonism.

Binding studies at human NPY receptors. Insurmountable antagonism might be caused, for example, by stabilization of receptor conformations with low affinity to the agonist (pNPY) or by a long-lasting (pseudo-irreversible) binding. Therefore, we performed equilibrium competition binding experiments with the radiolabeled agonist [^{3}H]propionyl-pNPY. Two variants of the competition binding experiment were performed. Firstly, the antagonists were added immediately after the agonist [^{3}H]propionyl-pNPY, and secondly, the cells were pre-incubated with the antagonists for 30 min. The binding constants (K_i values) of **5**, **6** and **38** obtained in these experiments are shown in Table 1, and competition binding curves are depicted in Figure 4. Pre-incubation of the cells with the antagonist did not result in a marked increase in apparent Y_1R affinity, that is, the antagonists did not show (pseudo-)irreversible binding after an incubation period of 2 h (equilibrium conditions). To gain insight into the mechanism causing the discrepancies between the result from binding studies and the insurmountable antagonism observed in the functional assay, a radiolabeled form of an insurmountable antagonist such as compound **38** was considered a useful molecular tool.

Table 1. Y_1 receptor affinities of pNPY and the argininamides 5-7, 16, 17, 28-30, 33, 36, 38, 40 and 41 determined by equilibrium competition binding with [^{3}H]2, [^{3}H]4, [^{3}H]propionyl-pNPY or [^{3}H]38.

Compound	K_i [nM] (Y_1R)			
	used radioligand:			
	[^{3}H]**2**[a]	[^{3}H]**4**[b]	[^{3}H]propionyl-pNPY[c]	[^{3}H]**38**[d]
pNPY	0.5[e]	0.7[f]	0.6[g]	0.41 ± 0.058[h] / 0.42 ± 0.054[i]
1a	1.3[e]	1.5[f]	1.5[g]	1.0 ± 0.019
5	0.17 ± 0.017	0.40 ± 0.082	0.06[j] 0.29 ± 0.016[k] / 0.24 ± 0.017[l]	--
6	0.21 ± 0.059	0.42 ± 0.12	0.3[g] 0.06[j] 0.28 ± 0.21[k] / 0.12 ± 0.007[l]	0.037 ± 0.009
7	--	0.49 ± 0.021	0.3[g]	--
16	4.5 ± 0.3	--	--	--
17	4.0 ± 0.20	--	--	--
28	6.9 ± 1.4	--	--	--
29	3.1 ± 0.63	--	--	--
30	41 ± 1.7	--	--	--
33	0.56 ± 0.062	0.34 ± 0.014	--	--
36	--	22 ± 3	--	--
38	--	0.31 ± 0.031	0.23 ± 0.026[k] / 0.10 ± 0.008[l]	0.077 ± 0.021
40	--	--	--	>1,000
41	400 ± 19[m]	--	--	--

[a]Determined by radioligand competition binding with [^{3}H]**2** (K_d = 1.2 nM, c = 1.5 nM) at SK-N-MC cells. [b]Determined by radioligand competition binding with [^{3}H]**4** (K_d = 2.0 nM, c = 2.0 nM) at SK-N-MC cells. [c]Determined by radioligand competition binding with [^{3}H]propionyl-pNPY (K_d = 1.9 nM, c = 5.0 nM) at SK-N-MC or HEL cells. [d]Determined by radioligand competition binding with [^{3}H]**38** (K_d = 0.044 nM, c = 0.15 nM) at SK-N-MC cells. [e-g]K_i values reported by: [e]Keller *et al.*,[11] [f]Keller *et al.*,[12] [g]Schneider *et al.*[18] [h/i]pNPY was either added immediately prior to [^{3}H]**38** (*h*) or 60 min after pre-incubation of the cells with [^{3}H]**38** (*i*) (K_i values not significantly different (P > 0.05)). [j]K_i value reported by Brennauer *et al.*[17] [k/l]The argininamides **5**, **6** and **38** were either added to the cells immediately after the radioligand (*k*) or pre-incubated with the cells (30 min) prior to the addition of [^{3}H]propionyl-pNPY (*l*) (**5**: K_i values not significantly different (P > 0.05), **6** and **38**: K_i values significantly different (P < 0.02)) [m]K_i value reported by Weiss *et al.*[20] Presented are mean values ± SEM from at least two independent experiments (performed in triplicate). Note: All K_i values were determined in our laboratory essentially according to the same experimental procedure. Because of the very high affinity of [^{3}H]**38** for the NPY Y_1 receptor, the binding assay was performed in a way to ensure

that the free radioligand concentration was not markedly decreased by binding to the receptor. Therefore, cells were kept below 80% confluency, and the volume per well was increased (500 µL, instead of 250 µL used for ligands with lower affinity such as [^{3}H]**2** and [^{3}H]**4**) to decrease the ratio of receptor-bound to free radioligand.

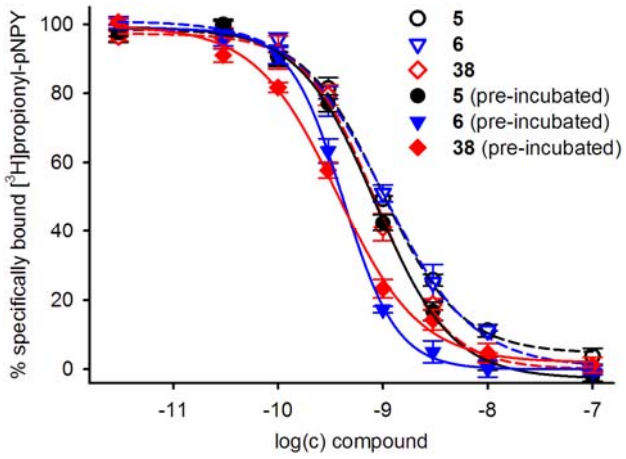


Figure 4. Displacement of [^{3}H]propionyl-pNPY (K_d = 1.9 nM, c = 5.0 nM) by argininamides **5**, **6** and **38** at SK-N-MC neuroblastoma cells in a competition binding experiment. The argininamides were either added immediately after the radioligand (open symbols, dashed lines) or cells were pre-incubated with the antagonists for a period of 30 min prior to the addition of the radioligand (filled symbols, solid lines). Pre-incubation with the argininamide resulted in a slight decrease in the equilibrium binding constant K_i (leftward shift of the curves) (compounds **6** and **38**) or did not result in a statistically significant difference in K_i values (compound **5**) (*cf.* Table 1). Mean values ± SEM from at least three independent experiments (performed in triplicate).

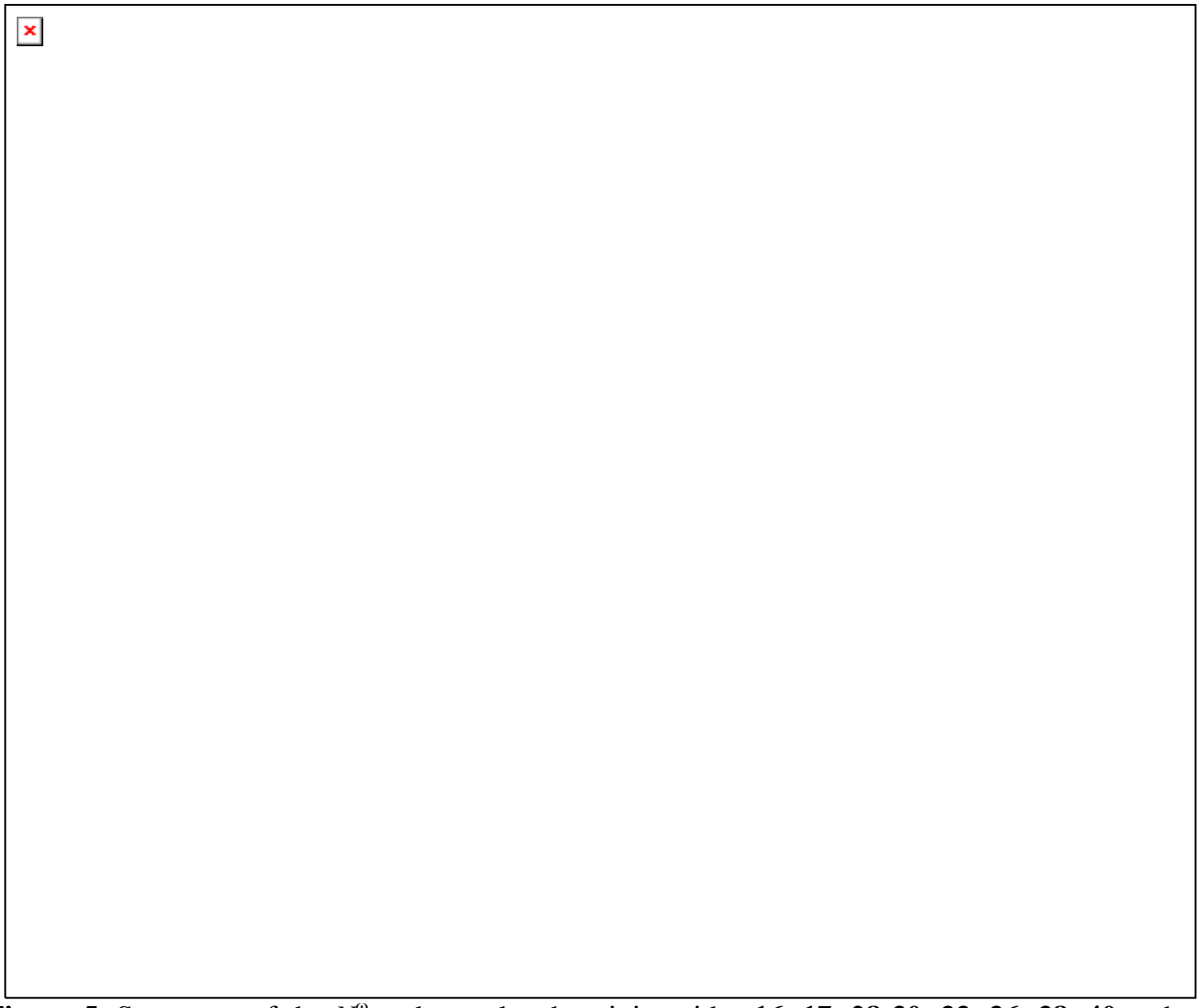


Figure 5. Structures of the N^{ω}-carbamoylated argininamides **16**, **17**, **28-30**, **33**, **36**, **38**, **40** and **41**.

Y_1R affinity data of the carbamoylated derivatives of **1a** (compounds **5-7**, **16**, **17**, **28-30**, **33**, **36**, **38** and **41**) (*cf*. Figure 5) were determined by competition binding with [^{3}H]**2** or [^{3}H]**4** at SK-N-MC neuroblastoma cells (K_i values presented in Table 1). Among the series of triazole derivatives (**16**, **17**, **28-30**, **33**, **41**) the highest Y_1R affinity resided in derivative **33**, the compound with the smallest substituent (*cf*. Figure 5). Argininamide **41**,[20] bearing the largest substituent in this series of compounds, exhibited the lowest Y_1R affinity, suggesting limited space in the binding region interacting with the moiety attached to the guanidine. The

propionamide **38**, which represents the shorter homolog of the previously reported radioligand [^{3}H]**4** (Figure 1), showed very high Y_1R affinity (K_i = 0.31 nM), comparable to that of compounds **5-7** (Table 1). Compound **38** is closely related to argininamide **7**: the length of the chain attached to the guanidine group is the same (8 atoms), the difference between the two ligands resides in the ester group (**7**) and amide group (**38**) (*cf.* Figures 1 and 5). When comparing amine **36** and propionamide **38** (Figure 5), propionylation of **36** led to a > 50-fold increase in Y_1R affinity (Table 1), whereas, in case of the shorter homologs **3** and **4**, respectively, propionylation had almost no effect on Y_1R affinity (Figure 1). Therefore, it can be speculated that the amide group in the substituent attached to the guanidine group in **38** contributes to receptor binding.

Argininamides **5-7**, **33**, **36** and **38** were characterized in terms of Y_1R selectivity by flow cytometric competition binding studies on CHO-hY_2, CHO-hY_4 and HEC-1B-hY_5 cells using the fluorescently labeled peptides Cy5-pNPY (Y_2R and Y_5R)[18,28] and Cy5-[K^4]hPP (Y_4R).[29] All of these compounds proved to be highly selective Y_1R ligands (Table 2). However, despite high Y_1R selectivity, the presented argininamides harbor potential 'off-target effects' in terms of binding to neuropeptide FF receptors as the parent compound **1a** was reported to bind to the $NPFF_1$ receptor (K_i = 12 nM) and the $NPFF_2$ receptor (K_i = 84 nM).[30] Therefore, selected argininamides with high Y_1R affinity (**6**, **33** and **38**) as well as **1a** were investigated in competition binding studies at membranes of CHO-h$NPFF_1$ and CHO-h$NPFF_2$ cells using the radiolabeled peptides [^{3}H]NPVF[31] and [^{3}H]EYF[31]. In contrast to the parent compound **1a**, the N^{ω}-carbamoylated derivatives **6**, **33** and **38** exhibited low $NPFF_1$ and $NPFF_2$ receptor affinities (K_i > 500 nM, Table 2, Supporting Information Figure S6) showing that the introduction of

carbamoyl residues at the guanidine group in **1a** leads to increased Y_1R specificity with respect to NPFF receptors.

Table 2. NPY receptor subtype selectivity data of argininamides 5-7, 33, 36 and 38 and K_i values of selected compounds determined at $NPFF_1$ and $NPFF_2$ receptors.

Compound	K_i [nM]					
	hY_1R[a]	hY_2R[b]	hY_4R[c]	hY_5R[b]	$hNPFF_1R$[d]	$hNPFF_2R$[e]
1a	1.5[f]	n.d.	n.d.	n.d.	18 ± 1.5	250 ± 21
5	0.40 ± 0.082	>3,000	>10,000	>10,000	n.d.	n.d.
6	0.42 ± 0.12	>3,000	>10,000	>10,000	1,100 ± 260	>3,000
7	0.49 ± 0.021	>3,000	>10,000	>10,000	n.d.	n.d.
33	0.34 ± 0.014	>10,000	>10,000	>10,000	630 ± 62	1,200 ± 240
36	22 ± 3	>10,000	>10,000	>10,000	n.d.	n.d.
38	0.31 ± 0.031	>3,000	>10,000	>10,000	1,000 ± 130	>3,000

[a]Determined by radioligand competition binding with [^{3}H]**4** (data from Table 1). [b]Determined by flow cytometry in a competition binding assay on CHO-hY_2 and HEC-1B-hY_5 cells using Cy5-pNPY as fluorescent ligand (K_d = 5.2 nM (Y_2R) and 4.4 nM (Y_5R), c = 5 nM). [c]Determined by flow cytometry in a competition binding assay on CHO-hY_4 cells with Cy5-[K^4]hPP (K_d = 5.6 nM, c = 3 nM) as fluorescent ligand. [d]Determined by radioligand competition binding with [^{3}H]NPVF (c = 0.54 nM) at CHO-$hNPFF_1$ cell membranes. [e]Determined by radioligand competition binding with [^{3}H]EYF (c = 0.93 nM) at CHO-$hNPFF_2$ cell membranes. [f]K_i value reported by Keller *et al.*[12] Data represent mean values ± SEM from at least three independent experiments (performed in triplicate (Y_1R) or duplicate ($NPFF_1R$ and $NPFF_2R$)), or the results from at least two independent experiments (performed in duplicate) (Y_2R, Y_4R and Y_5R).

Preparation and characterization of the radioligand [^{3}H]38. Aiming at the preparation of the high affinity propionamide **38** in its tritiated form, the stability of **38** in aqueous solution was investigated at pH 7 over 48 h. The compound showed excellent chemical stability (Figure 6). Stability studies were also performed with argininamides **5**, **6** and **33**. Whereas **5** and **33** showed

no decomposition, compound **6** proved to be unstable due to hydrolysis of the ester group (see Supporting Information Figures S2–S4).

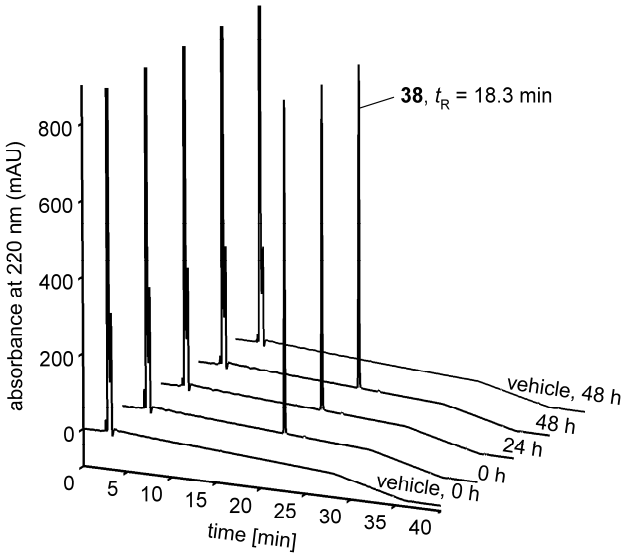


Figure 6. Reversed-phase HPLC analysis of argininamide **38** after incubation in PBS (pH 7.0) at 21 °C for up to 48 h. Compound **38** showed no decomposition.

The tritiated form of **38** was prepared by treating an excess of amine **36** with succinimidyl [^{3}H]propionate (Figure 7A) to afford [^{3}H]**38** with a radiochemical yield of 94% and a specific activity of 1.81 TBq/mmol. The radioligand was obtained with a radiochemical purity of 97% (Figure 7B) and proved to be stable when stored in ethanol at -20 °C (Figure 7C).

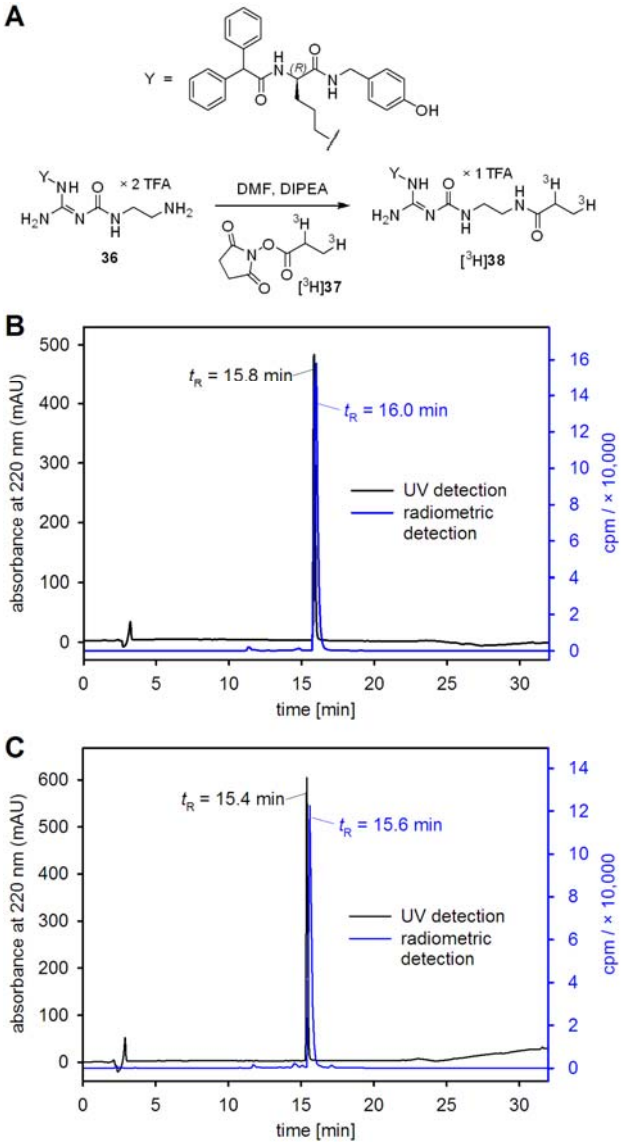


Figure 7. A: Synthesis of the radioligand [^{3}H]**38** by [^{3}H]propionylation of amine **36**. Radiochemical yield: 94%. B/C: RP-HPLC analysis (conditions see experimental section) of [^{3}H]**38** (0.7 µM), spiked with 'cold' **38** (20 µM), analyzed three days after synthesis (B) and after seven months of storage at -20 °C (C). Radiochemical purity: 97% and 95%, respectively. The minor shifts in t_R result from serial detection of the UV and the radiometric signal.

Saturation binding experiments with [^{3}H]**38** at SK-N-MC neuroblastoma cells and MCF-7-Y_1 breast cancer cells yielded binding constants of 0.044 nM and 0.14 nM, respectively (Figure 8, Table 3). A slightly lower affinity to Y_1 receptors expressed by MCF-7-Y_1 cells, compared to Y_1

receptors at SK-N-MC cells, was previously observed for the radioligands [^{3}H]**2** and [^{3}H]**4**, too.[11-12] The determined numbers of binding sites per cell (SK-N-MC: approx. 32,000 sites/cell, MCF-7-Y_1: approx. 130,000 sites/cell) were consistent with previously reported data.[10-12]

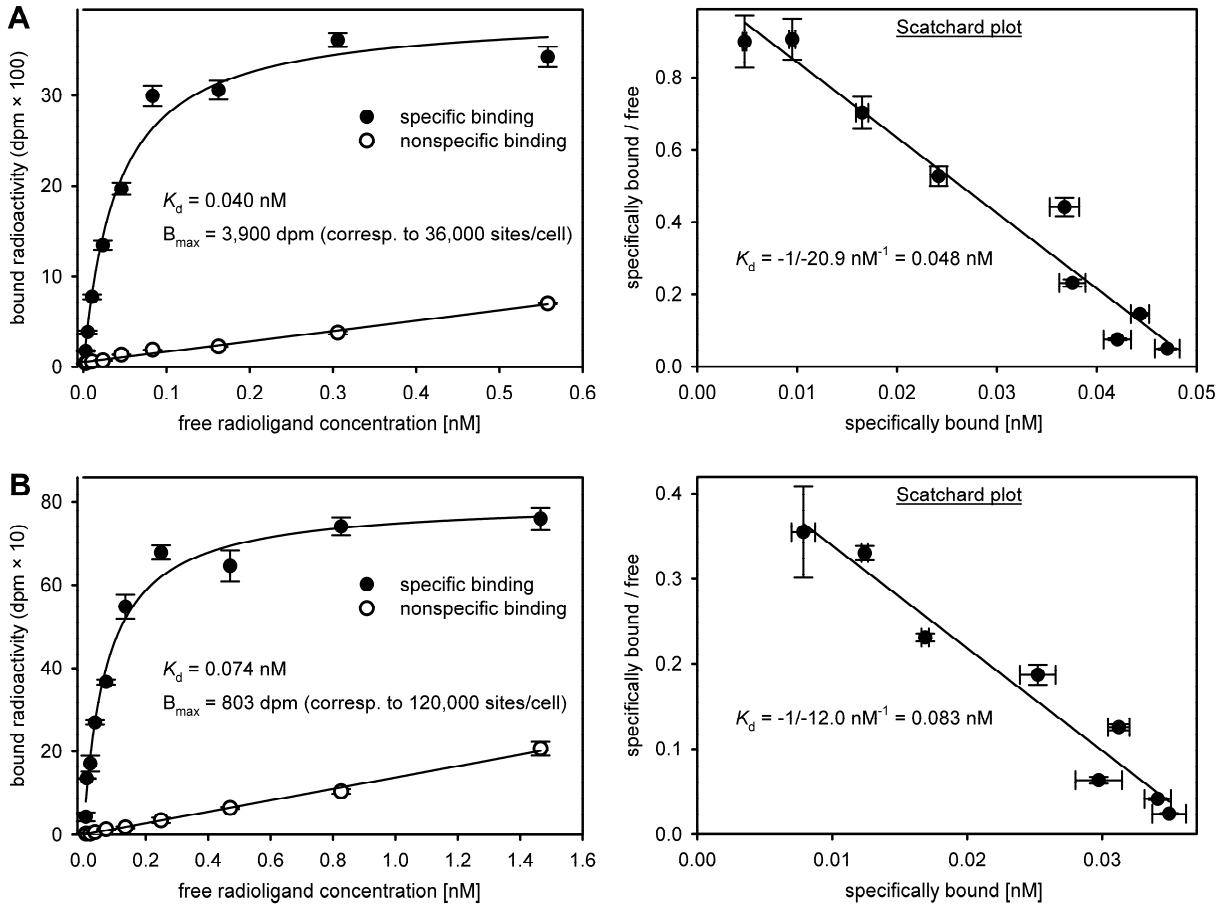


Figure 8. Representative saturation isotherms and Scatchard transformations of specific [^{3}H]**38** binding to SK-N-MC cells (panel A) and MCF-7-Y_1 cells (panel B). Experiments were performed in triplicate. Error bars of 'specific binding' and error bars in the Scatchard plot represent propagated errors calculated according to the Gaussian law of errors. Error bars of 'nonspecific binding' represent the SEM.

The association and dissociation kinetics of [^{3}H]**38** was determined at SK-N-MC cells analogous to the experiments performed previously with [^{3}H]**2** and [^{3}H]**4**.[11-12] Compared to the

higher homolog [^{3}H]**4**, reaching a plateau after 15 min, the association of [^{3}H]**38** was slower (plateau reached after approx. 40 min; Figure 9A). Likewise, with a half-life of 95 min, the dissociation of [^{3}H]**38** was markedly slower compared to [^{3}H]**4** ($t_{1/2}$ = 3 min; Figure 9B).

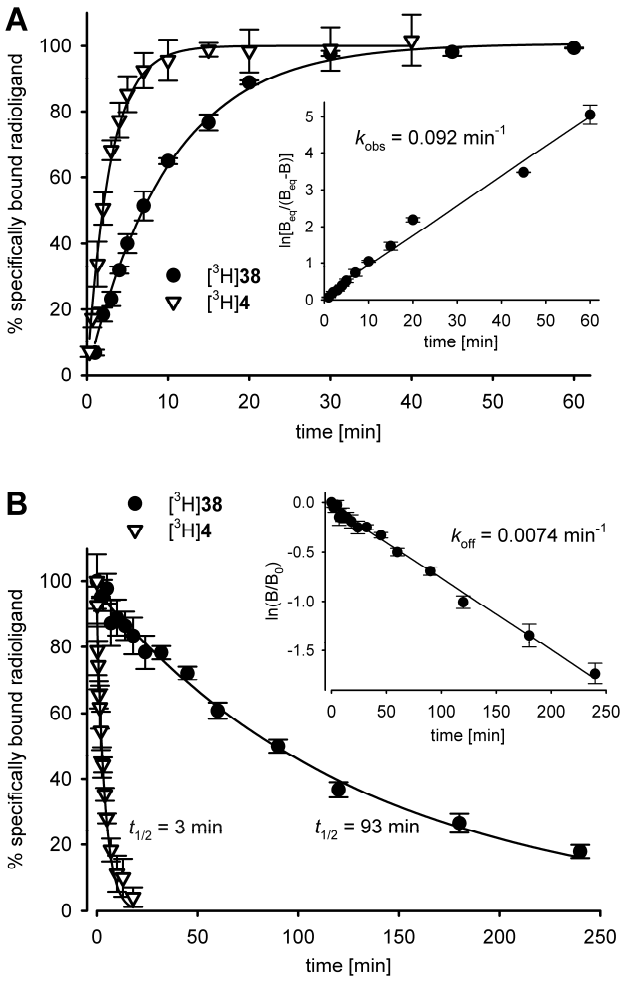


Figure 9. Association and dissociation kinetics of [^{3}H]**38** (in comparison to reference compound [^{3}H]**4**[12]), determined at SK-N-MC neuroblastoma cells at 22 °C. A: Radioligand (c = 0.3 nM) association to the Y_1R as a function of time. Inset: $\ln[B_{eq}/(B_{eq}-B)]$ versus time, slope = k_{obs} = 0.092 min^{-1}. Mean values ± SEM from three independent experiments (performed in triplicate). B: Radioligand (pre-incubation: 1 nM, 45 min) dissociation from the Y_1R as a function of time, monophasic exponential decay: $t_{1/2}$ = 93 min. Inset: $\ln(B/B_0)$ versus time, slope·(−1) = k_{off} = 0.0074 min^{-1}, $t_{1/2}$ = 95 min. Mean values ± SEM from four independent experiments (performed in triplicate).

The kinetically derived dissociation constant K_d(kin), calculated according to $K_d(kin) = k_{off}/k_{on}$, amounted to 0.026 nM being in good agreement with the K_d value obtained from saturation binding experiments (K_d = 0.044 nM). Complete dissociation of [^{3}H]**38**, best fitted by an equation describing a two-parameter mono-exponential decline (Figure 9B), is incompatible with the hypothesis of irreversible binding.

Table 3. NPY Y_1 receptor binding data of [^{3}H]38.

Saturation binding		Binding kinetics (SK-N-MC cells)		
K_d [nM][a]	K_d [nM][b]	k_{on} [$min^{-1} \cdot nM^{-1}$][c]	k_{off} [min^{-1}][d] $t_{1/2}$ [min]	K_d(kin) [nM][e]
0.044 ± 0.003	0.14 ± 0.017	0.28 ± 0.026	0.0074 ± 0.0007 95 ± 8	0.026 ± 0.005

[a]Equilibrium dissociation constant determined at SK-N-MC neuroblastoma cells; mean ± SEM from 10 independent experiments (performed in triplicate). [b]Equilibrium dissociation constant determined at MCF-7-Y_1 cells; mean ± SEM from 10 independent experiments (performed in triplicate). [c]Association rate constant ± propagated error, calculated from k_{obs} (0.092 ± 0.0072 min^{-1}, mean ± SEM from 3 independent experiments (performed in triplicate)), k_{off} (0.0074 ± 0.0007 min^{-1}) and the applied radioligand concentration (0.3 nM). [d]Dissociation rate constant and derived half-life; mean ± SEM from 4 independent experiments (performed in triplicate). [e]Kinetically derived dissociation constant ± propagated error ($K_d(kin) = k_{off}/k_{on}$).

In competition binding experiments with [^{3}H]**38**, the K_i values of compounds **1a**, **6**, **33**, the fluorescently labeled argininamide **40** and pNPY were determined at SK-N-MC cells (Figure 10, Table 1). The K_i value of 1.0 nM obtained for the parent compound **1a** was in excellent agreement with previously determined K_i values using the radioligands [^{3}H]**2**, [^{3}H]**4** and [^{3}H]propionyl-pNPY (*cf.* Table 1). The K_i value of 0.077 nM determined for **38** is in good accordance with the K_d value of [^{3}H]**38** (Table 1). The fluorescently labeled compound **40** was incapable of displacing [^{3}H]**38** (K_i > 1,000 nM), which, at first sight, appears consistent with the aforementioned decrease in Y_1R affinity by the introduction of bulky moieties in the N^{ω}-

substituent (compound **41**, *cf*. Figure 5 and Table 1). However, the length of the spacer and the chemical nature of both the spacer and the fluorophore, are critical for Y_1R affinity as becomes obvious from previously reported structure-activity relationships of fluorescent ligands derived from **1a**.[15,32]

Competition binding experiments at the Y_1R with [^{3}H]**38** and pNPY resulted in a complete displacement of the labeled antagonist (Figure 10B) affording a K_i value of pNPY in the expected range (0.41 nM). This is in contrast to a recently reported competition binding experiment at the Y_2R with an insurmountable radiolabeled argininamide-type Y_2R antagonist and pNPY, where the displacement curve was biphasic and dramatically shifted to approximately 1000-fold higher pNPY concentrations than expected from the K_d value of the peptide.[23] The fact that a pre-incubation of the Y_1 receptor with [^{3}H]**38** prior to the addition of the competitor pNPY did not affect the K_i value of the peptide (Figure 10B) further support fully reversible binding of argininamide-type Y_1R antagonists such as **5-7**, **33** and **38** to the Y_1 receptor.

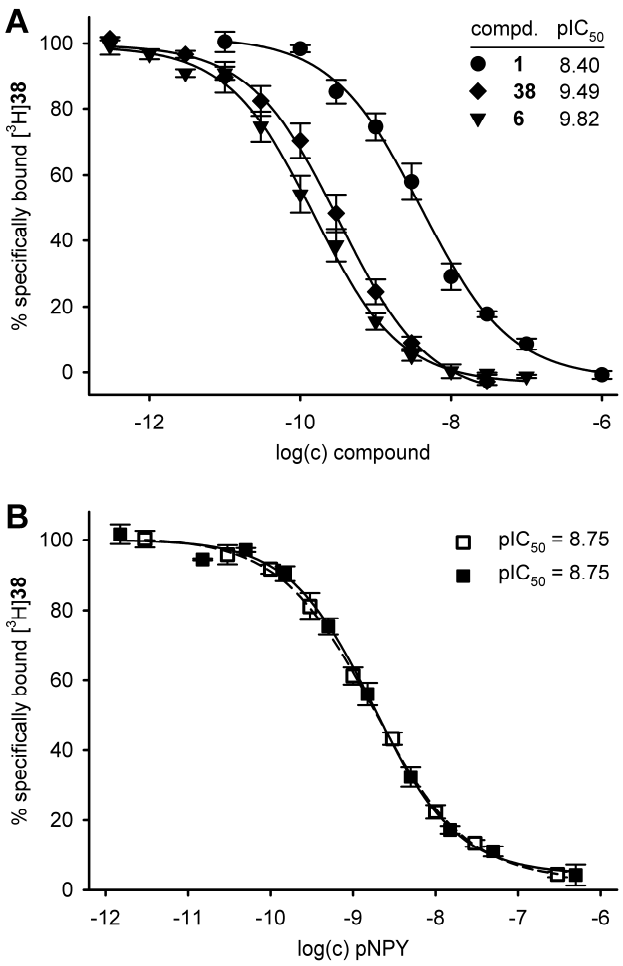


Figure 10. Competition binding experiments performed with [^{3}H]**38** at SK-N-MC cells. A: Displacement of [^{3}H]**38** (K_d = 0.044 nM, c = 0.15 nM) by argininamides **1a**, **6** and **38**. Mean values ± SEM from at least three independent experiments (performed in triplicate). B: Displacement of [^{3}H]**38** by pNPY. The peptide was either added immediately prior to the radioligand (open squares, dashed line) or 60 min after pre-incubation of the cells with the radioligand (filled squares, solid line). The affinity of pNPY was not affected by pre-incubation of the cells with [^{3}H]**38**. Mean values ± SEM from at least four independent experiments (performed in triplicate).

Conclusion

Unlike the parent compound, argininamide **1a**, N^{ω}-carbamoylated argininamide-type Y_1R antagonists caused a concentration- and time-dependent depression of the concentration-response curve of pNPY, indicative of insurmountable antagonism, in the Fura-2 Ca^{2+} assay. In contrast to

the functional assay, radioligand binding studies using the tritiated agonist [^{3}H]propionyl-pNPY gave no hint to irreversible Y_1R binding. Inversely, the radiolabeled form of such an antagonist, [^{3}H]**38**, proved to be completely displaceable by pNPY (K_i = 0.41 nM) under equilibrium conditions, irrespective of pre-incubation of the cells with [^{3}H]**38**, suggesting the peptidic agonist and the nonpeptide antagonist to compete for overlapping binding sites. With a K_d of 0.044 nM (SK-N-MC cells), [^{3}H]**38** proved to be by far superior to the previously reported higher homolog [^{3}H]**4** (K_d = 2.0 nM),[12] bearing a tetramethylene instead of an ethylene spacer. Most interestingly, [^{3}H]**38** exhibited a strongly increased target residence time (half-life of 95 min) compared to [^{3}H]**4** ($t_{1/2}$ = 3 min). As Y_1R binding was fully reversible, the strong depression of the concentration-response curves in the functional assay, suggesting insurmountable antagonism, has to be attributed to the slow dissociation of [^{3}H]**38**. With respect to affinity, NPY receptor subtype selectivity, and specificity with respect to NPFF receptors, this new radioligand represents an outstanding molecular tool for the study of the Y_1R. In combination with related argininamide-type antagonists, [^{3}H]**38** may be useful to gain deeper insight into the structural determinants of increased drug-target residence time, a generally important issue for lead optimization today. The presented high affinity Y_1R antagonists will be studied in more detail, e.g., in binding studies at receptor mutants to explore the binding mode, and harbor the potential of being used for the crystallization of receptor-ligand complexes.

Experimental Section

General experimental conditions. Solvents and reagents (analytical grade), isocyanates **8** and **11** (Sigma-Aldrich Chemie, Munich, Germany), isocyanate **12** (ABCR, Karlsruhe, Germany), 1,4-diaminobenzene (Sigma-Aldrich), propargylbromide (Sigma-Aldrich) and amine **34** (ABCR) were purchased from commercial suppliers and used without further purification. Technical grade solvents (EtOAc), light petroleum (40-60 °C) and dichloromethane) were distilled before use. Acetonitrile for HPLC (gradient grade) was from Merck (Darmstadt, Germany). Compounds **9**,[33] **15**,[11] **20**,[34] **18**,[35] **19**,[36] **21**[37] and **37**[11] were prepared according to published procedures. Porcine NPY (pNPY) and human pancreatic polypeptide (hPP) were a gift from Prof. Dr. C. Cabrele (University Salzburg, Salzburg, Austria). The syntheses of the fluorescently labeled peptides Cy5-pNPY[18] and Cy5-[K^4]hPP[29] were described elsewhere. [^{3}H]propionyl-pNPY (specific activity: 27.4 Ci/mmol) was prepared in house by labeling of pNPY with commercially available succinimidyl [2,3-^{3}H]propionate (see Supporting Information). Millipore water was used throughout for the preparation of buffers and HPLC eluents. 1.5- or 2-mL polypropylene reaction vessels with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the preparation and storage of stock solutions, for the synthesis of the radioligand [^{3}H]**38** and the fluorescently labeled compound **40**, and for the investigation of chemical stabilities. Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates. For column chromatography silica gel Geduran 60 (0.063-0.200 µm, Merck) or Silica Gel 60 (40-63 µm, Merck) (flash chromatography) was used. In case of the purification of acid sensitive compounds (e.g. Boc protection group) the stationary phase was conditioned with the respective solvent containing 0.5% triethylamine. The purity of final compounds was determined by RP-HPLC and

was ≥ 95% throughout. In addition, the purity of compounds used for detailed pharmacological studies was proven by elemental analysis (compounds **5-7**, **33**, **36** and **38**).

Melting points were determined with a Büchi 510 apparatus (Büchi, Essen, Germany) (compounds **10**, **13**, **14**, **35**) or with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK) (compounds **22**, **24**-**27**, **31**, **32**) and are uncorrected. Specific optical rotations at 589 nm (Na-D line) were measured on a Polarimeter P8000-T equipped with an electronic Peltier thermostat PT31 (A. KRÜSS Optronic, Hamburg, Germany) using a micro-cuvette (layer thickness: 100 mm, volume: 1 mL, thermostated at 20 °C) and acetonitrile/H_2O 70:30 (v/v) as solvent. IR spectra were measured on a NICOLET 380 FT-IR spectrophotometer (Thermo Scientific, Waltham, MA). NMR spectra were recorded on a Bruker Avance 300 instrument (7.05 T, ^{1}H: 300 MHz, ^{13}C: 75 MHz), a Bruker Avance 400 instrument (9.40 T, ^{1}H: 400 MHz, ^{13}C: 100 MHz) or a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H: 600 MHz, ^{13}C: 150 MHz) (Bruker, Karlsruhe, Germany). Elemental analysis was performed with a Vario MICRO Cube elemental analyzer (Elementar Analysensysteme, Hanau, Germany). Low-resolution mass spectrometry (MS) was performed on a Finnigan ThermoQuest TSQ 7000 instrument (Thermo Finnigan, San Jose, CA) equipped with an electrospray ionization (ESI) source and coupled to an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA). The following LC method was used: Column: Luna C18, 2.5 µm, 50 × 2 mm HST (Phenomenex, Aschaffenburg, Germany); column temperature: 40 °C; flow: 0.40 mL/min; solvent A: MeCN, solvent B: 0.1% aq formic acid; gradient program: 0–1 min: A/B 5:95, 1–8 min: 5:95–98:2, 8–11 min: 98:2, 11–12 min: 98:2–5:95, 12–15 min: 5:95. High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI source. Preparative HPLC

was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18, 5 µm, 250 × 21 mm (Macherey-Nagel, Dueren, Germany), a Kinetex XB C18, 5 µm, 250 × 21 mm (Phenomenex, Aschaffenburg, Germany) or a Puriflash C18HQ Flash Column, 15 µm, 120 g (Interchim, France) served as RP-columns at flow rates of 18, 20 and 50 mL/min, respectively. Mixtures of acetonitrile and 0.1% or 0.2% aq TFA were used as mobile phase and a detection wavelength of 220 nm was used throughout. Acetonitrile was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilisation (Christ alpha 2-4 LD lyophilisation apparatus (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) equipped with a Vacuubrand RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany)). Analytical HPLC analysis of compounds **5-7**, **17**, **28**, **29**, **33**, **36** and **38** (concentrations *ca* 50 µM) was performed with a system from Thermo Separation Products composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, Phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector. A Eurospher-100 C18, 5 µm, 250 × 4 mm (Knauer, Berlin, Germany) served as RP-column at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.05% aq TFA (B) were used as mobile phase. The following linear gradient was applied: 0–30 min: A/B 20:80–95:5, 30–40 min: 95:5. An oven temperature of 30 °C, an injection volume of 100 µL and a detection wavelength of 220 nm were used throughout. Analytical HPLC analysis of compounds **30** and **40** (concentration *ca* 100 and 70 µM, respectively) was performed on a system from Agilent Technologies composed of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity Autosampler, a 1290 Infinity Thermostated Column Compartment and a 1260 Infinity Diode Array Detector. A Kinetex XB C18, 2.5 µm, 100 × 3 mm (Phenomenex) served as stationary phase at a flow rate of 0.6 mL/min. Mixtures of acetonitrile (A) and 0.05% aq TFA

(B) were used as mobile phase. The following linear gradient was applied: 0–15 min: A/B 20:80–50:50, 15–18 min: 50:50–95:5, 18–23 min: 95:5. The oven temperature was 25 °C and the injection volume was 20 µL. Detection was performed at 220 nm and in case of **40** additionally at 500 nm.

Chemistry: Experimental protocols and analytical data.

Compounds 17 and 22-33

General procedure A. Azide **18** or **19** (2.0 mmol) and the Boc-protected alkyne derivative **20** or **21** (2.0 mmol) were dissolved in MeOH (5 mL). A solution of ascorbic acid (35 mg, 0.2 mmol) in water (0.5 mL) was added followed by the addition of $CuSO_4 \cdot 5\ H_2O$ (25 mg, 0.1 mmol) dissolved in water (0.5 mL). The mixture was stirred at 60 °C for 1 h. The Boc-protected intermediate was isolated by column chromatography and dissolved in MeOH/concentrated aq HCl 3:1 (v/v) (4 mL) (**22**, **24**) or in CH_2Cl_2/TFA 2:1 (v/v) (4 mL) (**23**). The mixture was stirred at rt for 1 h and the solvent was removed *in vacuo* to afford the product as hydrochloride or hydrotrifluoroacetate.

General procedure B. Triphosgene (116 mg, 0.39 mmol) was dissolved in 10 mL of CH_2Cl_2 (synthesis of **25-27**) or MeCN (synthesis of **32**) and the solution was cooled in an ice bath. Under vigorous stirring, a solution of the respective amine (**22-24** or **31**) (1.00 mmol) and DIPEA (396 mg, 3.07 mmol) in the same solvent was added dropwise over a period of 20 min. The mixture was warmed up to ambient temperature, a solution of compound **9** (200 mg, 1.00 mmol) in CH_2Cl_2 (10 mL) was slowly added over a period of 10 min, and stirring was continued at ambient temperature overnight. The product was purified by column chromatography.

General procedure C. The respective *S*-methylisothiourea derivative (**25-27** or **32**) (0.2 mmol), compound **15** (0.2 mmol) and $HgCl_2$ (0.2 mmol) were dissolved separately in small amounts of DMF (1-2 mL) and the solutions were combined under an atmosphere of nitrogen. Triethylamine (2.0 mmol) was added under stirring and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2. The precipitate of mercury salts was filtered off and the filtrate was concentrated. The Boc/*t*Bu-protected intermediate was isolated by column chromatography (eluent: light petroleum/EtOAc mixtures) and dissolved in CH_2Cl_2/TFA 1:1 (v/v) (5 mL). The mixture was stirred at rt for 2 h. The solvent was removed under reduced pressure, and the oily residue was repeatedly dissolved in CH_2Cl_2 followed by evaporation of the solvent. The product was purified by preparative HPLC except for compound **30**.

(*R*)-N^{ω}-Aminocarbonyl-N^{α}-diphenylacetyl-(4-hydroxybenzyl)argininamide hydrotrifluoroacetate (17). Azide **16** (6.2 mg, 7.5 μg) was dissolved in MeOH (5 mL) and a 10% Pd/C catalyst (5 mg) was added under an atmosphere of argon. The mixture was stirred under 1 bar of hydrogen pressure for 2 h. The catalyst was removed by filtration over Celite and the solvent was removed under reduced pressure. The product was purified by preparative HPLC and obtained as a white solid (6.0 mg, quantitative). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.34-1.49 (m, 3H), 1.60-1.75 (m, 1H), 3.13-3.24 (m, 2H), 4.06-4.22 (m, 2H), 4.28-4.38 (m, 1H), 4.42 (d, 2H, *J* 5.3 Hz), 5.12 (s, 1H), 6.64-6.73 (m, 4H), 6.96-7.03 (m, 2H), 7.15-7.32 (m, 11H), 7.41-7.48 (m, 2H), 8.08 (br s, 1H), 8.30-8.48 (m, 4H), 8.50 (d, 1H, *J* 8.2 Hz), 8.88 (br s, 1H), 9.29 (br s, 1H), 9.62 (br s, 1H). RP-HPLC (220 nm): 95% (t_R = 12.5 min, *k* = 3.6). MS (LC-MS,

ESI, t_R = 5.27 min): *m/z* (%) 689 (100) $[M+H]^+$, 386 (50), 345.5 (80) $[M+2H]^{2+}$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{41}N_{10}O_4]^+$ 689.3307, found: 689.3312. $C_{37}H_{40}N_{10}O_4 \cdot C_2HF_3O_2$ (688.78 + 114.02).

(1-Phenyl-1*H*-1,2,3-triazol-4-yl)methanamine hydrochloride (22).[21] The compound was prepared from **18** and **20** according to general procedure A and obtained as white solid (362 mg, 86%) mp 244 °C. ^{1}H-NMR (300 MHz, D_2O): δ (ppm) 4.40 (s, 2H), 7.50-7.65 (m, 3H), 7.65-7.80 (m, 2H), 8.52 (s, 1H). ^{13}C-NMR (75 MHz, D_2O): δ (ppm) 34.0, 121.0, 123.8, 129.7, 129.9, 136.0, 140.3. MS (LC-MS, ESI, t_R = 0.88 min): *m/z* (%) 349 (30) $[2M+H]^+$, 175 (100) $[M+H]^+$. $C_9H_{10}N_4 \cdot HCl$ (174.20 + 36.46).

[1-(3-Azidophenyl)-1*H*-1,2,3-triazol-4-yl]methanamine hydrotrifluoroacetate (23). The compound was prepared from **19** and **20** according to general procedure A and obtained as yellow oil (250 mg, 38%). ^{1}H-NMR (300 MHz, D_2O): δ (ppm) 4.27 (s, 2H), 7.00-7.10 (m, 1H), 7.20-7.26 (m, 1H), 7.30-7.45 (m, 2H), 8.38 (s, 1H). ^{13}C-NMR (75 MHz, D_2O): δ (ppm) 33.8, 110.5, 116.1, 118.9, 122.9, 130.8, 136.8, 140.4, 141.1. MS (LC-MS, ESI, t_R = 2.93 min): *m/z* (%) 431 (100) $[2M+H]^+$, 216 (100) $[M+H]^+$. $C_9H_9N_7 \cdot C_2HF_3O_2$ (215.21 + 114.02).

***N*-Methyl-1-(1-phenyl-1*H*-1,2,3-triazol-4-yl)methanamine hydrochloride (24).** The compound was prepared from **18** and **21** according to general procedure A and obtained as white solid (364 mg, 81%) mp 228 °C. ^{1}H-NMR (300 MHz, D_2O): δ (ppm) 2.73 (s, 3H), 4.38 (s, 2H), 7.40-7.58 (m, 3H), 7.60-7.70 (m, 2H), 8.50 (s, 1H). ^{13}C-NMR (75 MHz, D_2O): δ (ppm) 32.1,

42.6, 121.1, 124.8, 129.8, 130.0, 136.0, 138.5. MS (LC-MS, ESI, t_R = 1.04 min): *m/z* (%) 377 (35) $[2M+H]^+$, 189 (100) $[M+H]^+$. $C_{10}H_{12}N_4 \cdot HCl$ (188.23 + 36.46).

***N-tert*-Butoxycarbonyl-*N'*-[(1-phenyl-1*H*-1,2.3-triazol-4-yl)methyl]aminocarbonyl-*S*-methylisothiourea (25).** The compound was prepared from **9** and **22** according to general procedure B. Column chromatography: eluent: light petroleum/EtOAc 3:1 to 2:3 (R_f = 0.3 for light petroleum/EtOAc 3:2). The product was obtained as white solid (308 mg, 79%) mp 157 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.48 (s, 9H), 2.30 (s, 3H), 4.55-4.65 (d, 2H, *J* 6.2 Hz), 6.24 (s, 1H), 7.35-7.60 (m, 3H), 7.60-7.80 (m, 2H), 7.80 (s, 1H), 12.21 (br s, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 14.4, 28.0, 35.6, 82.8, 120.4, 120.6, 128.8, 129.8, 137.0, 145.6, 160.0, 161.8, 168.3. MS (LC-MS, ESI, t_R = 7.51 min): *m/z* (%) 781 (60) $[2M+H]^+$, 391 (100) $[M+H]^+$. $C_{17}H_{22}N_6O_3S$ (390.46).

***N-tert*-Butoxycarbonyl-*N'*-{[1-(3-azidophenyl)-1*H*-1,2.3-triazol-4-yl]methyl}aminocarbonyl-*S*-methylisothiourea (26).** The compound was prepared from **9** and **23** according to general procedure B. Column chromatography: eluent: light petroleum/EtOAc 3:1 to 2:3 (R_f = 0.3 for light petroleum/EtOAc 3:2). The product was obtained as white solid (164 mg, 38%) mp 128 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.48 (s, 9H), 2.30 (s, 3H), 4.55-4.65 (d, 2H, *J* 6.2 Hz), 6.24 (s, 1H), 7.00-7.10 (m, 1H), 7.20-7.26 (m, 1H), 7.30-7.45 (m, 2H), 7.70 (s, 1H), 12.18 (br s, 1H). MS (LC-MS, ESI, t_R = 7.88 min): *m/z* (%) 863 (60) $[2M+H]^+$, 432 (100) $[M+H]^+$. $C_{17}H_{21}N_9O_3S$ (431.47).

***N-tert*-Butoxycarbonyl-*N'*-[(1-phenyl-1*H*-1,2,3-triazol-4-yl)methyl]methylaminocarbonyl-*S*-methylisothiourea (27).** The compound was prepared from **9** and **24** according to general procedure B. Column chromatography: eluent: light petroleum/EtOAc 3:1 to 2:3 (R_f = 0.3 for light petroleum/EtOAc 3:2). The product was obtained as white solid (144 mg, 25%) mp 145 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.49 (s, 9H), 2.33 (d, 3H, *J* 4.8 Hz), 3.00-3.30 (m, 3H), 4.65-5.30 (m, 2H), 7.35-7.60 (m, 3H), 7.65-7.75 (m, 2H), 7.75-8.05 (m, 1H), 12.30-12.55 (m, 1H). MS (LC-MS, ESI, t_R = 7.95 min): *m/z* (%) 809 (20) $[2M+H]^+$, 405 (100) $[M+H]^+$. $C_{18}H_{24}N_6O_3S$ (404.49).

(*R*)-N^{α}-Diphenylacetyl-N^{ω}-[(1-phenyltriazol-4-yl)methyl]aminocarbonyl-(4-hydroxybenzyl)argininamide hydrotrifluoroacetate (28). The compound was prepared from **15** and **25** according to general procedure C and obtained as white solid (90 mg, 57%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.34-1.61 (m, 3H), 1.61-1.77 (m, 1H), 3.12-3.28 (m, 2H), 4.05-4.23 (m, 2H), 4.28-4.39 (m, 1H), 4.47 (d, 2H, *J* 5.3 Hz), 5.12 (s, 1H), 6.64-6.71 (m, 2H), 6.96-7.03 (m, 2H), 7.14-7.33 (m, 10H), 7.45-7.53 (m, 1H), 7.55-7.64 (m, 2H), 7.84-7.92 (m, 2H), 8.11 (br s, 1H), 8.39 (t, 1H, *J* 5.8 Hz), 8.41-8.60 (m, 3H), 8.70 (s, 1H), 8.96 (br s, 1H), 9.27 (br s, 1H), 10.08 (br s, 1H). RP-HPLC (220 nm): 99% (t_R = 16.0 min, *k* = 4.9). MS (LC-MS, ESI, t_R = 5.63 min): *m/z* (%) 674 (100) $[M+H]^+$, 337.5 (10) $[M+2H]^{2+}$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{40}N_9O_4]^+$ 674.3198, found: 674.3201. $C_{37}H_{39}N_9O_4 \cdot C_2HF_3O_2$ (673.76 + 114.02).

(*R*)-N^{ω}-{[1-(3-Azidophenyl)triazol-4-yl]methyl}aminocarbonyl-N^{α}-diphenylacetyl-(4-hydroxybenzyl)argininamide hydrotrifluoroacetate (29). The compound was prepared from **15** and **26** according to general procedure C and obtained as white solid (166 mg, 21%). ^{1}H-

NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.32-1.60 (m, 3H), 1.60-1.76 (m, 1H), 3.11-3.26 (m, 2H), 4.05-4.22 (m, 2H), 4.28-4.39 (m, 1H), 4.47 (d, 2H, *J* 5.3 Hz), 5.12 (s, 1H), 6.63-6.70 (m, 2H), 6.96-7.03 (m, 2H), 7.15-7.33 (m, 11H), 7.58-7.66 (m, 2H), 7.69-7.76 (m, 1H), 8.11 (br s, 1H), 8.38 (t, 1H, *J* 5.8 Hz), 8.46 (br s, 2H), 8.50 (d, 1H, *J* 8.1 Hz), 8.79 (s, 1H), 8.91 (br s, 1H), 9.30 (br s, 1H), 9.91 (br s, 1H). RP-HPLC (220 nm): 99% (t_R = 17.5 min, *k* = 5.5). MS (LC-MS, ESI, t_R = 5.78 min): *m/z* (%) 715 (100) $[M+H]^+$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{39}N_{12}O_4]^+$ 715.3212, found: 715.3217. $C_{37}H_{38}N_{12}O_4 \cdot C_2HF_3O_2$ (714.78 + 114.02).

(*R*)-N^{α}-Diphenylacetyl-N^{ω}-[(1-phenyltriazol-4-yl)methyl]methylaminocarbonyl-(4-hydroxybenzyl)argininamide hydrotrifluoroacetate (30). The compound was prepared from **15** and **27** according to general procedure C and obtained as white solid (160 mg, 72%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.34-1.62 (m, 3H), 1.62-1.78 (m, 1H), 3.06 (s, 3H), 3.18-3.30 (m, 2H), 4.06-4.23 (m, 2H), 4.29-4.40 (m, 1H), 4.69 (s, 2H), 5.12 (s, 1H), 6.64-6.70 (m, 2H), 6.96-7.04 (m, 2H), 7.15-7.33 (m, 10H), 7.46-7.54 (m, 1H), 7.56-7.65 (m, 2H), 7.85-7.93 (m, 2H), 8.38 (t, 1H, *J* 5.8 Hz), 8.50 (d, 1H, *J* 8.1 Hz), 8.58 (br s, 2H), 8.77 (s, 1H), 8.97 (br s, 1H), 9.31 (br s, 1H), 9.78 (br s, 1H). RP-HPLC (220 nm): 96% (t_R = 12.5 min, *k* = 15.4). MS (LC-MS, ESI, t_R = 5.70 min): *m/z* (%) 688 (100) $[M+H]^+$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{38}H_{42}N_9O_4]^+$ 688.3354, found: 688.3362. $C_{38}H_{41}N_9O_4 \cdot C_2HF_3O_2$ (687.79 + 114.02).

(1-Methyl-1*H*-1,2,3-triazol-4-yl)methanamine hydrochloride (31).[22] NaN_3 (714 mg, 11.0 mmol) was dissolved in water (2 mL) and diethyl ether (4 mL) was added followed by the addition of tetrabutylammonium tetrafluoroborate (phase-transfer catalyst) (90 mg, 0.27 mmol) and methyl iodide (780 mg, 5.49 mmol). The mixture was stirred vigorously at rt for 2 d. The

organic phase was separated and the aqueous phase washed with diethyl ether (2 × 2 mL). The extracts were combined and a solution of alkyne **20** (510 mg, 3.29 mmol) in MeOH (3 mL) was added followed by the addition of ascorbic acid (95 mg, 0.54 mmol) dissolved in water (0.8 mL) and $CuSO_4 \cdot 5\ H_2O$ (68 mg, 0.27 mmol) dissolved in water (0.8 mL). The mixture was stirred vigorously at rt overnight. The solvent was evaporated, the residue dissolved in 1 mL of $CHCl_3$/EtOH 19:1 (v/v), and the solution subjected to column chromatography (eluent: $CHCl_3$/EtOH 19:1 to 9:1, R_f = 0.3 for $CHCl_3$/EtOH 9:1) to afford the Boc-protected intermediate as a white solid, which was dissolved in MeOH/concentrated aq HCl 3:1 (4 mL). The mixture was stirred at rt for 30 min and the solvent was removed *in vacuo* to yield the product as pale yellow solid (352 mg, 72%) mp 172 °C. ^{1}H-NMR (300 MHz, D_2O): δ (ppm) 4.10 (s, 3H), 4.30 (s, 2H), 8.02 (s, 1H). ^{13}C-NMR (75 MHz, D_2O): δ (ppm) 34.0, 36.7, 126.2. MS (LC-MS, ESI, t_R = 0.32 min): *m/z* (%) 225 (100) $[2M+H]^+$, 113 (60) $[M+H]^+$. $C_4H_8N_4 \cdot HCl$ (112.13 + 36.46).

***N-tert*-Butoxycarbonyl-*N'*-[(1-methyl-1*H*-1,2,3-triazol-4-yl)methyl]aminocarbonyl-*S*-methylisothiourea (32).** The compound was prepared from **9** and **31** according to general procedure B. Column chromatography: eluent: EtOAc (R_f = 0.26). The product was obtained as white solid (735 mg, 78%) mp 138 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.49 (s, 9H), 2.33 (s, 3H), 4.08 (s, 3H), 4.40-4.80 (m, 2H), 6.40 (br s, 1H), 7.58 (br s, 1H), 12.28 (br s, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 14.3, 28.0, 35.5, 36.7, 82.7, 123.0, 145.1, 151.0, 161.8, 168.0. MS (LC-MS, ESI, t_R = 6.27 min): *m/z* (%) 657 (100) $[2M+H]^+$, 328 (75) $[M+H]^+$. $C_{12}H_{20}N_6O_3S$ (328.39).

(*R*)-N^{α}-Diphenylacetyl-N^{ω}-[(1-methyltriazol-4-yl)methyl]aminocarbonyl-(4-hydroxybenzyl)argininamide dihydrate hydrotrifluoroacetate (33). The compound was prepared from **15** and **32** according to general procedure C and obtained as white solid (80 mg, 55%). Anal. calcd. for $C_{32}H_{37}N_9O_4 \cdot C_2HF_3O_2 \cdot H_4O_2$: C 53.61, H 5.56, N 16.55; found: C 53.77, H 5.37, N 16.32. $[\alpha]_D^{20}$ 10.5 (*c* 1.1, MeCN/H_2O 7:3 v/v). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ (ppm) 1.35-1.50 (m, 2H), 1.50-1.58 (m, 1H), 1.64-1.72 (m, 1H), 3.14-3.25 (m, 2H), 4.01 (s, 3H), 4.09-4.20 (m, 2H), 4.31-4.38 (m, 3H), 5.12 (s, 1H), 6.65-6.69 (m, 2H), 6.98-7.02 (m, 2H), 7.19-7.25 (m, 2H), 7.26-7.32 (m, 8H), 7.93 (s, 1H), 7.99 (br s, 1H), 8.36 (t, 1H, *J* 5.9 Hz), 8.45 (br s, 2H), 8.48 (d, 1H, *J* 8.1 Hz), 8.93 (br s, 1H), 9.30 (br s, 1H), 9.99 (br s, 1H). ^{13}C-NMR (150 MHz, [D_6]DMSO): δ (ppm) 24.5, 29.4, 34.8, 36.2, 40.4, 41.6, 52.3, 55.9, 115.0, 115.6 (TFA), 117.6 (TFA), 123.7, 126.57, 126.59, 128.16, 128.19, 128.4, 128.48, 128.51, 129.1, 140.3, 140.4, 144.0, 153.5, 153.6, 156.3, 158.4 (q, *J* 33.2 Hz) (TFA), 170.95, 171.01. RP-HPLC (220 nm): 98% (t_R = 12.5 min, *k* = 3.6). MS (LC-MS, ESI, t_R = 5.15 min): *m/z* (%) 1223 (5) $[2M+H]^+$, 612 (100) $[M+H]^+$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{32}H_{38}N_9O_4]^+$ 612.3041, found: 612.3043. $C_{32}H_{37}N_9O_4 \cdot C_2HF_3O_2 \cdot H_4O_2$ (611.69 + 114.02 + 36.03).

Compounds 35, 36, 38 and 40

***N*-*tert*-Butoxycarbonyl-*N'*-[2-(*tert*-butoxycarbonylamino)ethyl]aminocarbonyl-*S*-methylisothiourea (35).**[23] The reaction was carried out under argon in a two-necked round bottom flask equipped with a pressure equalizing dropping funnel. Under cooling with water a solution of amine **34** (3.0 g, 18.7 mmol) and DIPEA (7.26 g, 56.2 mmol) in anhydrous CH_2Cl_2 (35 mL) was added dropwise to a solution of triphosgene (2.78 g, 9.36 mmol) in anhydrous CH_2Cl_2 (25 mL) over a period of 30 min. *N*-Boc-*S*-methylisothiourea (**7**) (3.92 g, 20.6 mmol)

was added and stirring was continued at rt for 1 h. The volatiles were removed under reduced pressure and the residue was subjected to column chromatography (eluent: CH_2Cl_2/EtOAc 50:1 to 3:1). The eluate was evaporated under reduced pressure, CH_2Cl_2 (50 mL) was added, the solution evaporated, and this process repeated. Product **35** was obtained as colorless oil, which crystallized during storage at -20 °C to give a white crystalline solid (4.64 g, 66%) mp 136-138 °C. R_f = 0.4 (CH_2Cl_2/EtOAc 5:1). ^{1}H-NMR (400 MHz, $CDCl_3$) (major conformer): δ (ppm) 1.40 (s, 9H), 1.45 (s, 9H), 2.30 (s, 3H), 3.20-3.35 (m, 4H), 4.95 (br s, 1H), 6.14 (br s, 1H), 12.26 (br s, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$) (major conformer): δ (ppm) 14.5, 28.1, 28.4, 40.4, 40.8, 79.6, 83.0, 151.0, 156.5, 161.9, 168.2. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{15}H_{29}N_4O_5S]^+$ 377.1853, found: 377.1856. $C_{15}H_{28}N_4O_5S$ (376.47).

(*R*)-N^{ω}-(2-Aminoethyl)aminocarbonyl-N^{α}-diphenylacetyl-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) dihydrate (36). $HgCl_2$ (452 mg, 1.67 mmol) and DIPEA (359 mg, 2.78 mmol) were added to a stirred solution of amine **15** (542 mg, 1.11 mmol) and compound **35** (460 mg, 1.22 mmol) in CH_2Cl_2 (10 mL), and stirring was continued at rt for 1.5 h. Solid material was removed by vacuum filtration. The solvent was evaporated and the residue subjected to column chromatography (eluent: CH_2Cl_2/EtOAc 3:1 to 1:1) to isolate the Boc/*t*Bu-protected intermediate as colorless glass (R_f = 0.3 for CH_2Cl_2/EtOAc 2:1), which was dissolved in CH_2Cl_2/TFA/H_2O 10:10:1 (5 mL). The mixture was stirred at rt for 2 h. CH_2Cl_2 (30 mL) was added, the volatiles were evaporated and the process repeated. The product was purified by preparative HPLC (column: Puriflash C18HQ 120 g; gradient: 0–30 min: MeCN/0.2% aq TFA 14:86–48:52, t_R = 15 min). Lyophilisation of the eluate afforded product **36** as white fluffy solid (595 mg, 65%). Anal. calcd. for $C_{30}H_{37}N_7O_4 \cdot C_4H_2F_6O_4 \cdot H_4O_2$: C 49.57, H 5.25, N 11.90;

found: C 49.70, H 5.12, N 11.80. IR (KBr) 3300, 3085, 2950, 1675, 1540, 1515, 1205, 1140 cm^{-1}. ^{1}H-NMR (600 MHz, [D_6]DMSO): δ (ppm) 1.36-1.51 (m, 2H), 1.51-1.59 (m, 1H), 1.64-1.73 (m, 1H), 2.88-2.97 (m, 2H), 3.17-3.26 (m, 2H), 3.32-3.40 (m, 2H), 4.09-4.20 (m, 2H), 4.31-4.37 (m, 1H), 5.13 (s, 1H), 6.65-6.70 (m, 2H), 6.98-7.02 (m, 2H), 7.19-7.25 (m, 2H), 7.26-7.32 (m, 8H), 7.61 (br s, 1H), 7.89 (br s, 3H), 8.36 (t, 1H, *J* 5.8 Hz), 8.49 (d, 1H, *J* 8.1 Hz), *ca* 8.52 (br s, 2H), 9.05 (br s, 1H), 9.34 (br s, 1H), 10.82 (br s, 1H). ^{13}C-NMR (150 MHz, [D_6]DMSO): δ (ppm) 24.6, 29.4, 37.2, 38.5, 40.4, 41.6, 52.3, 55.9, 115.0, 116.9 (q, *J* 298 Hz) (TFA), 126.58, 126.61, 128.17, 128.21, 128.4, 128.50, 128.52, 129.1, 140.3, 140.5, 153.7, 154.4, 156.3, 159.0 (q, *J* 32.4 Hz) (TFA), 170.98, 171.05. RP-HPLC (220 nm): 99% (t_R = 10.1 min, k = 2.7). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{30}H_{38}N_7O_4]^+$ 560.2980, found: 560.2980. $C_{30}H_{37}N_7O_4 \cdot C_4H_2F_6O_4 \cdot H_4O_2$ (559.66 + 228.03 + 36.03).

(*R*)-N^{α}-Diphenylacetyl-N^{ω}-(propionylaminoethyl)aminocarbonyl-(4-hydroxybenzyl)argininamide dihydrate hydrotrifluoroacetate (38). Succinimidyl propionate (**37**) (49 mg, 0.29 mmol) and DIPEA (111 mg, 0.86 mmol) were added to a solution of compound **36** (225 mg, 0.27 mmol) in anhydrous DMF (1.8 mL). The mixture was stirred at rt for 1.5 h and acidified by addition of 10% aq TFA (430 µL). The product was purified by preparative HPLC (column: Puriflash C18HQ 120 g, gradient: 0–30 min: MeCN/0.2% aq TFA 19:81–57:43, t_R = 14.5 min). Lyophilisation of the eluate afforded **38** as white fluffy solid (196 mg, 83%). Anal. calcd. for $C_{33}H_{41}N_7O_5 \cdot C_2HF_3O_2 \cdot H_4O_2$: C 54.90, H 6.05, N 12.80; found: C 54.76, H 5.72, N 12.47. IR (KBr) 3300, 3090, 3030, 2940, 1725, 1650, 1540, 1520, 1205, 1140 cm^{-1}. $[\alpha]_D^{20}$ 10.0 (*c* 1.4, MeCN/H_2O 7:3 v/v). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ (ppm) 0.99 (t, 3H, *J* 7.6 Hz), 1.36-1.50 (m, 2H), 1.51-1.58 (m, 1H), 1.64-1.72 (m, 1H), 2.07 (t, 2H, *J* 7.6 Hz),

3.12-3.18 (m, 4H), 3.18-3.23 (m, 2H), 4.09-4.20 (m, 2H), 4.30-4.37 (m, 1H), 5.13 (s, 1H), 6.65-6.69 (m, 2H), 6.98-7.02 (m, 2H), 7.19-7.25 (m, 2H), 7.26-7.31 (m, 8H), 7.50 (br s, 1H), 7.86 (br s, 1H), 8.36 (t, 1H, *J* 5.8 Hz), 8.42 (br s, 2H), 8.48 (d, 1H, *J* 8.2 Hz), 8.94 (br s, 1H), 9.31 (br s, 1H), 10.16 (br s, 1H). ^{13}C-NMR (150 MHz, [D$_6$]DMSO): δ (ppm) 9.8, 24.6, 28.5, 29.4, 38.1, 39.1, 40.3, 41.6, 52.3, 55.9, 115.0, 115.8 (TFA), 117.8 (TFA), 126.57, 126.61, 128.16, 128.20, 128.4, 128.49, 128.52, 129.1, 140.3, 140.5, 153.6, 153.8, 156.3, 158.8 (q, *J* 32.4 Hz) (TFA), 170.97, 171.02, 173.3. RP-HPLC (220 nm): 99% (t_R = 12.6 min, k = 3.7). HRMS (ESI): *m/z* [*M*+H]$^+$ calcd. for [$C_{33}H_{42}N_7O_5$]$^+$ 616.3242, found: 616.3242. $C_{33}H_{41}N_7O_5 \cdot C_2HF_3O_2 \cdot H_4O_2$ (615.72 + 114.02 + 36.03).

Preparation of (*R*)-N^{α}-diphenylacetyl-N^{ω}-[2-([2,3-^{3}H]propionylamino)ethyl]aminocarbonyl-(4-hydroxybenzyl)argininamide ([^{3}H]38). Amine **36** (0.5 mg, 607 nmol) was dissolved in DMF/DIPEA 30:1 (v/v) (60 µL) in a 1.5-mL reaction vessel with screw cap, a solution of succinimidyl [^{3}H]propionate (specific activity according to the supplier: 80 Ci/mmol, purchased from American Radiolabeled Chemicals (St. Louis, MO, USA) via Hartmann Analytics (Braunschweig, Germany)) (6 mCi, 12.8 µg, 75 nmol) in EtOAc (0.6 mL) was added and the volatile components were removed in a vacuum concentrator (about 45 min at *ca* 35 °C). DMF/DIPEA 10:1 (v/v) (10 µL) was added and the vessel was shaken at rt for 3 h. The mixture was acidified by the addition of 2% aq TFA (90 µL). Acetonitrile/0.05% aq TFA 20:80 (v/v) (60 µL) was added and an aliquot of 0.5 µL (diluted with 100 µL of mobile phase) was analyzed by RP-HPLC using a system from Waters (Eschborn, Germany) consisting of two pumps 510, a pump control module, a 486 UV/VIS detector, and a Flow-one beta series A-500 radiodetector (Packard, Meriden, USA). A Luna C18(2), 3 µm, 150 × 4.6 mm

(Phenomenex, Aschaffenburg, Germany) served as stationary phase. Mixtures of acetonitrile supplemented with 0.04% TFA (A) and 0.05% aq TFA (B) were used as mobile phase. The flow rate of the mobile phase and the liquid scintillator (acetonitrile/Rotiszint eco plus (Carl Roth, Karlsruhe, Germany) 15:85 v/v) was set to 0.8 and 4.0 mL/min, respectively. The following linear gradient was applied: 0–20 min: A/B 20:80–43:57, 20–22 min: 43:57–95:5, 22–32 min: 95:5. The retention time of the product was 17.4 min (UV detection, 220 nm) and 17.5 min (radiometric detection), respectively. [^{3}H]**38** was isolated using the same HPLC system and a YMC-Triart C18, 5 µm, 250 × 6.0 mm (YMC Europe, Dinslaken, Germany) at a flow rate of 1.4 mL/min (linear gradient: 0–20 min: A/B 20:80–47:53, 20–22 min: 47:53–95:5, 22–32 min: 95:5). Two HPLC runs (without radiometric detection) were performed and the product (t_R = 20.1 min) was collected in the same 2-mL reaction vessel with screw cap. The combined product fractions were concentrated to a volume of *ca* 100 µL in a vacuum concentrator, ethanol containing 200 µM TFA (900 µL) was added, and the solution was transferred into two 3-mL borosilicate glass vials with conical bottom (Wheaton NextGen 3-mL V-vials) (500 and 480 µL). The reaction vessel was rinsed twice with EtOH and the washings were transferred to the 3-mL glass vials to reach a volume of 1.5 mL in each vessel. For the quantification of [^{3}H]**38**, a four-point calibration was performed with **38** (0.3, 0.6, 1.0 and 1.5 µM; injection volume: 100 µL) using the Luna C18(2) (see above) as stationary phase at a flow rate of 0.8 mL/min (linear gradient: 0–20 min: A/B 20:80–49:51, 20–22 min: 49:51–95:5, 22–32 min: 95:5; UV detection: 220 nm). Two times an aliquot (3 µL) of the EtOH solution of [^{3}H]**38** (total volume = 1,500 µL) was added to 127 µL of acetonitrile/0.05% aq TFA 20:80 (v/v), 100 µL of this solution were analyzed by HPLC and five times 3 µL were counted in 3 mL of scintillator (Rotiszint eco plus) with a Beckman LS 6500 liquid scintillation counter (Beckmann-Coulter, Munich, Germany).

The molarity of the solution of [^{3}H]**38** in EtOH was calculated from the mean of the peak areas and the linear calibration curve obtained from the peak areas of the standards. To determine the radiochemical purity and to proof the identity, a 0.7 µM solution of [^{3}H]**38** (100 µL) spiked with **38** (20 µM) was analyzed by RP-HPLC using the column, flow rate, gradient, injection volume and UV detection as for the quantification, and additionally radiometric detection (radiochemical purity: 97%, *cf*. Figure 7B). This analysis was repeated after seven months of storage at −20 °C (*cf*. Figure 7C). Calculated specific activity: 1.81 TBq/mmol (48.9 Ci/mmol). The activity concentration was adjusted to 37 MBq/mL (1.0 mCi/mL) by addition of EtOH yielding a molarity of 20.4 µM (final total volume: 5.655 mL). Storage conditions: −20 °C. Yield (hydrotrifluoroacetate of [^{3}H]**38**): 84 µg, 115 nmol. Radiochemical yield: 5.66 mCi (209.2 MBq), 94%.

(*R*)-N^{α}-Diphenylacetyl-N^{ω}-(2-{*N*-[6-(6-{2-[7-(diethylamino)-2-oxo-2*H*-chromen-3-yl]vinyl}-3-sulfonato-pyridin-1-ium-1-yl)hexanoyl]amino}ethyl)aminocarbonyl-(4-hydroxybenzyl)argininamide hydrotrifluoroacetate (40). A solution of Fluorescence Red Mega 480 succinimidyl ester (**39**) (1 mg, 1.63 µmol) in anhydrous DMF (15 µL) was added to a solution of amine **36** (9.0 mg, 11.4 µmol) and DIPEA (5.9 mg, 45.7 µmol) in DMF (100 µL) in a 1.5-mL reaction vessel with screw cap and the vessel was shaken at rt in the dark for 30 min. 10% aqueous TFA (42 µL) was added and the product was purified by preparative HPLC (column: Kinetex 250 × 21 mm, gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–40:60, 25–40 min: 40:60–53:47, t_R = 31 min). Lyophilisation of the eluate yielded product **40** as a dark red solid (1.32 mg, 69%). RP-HPLC (220 and 500 nm): 99% (t_R = 11.2 min, k = 13.7). HRMS (ESI):

m/z $[M+H]^+$ calcd. for $[C_{56}H_{66}N_9O_{10}S]^+$ 1056.4648, found: 1056.4650. $C_{56}H_{65}N_9O_{10}S \cdot C_2HF_3O_2$ (1056.23 + 114.02).

Investigation of the chemical stability of compounds 5, 6, 33 and 38. The chemical stability of compounds **5**, **6**, **33** and **38** was investigated in PBS (pH 7.0) at 21 °C. Incubation was started by addition of a 2 mM solution of the compounds in DMSO/water 20:80 (v/v) (25 µL) to PBS (1:5 diluted with water) (475 µL) to give a final concentration of 100 µM. After 0, 24 and 48 h, as well as after 8 days in case of **6**, an aliquot (80 µL) was taken and diluted with acetonitrile/water/1% aq TFA 5:1:4 (v/v/v) (80 µL). An aliquot (100 µL) of the resulting solution (pH < 3) was analyzed by RP-HPLC using a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, Phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector) with a Gemini-NX C18, 5 µm, 250 × 4.6 mm (Phenomenex, Aschaffenburg, Germany) as stationary phase (flow rate: 1 mL/min). Mixtures of acetonitrile (A) and 0.05% aq TFA (B) were used as mobile phase. The following linear gradient was applied: 0–25 min: A/B 10:90–50:50, 25–33 min: 50:50–95:5, 33–40 min: 95:5. The oven temperature was 30 °C and the detection wavelength was set to 220 nm.

Fura-2 calcium assay (Y_1R). The Fura assay was performed with HEL cells as previously described using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany).[33,38] In time-dependent investigations the intracellular Ca^{2+} mobilization induced by 10 nM pNPY or by 300 nM pNPY was measured in the presence of increasing concentrations of antagonist and in the presence of one defined concentration of antagonist, respectively. The cells were incubated with the antagonist for different periods of time prior to the addition of the

agonist (the minimal pre-incubation period was 10 s). For the determination of pNPY concentration-effect curves in the presence of antagonist, and for the concentration-dependent inhibition of the maximal pNPY response (c = 300 nM), the cells were pre-incubated with the antagonist for 15 min.

Radioligand binding assay (Y_1R). All radioligand binding experiments were performed at 22 ± 1 °C. Radioligand competition binding experiments with the radioligands [^{3}H]**2**, [^{3}H]**4** and [^{3}H]propionyl-pNPY were performed at intact Y_1R expressing SK-N-MC neuroblastoma cells as previously described (the incubation period was 2 h in case of [^{3}H]propionyl-pNPY).[12] Nonspecific binding was determined in the presence of 500-fold excess of pNPY ([^{3}H]**2**, [^{3}H]**4**) or 500-fold excess of **1a** ([^{3}H]propionyl-pNPY). The K_d value of [^{3}H]propionyl-pNPY was determined by saturation binding at SK-N-MC cells and amounted to 1.9 ± 0.26 nM (two independent experiments, performed in triplicate). Saturation and competition binding experiments with [^{3}H]**38** at SK-N-MC cells were essentially performed as previously described for [^{3}H]**4**,[12] with the following modifications: Taking into account the very high affinity of [^{3}H]**38** for the NPY Y_1 receptor, the binding assay was performed in a manner ensuring that the free radioligand concentration was not markedly decreased by binding to the receptor. For this purpose, cells were cultured below 80% confluency and the volume per well was increased from 250 µL to 750 µL in case of saturation binding and to 500 µL for competition binding to decrease the ratio of receptor-bound to free radioligand.

Saturation binding. The cells were washed with 750 µL of buffer and covered with binding buffer (600 µL) followed by the addition of binding buffer (75 µL) and binding buffer (75 µL) containing the radioligand 10-fold concentrated (for the determination of total binding), or, to

determine nonspecific binding, binding buffer (75 µL) containing the competitor **1b** 10-fold concentrated (1,000-fold excess to [^{3}H]**38**) and binding buffer (75 µL) containing the radioligand 10-fold concentrated. The incubation period was 90 min. To determine K_d values, 'specifically bound radioligand' was plotted against the 'free radioligand concentration' (not the total radioligand concentration), to account for the decrease in radioligand concentration.

Competition binding. Cells were covered with 400 µL of binding buffer followed by the addition of two 50-µL aliquots of binding buffer (neat, containing the radioligand 10-fold concentrated, or containing the competitor 10-fold concentrated). Nonspecific binding was determined in the presence of **1b** (500-fold excess to [^{3}H]**38**) and the incubation period was 2 h (beginning with the addition of the radioligand).

The investigation of the binding kinetics of [^{3}H]**38** at SK-N-MC cells was performed as previously described for [^{3}H]**4** using pNPY (500-fold excess) to determine nonspecific binding.[12] For association experiments the radioligand concentration was 0.3 nM. In case of dissociation experiments cells were incubated with 1.0 nM [^{3}H]**38** for 60 min. The washing step after pre-incubation of the cells with radioligand was omitted, that is, the radioligand solution was removed by suction and the cells were directly covered with binding buffer (250 µL) containing **38** (200 nM).

Saturation binding experiments with [^{3}H]**38** at MCF-7-Y_1 cells[12] were performed in tissue culture treated white 96-well plates with clear bottom (Corning Inc. Life Sciences, Tewksbury, MA; Corning cat. no. 3610) using the washing buffer and binding buffer as described for [^{3}H]**4**.[12] Cells were seeded two days prior to the experiment. On the day of the experiment confluency of the cells was 50-70%. The culture medium was removed by suction, the cells were washed with buffer (200 µL) and covered with binding buffer (160 µL). For total binding, binding buffer (20

μL) and binding buffer (20 μL) containing the radioligand 10-fold concentrated were added. For the determination of nonspecific binding, binding buffer (20 μL) containing **1b** 10-fold concentrated (1,000-fold excess to [^{3}H]**38**) and binding buffer (20 μL) containing the radioligand 10-fold concentrated were added. The plates were gently shaken during incubation at 22 ± 1 °C for 90 min. After incubation the liquid was removed by suction, the cells were washed twice with ice-cold buffer (200 μL; washing period < 2 min) and lysis solution (urea (8 M), acetic acid (3 M) and Triton-X-100 (1%) in water) (25 μL) was added. The plates were shaken for at least 20 min, liquid scintillator (Optiphase Supermix, PerkinElmer, Rodgau, Germany) (200 μL) was added, and the plates were sealed with a transparent sealing tape (permanent seal for microplates, PerkinElmer, prod. no. 1450–461). The plates were turned up-side down several times in order to achieve complete mixing of scintillator and lysis solution. The samples were kept in the dark for at least 1 h prior to the measurement of radioactivity (dpm) with a MicroBeta2 plate counter (PerkinElmer).

Radioligand competition binding at human $NPFF_1$ and $NPFF_2$ receptors. Radioligand competition binding studies were performed with the radiolabeled peptides [^{3}H]NPVF[31] ($NPFF_1$ receptor) and [^{3}H]EYF[31] ($NPFF_2$ receptor) using membranes of CHO cells expressing the $hNPFF_1$ (CHO-$hNPFF_1$ cells)[30] or the $hNPFF_2$ receptor (CHO-$hNPFF_2$ cells)[30] as reported previously.[31] The K_d values of [^{3}H]NPVF and [^{3}H]EYF, determined under the same conditions by saturation binding, amounted to 2.8 and 6.9 nM, respectively. Determined binding data of the reference peptide NPFF ($hNPFF_1R$: K_i = 25 ± 7.8 nM, $hNPFF_2R$: K_i = 1.1 ± 0.15 nM (mean ± SEM from three independent experiments, performed in duplicate)) were in good agreement with reported data.[30-31]

Flow cytometric binding assays – general conditions. Flow cytometric binding assays were performed on a FACSCalibur™ flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm) (settings: FSC: E-1, SSC: 280 V, Fl-4: 700-800 V). Samples were incubated in 1.5-mL reaction vessels (Sarstedt, Nümbrecht, Germany) at 22 °C in the dark for 2 h. Measurements were stopped after counting of 10,000-15,000 gated events. Raw data were processed with the aid of the FlowJo Data Analysis Software version 10 (FLOWJO, Ashland, OR).

Flow cytometric binding assays (Y_2R and Y_5R). Flow cytometric binding assays on CHO-hY_2 and HEC-1B-hY_5 cells using Cy5-pNPY (K_d = 5.2 nM (Y_2R),[28] K_i = 4.4 nM (Y_5R)[18]) as labeled ligands were performed as described previously.[18,28] Two or three independent experiments were performed in duplicate. The cell density in loading buffer was $1 \cdot 10^6$ cells/mL (Y_2R) or $0.5 \cdot 10^6$ cells/mL (Y_5R).

Flow cytometric binding assay (Y_4R). The Y_4R competition binding assay on CHO-hY_4-Gq_{i5}-mtAEQ cells using Cy5-[K^4]hPP (K_d = 5.6 nM)[29] as labeled ligand was essentially performed as previously described with minor modifications.[32] Cy5-[K^4]-hPP (3 nM) was used instead of S0586-[K^4]hPP (10 nM). The cell density in loading buffer was $1 \cdot 10^6$ cells/mL. Nonspecific binding was determined by addition of hPP at a concentration of 1 µM. Two or three independent experiments were performed in duplicate.

Data processing. Specific optical rotation was calculated according to $[\alpha] = 100 \cdot \alpha/(c \cdot l)$, for which α = measured angle of rotation, c = concentration [g/100 mL], and l = length of the cuvette [dm]. Retention (capacity) factors k were calculated from retention times (t_R) according to $k = (t_R - t_0)/t_0$ (t_0 = dead time). Concentration-dependent inhibition data from the Fura-2 calcium assay

(% Ca^{2+} response induced by 10 nM (*cf.* Figure 2) and 300 nM (*cf.* Figure 3B) pNPY plotted against log(concentration antagonist)) were analyzed by four-parameter sigmoidal fits (SigmaPlot 11.0, Systat Software Inc., Chicago, IL) (Figure 2) and seven-parameter biphasic sigmoidal fits (GraphPad Prism Software 5.0, GraphPad Software, San Diego, CA) (Figure 3B), respectively, to obtain pIC_{50} values. Concentration-response curves of pNPY (*cf.* Figures 3A and S5) were obtained by analyzing the data with four-parameter sigmoidal fits (SigmaPlot 11.0). Time-dependent inhibition data from the Fura-2 assay (% Ca^{2+} response elicited by 300 nM pNPY in the presence of antagonist plotted against time) (*cf.* Figure 3C) were analyzed by a three-parameter equation describing a monophasic exponential decline (SigmaPlot 11.0). Specific binding data from radioligand competition binding experiments performed at SK-N-MC cells (Y_1R) were plotted as % (100% = bound radioligand in the absence of competitor) over log(concentration competitor) and analyzed by four-parameter sigmoidal fits (SigmaPlot 11.0). In case of radioligand competition binding experiments at $NPFF_1$ and $NPFF_2$ receptors, specific binding data were plotted as % over log(concentration competitor) and analyzed by four-parameter sigmoidal fits (GraphPad Prism 5.0) (constraints: slope factor = -1, upper curve plateau (top) = 100% and lower curve plateau (bottom) < 5). Resulting IC_{50} values were converted to K_i values according to the Cheng–Prusoff equation[39] using the K_d value of the respective radioligand ([^{3}H]**2**: K_d = 1.2 nM, [^{3}H]**4**: K_d = 2.0 nM, [^{3}H]**38**: K_d = 0.044 nM, [^{3}H]propionyl-pNPY: K_d = 1.9 nM, [^{3}H]NPVF: K_d = 2.76 nM, [^{3}H]EYF: K_d = 6.93 nM). Specific binding data (dpm) from saturation binding experiments were plotted against the free radioligand concentration and analyzed by an equation describing hyperbolic binding (ligand binding - one site saturation fit, SigmaPlot 11.0) to obtain K_d and B_{max} values. The free radioligand concentration (nM) was calculated by subtracting the amount of bound radioligand (nM)

(calculated from the specifically bound radioligand in dpm, the specific activity and the volume per well) from the total radioligand concentration. The number of (specific) binding sites per cell (sites/cell) was calculated according to the equation: sites/cell = $B_{max}/60/a_{spec}\cdot N_A/N_{cell}$, where B_{max} is the maximum number of binding sites per well in dpm (obtained from the ligand binding - one-site saturation fit), a_{spec} is the specific activity of the radioligand given in Bq mol^{-1}, N_A is the Avogadro constant in mol^{-1}, and N_{cell} is the number of cells per well. Specific binding data from radioligand association experiments were analyzed by a two-parameter equation describing an exponential rise to a maximum (SigmaPlot 11.0), and the resulting B_{eq} value (maximum of specifically bound radioligand) was used to calculate specifically bound radioligand (B) in % (*cf.* Figure 8A). The observed association rate constant k_{obs} was obtained by plotting $\ln(B_{eq}/[B_{eq}-B])$ against time and analysis by linear regression. Data from radioligand dissociation experiments (% specifically bound radioligand (B) plotted over time) were analyzed by a two-parameter equation describing a monophasic exponential decline (SigmaPlot 11.0). The dissociation rate constant k_{off} was obtained by plotting $\ln(B/B_0)$ (B_0 = initially bound radioligand) against time and analysis by linear regression. The association rate constant (k_{on}) was calculated from k_{obs}, k_{off} and the radioligand concentration ([RL]) according to the correlation: $k_{on} = (k_{obs}-k_{off})/[RL]$. Propagated errors were calculated according to the Gaussian law of errors. Statistical significance (comparison of two given K_i values) was assessed by a Welch two-sample t-test for unpaired samples.

ASSOCIATED CONTENT

Supporting Information. Synthesis protocols and analytical data of compounds **5-7**, **10**, **13** and **14**; preparation of [^{3}H]propionyl-pNPY; chemical stability of argininamides **5**, **6** and **33**;

concentration-response curves of pNPY in the presence of argininamide **16** (Figure S5); displacement curves of [^{3}H]NPVF and [^{3}H]EYF from competition binding experiments with **1a**, **6**, **33** and **38** at membranes of CHO-hNPFF$_1$ and CHO-hNPFF$_2$ cells, respectively (Figure S6); ^{1}H-NMR spectra of compounds **5-7**, **17**, **28-30**, **33**, **36** and **38**; ^{13}C-NMR spectra of compounds **5-7**, **33**, **36** and **38**; RP-HPLC chromatograms of compounds **5-7**, **17**, **28**-**30**, **33**, **36**, **38** and **40**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

br s, broad singlet, CH_2Cl_2, dichloromethane; DIPEA, diisopropylethylamine; dpm, disintegrations per minute; EtOAc, ethyl acetate; HEL cells, human erythroleukemia cells; hPP, human pancreatic polypeptide; *k*, retention (or capacity) factor (HPLC), K_d, dissociation (or binding) constant obtained from a saturation binding experiment; K_i, dissociation (or binding) constant obtained from a competition binding experiment; MeCN, acetonitrile; NPFF, neuropeptide FF; NPY, neuropeptide Y; pNPY, porcine NPY; RP-HPLC, reversed-phase HPLC; SEM, standard error of the mean; Y_1R, NPY Y_1 receptor

REFERENCES

(1) Brothers, S. P.; Wahlestedt, C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* **2010**, *2*, 429-439.

(2) Yulyaningsih, E.; Zhang, L.; Herzog, H.; Sainsbury, A. NPY receptors as potential targets for anti-obesity drug development. *Br. J. Pharmacol.* **2011**, *163*, 1170-1202.

(3) Dumont, Y.; Quirion, R. NPY. In *Handb. Biol. Act. Pept.*, 2nd ed.; Kastin, A. J., Ed. Academic Press: San Diego, 2013; pp 883-888.

(4) Reubi, J. C.; Gugger, M.; Waser, B.; Schaer, J. C. Y(1)-mediated effect of neuropeptide Y in cancer: breast carcinomas as targets. *Cancer Res.* **2001**, *61*, 4636-4641.

(5) Körner, M.; Reubi, J. C. NPY receptors in human cancer: a review of current knowledge. *Peptides* **2007**, *28*, 419-425.

(6) Reubi, J. C.; Maecke, H. R. Peptide-based probes for cancer imaging. *J. Nucl. Med.* **2008**, *49*, 1735-1738.

(7) Memminger, M.; Keller, M.; Lopuch, M.; Pop, N.; Bernhardt, G.; von Angerer, E.; Buschauer, A. The neuropeptide Y Y_1 receptor: a diagnostic marker? Expression in MCF-7 breast cancer cells is down-regulated by antiestrogens in vitro and in xenografts. *PLoS One* **2012**, *7*, e51032.

(8) Morgat, C.; Mishra, A. K.; Varshney, R.; Allard, M.; Fernandez, P.; Hindie, E. Targeting neuropeptide receptors for cancer imaging and therapy: perspectives with bombesin, neurotensin, and neuropeptide-Y receptors. *J. Nucl. Med.* **2014**, *55*, 1650-1657.

(9) Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. The first highly potent and selective non-peptide neuropeptide Y Y_1 receptor antagonist: BIBP3226. *Eur. J. Pharmacol.* **1994**, *271*, R11-13.

(10) Entzeroth, M.; Braunger, H.; Eberlein, W.; Engel, W.; Rudolf, K.; Wienen, W.; Wieland, H. A.; Willim, K.-D.; Doods, H. N. Labeling of neuropeptide Y receptors in SK-N-MC cells using the novel, nonpeptide Y1 receptor-selective antagonist [^{3}H]BIBP3226. *Eur. J. Pharmacol.* **1995**, *278*, 239-242.

(11) Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Guanidine-acylguanidine bioisosteric approach in the design of radioligands: synthesis of a tritium-labeled N^G-propionylargininamide ([^{3}H]-UR-MK114) as a highly potent and selective neuropeptide Y Y_1 receptor antagonist. *J. Med. Chem.* **2008**, *51*, 8168-8172.

(12) Keller, M.; Bernhardt, G.; Buschauer, A. [^{3}H]UR-MK136: a highly potent and selective radioligand for neuropeptide Y Y_1 receptors. *ChemMedChem* **2011**, *6*, 1566-1571.

(13) Wieland, H. A.; Engel, W.; Eberlein, W.; Rudolf, K.; Doods, H. N. Subtype selectivity of the novel nonpeptide neuropeptide Y Y_1 receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.* **1998**, *125*, 549-555.

(14) Keller, M.; Schindler, L.; Bernhardt, G.; Buschauer, A. Toward labeled argininamide-type NPY Y_1 receptor antagonists: identification of a favorable propionylation site in BIBO3304. *Arch. Pharm.* **2015**, *348*, 390-398.

(15) Keller, M.; Erdmann, D.; Pop, N.; Pluym, N.; Teng, S.; Bernhardt, G.; Buschauer, A. Red-fluorescent argininamide-type NPY Y_1 receptor antagonists as pharmacological tools. *Bioorg. Med. Chem.* **2011**, *19*, 2859-2878.

(16) Kagermeier, N.; Werner, K.; Keller, M.; Baumeister, P.; Bernhardt, G.; Seifert, R.; Buschauer, A. Dimeric carbamoylguanidine-type histamine H_2 receptor ligands: a new class of potent and selective agonists. *Bioorg. Med. Chem.* **2015**, *23*, 3957–3969.

(17) Brennauer, A.; Dove, S.; Buschauer, A. Structure-activity relationships of nonpeptide neuropeptide Y receptor antagonists. In *Handb. Exp. Pharmacol.*, Michel, M. C., Ed., 2004; Vol. 162, pp 506-537.

(18) Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. A simple and powerful flow cytometric method for the simultaneous determination of

multiple parameters at G protein-coupled receptor subtypes. *Chembiochem* **2006**, *7*, 1400-1409.

(19) Pluym, N.; Brennauer, A.; Keller, M.; Ziemek, R.; Pop, N.; Bernhardt, G.; Buschauer, A. Application of the guanidine-acylguanidine bioisosteric approach to argininamide-type NPY Y_2 receptor antagonists. *ChemMedChem* **2011**, *6*, 1727-1738.

(20) Weiss, S.; Bernhardt, G.; Buschauer, A.; Koenig, B. Synthesis and characterization of DMAP-modified NPY Y_1 receptor antagonists as ACYL-transfer catalysts. *Collect. Czech. Chem. Commun.* **2011**, *76*, 763-780.

(21) Molteni, G.; Bianchi, C. L.; Marinoni, G.; Santo, N.; Ponti, A. Cu/Cu-oxide nanoparticles as catalyst in the "click" azide-alkyne cycloaddition. *New J. Chem.* **2006**, *30*, 1137-1139.

(22) Ancliff, R. A.; Cook, C. M.; Eldred, C. D.; Gore, P. M.; Harrison, L. A.; Hayes, M. A.; Hodgson, S. T.; Judd, D. B.; Keeling, S. E.; Lewell, X. Q.; Mills, G.; Robertson, G. M.; Swanson, S.; Walker, A. J.; Wilkinson, M. Preparation of morpholinylmethylureas as CCR-3 antagonists. WO 2003082861-A2, 2003; Glaxo Group Limited, UK.

(23) Pluym, N.; Baumeister, P.; Keller, M.; Bernhardt, G.; Buschauer, A. [^{3}H]UR-PLN196: a selective nonpeptide radioligand and insurmountable antagonist for the neuropeptide Y Y_2 receptor. *ChemMedChem* **2013**, *8*, 587-593.

(24) Vauquelin, G.; Van Liefde, I.; Vanderheyden, P. Models and methods for studying insurmountable antagonism. *Trends Pharmacol. Sci.* **2002**, *23*, 514-518.

(25) Vauquelin, G.; Van Liefde, I.; Birzbier, B. B.; Vanderheyden, P. M. L. New insights in insurmountable antagonism. *Fundam. Clin. Pharmacol.* **2002**, *16*, 263-272.

(26) Guo, D.; Hillger, J. M.; Ijzerman, A. P.; Heitman, L. H. Drug-target residence time - a case for G protein-coupled receptors. *Med. Res. Rev.* **2014**, *34*, 856-892.

(27) Kenakin, T. P. *A pharmacology primer: theory, applications, and methods*; 3rd ed., Elsevier Academic Press: Burlington, 2009.

(28) Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y_2 receptor ligands. *Eur. J. Pharmacol.* **2006**, *551*, 10-18.

(29) Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of affinity and activity of ligands at the human neuropeptide Y Y_4 receptor by flow cytometry and aequorin luminescence. *J. Recept. Signal Transduct.* **2007**, *27*, 217-233.

(30) Mollereau, C.; Mazarguil, H.; Marcus, D.; Quelven, I.; Kotani, M.; Lannoy, V.; Dumont, Y.; Quirion, R.; Detheux, M.; Parmentier, M.; Zajac, J.-M. Pharmacological characterization of human $NPFF_1$ and $NPFF_2$ receptors expressed in CHO cells by using NPY Y_1 receptor antagonists. *Eur. J. Pharmacol.* **2002**, *451*, 245-256.

(31) Talmont, F.; Garcia, L. P.; Mazarguil, H.; Zajac, J.-M.; Mollereau, C. Characterization of two novel tritiated radioligands for labelling neuropeptide FF ($NPFF_1$ and $NPFF_2$) receptors. *Neurochem. Int.* **2009**, *55*, 815-819.

(32) Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. Synthesis and characterization of the first fluorescent nonpeptide NPY Y1 receptor antagonist. *Chembiochem* **2007**, *8*, 1981-1988.

(33) Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Koenig, B. Modular synthesis of non-peptidic bivalent NPY Y_1 receptor antagonists. *Bioorg. Med. Chem.* **2008**, *16*, 9858-9866.

(34) Qiao, C.; Jeon, H.-B.; Sayre, L. M. Selective inhibition of bovine plasma amine oxidase by homopropargylamine, a new inactivator motif. *J. Am. Chem. Soc.* **2004**, *126*, 8038-8045.

(35) Ciocoiu, C. C.; Nikolic, N.; Nguyen, H. H.; Thoresen, G. H.; Aasen, A. J.; Hansen, T. V. Synthesis and dual PPARα/δ agonist effects of 1,4-disubstituted 1,2,3-triazole analogues of GW 501516. *Eur. J. Med. Chem.* **2010**, *45*, 3047-3055.

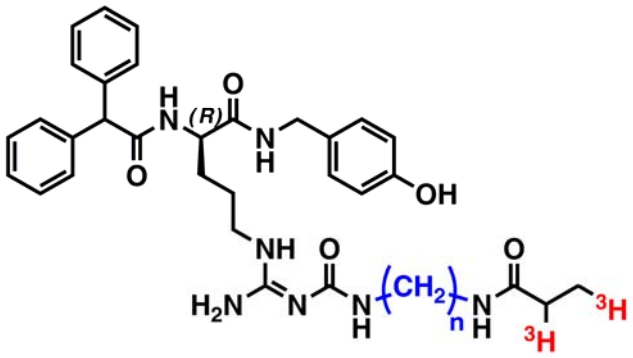
(36) Latyshev, G. V.; Baranov, M. S.; Kazantsev, A. V.; Averin, A. D.; Lukashev, N. V.; Beletskaya, I. P. Copper-catalyzed [1,3]-dipolar cycloaddition for the synthesis of macrocycles containing acyclic, aromatic and steroidal moieties. *Synthesis* **2009**, 2605-2615.

(37) Bradbury, B. J.; Baumgold, J.; Jacobson, K. A. Functionalized congener approach for the design of novel muscarinic agents. Synthesis and pharmacological evaluation of N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl] amides. *J. Med. Chem.* **1990**, *33*, 741-748.

(38) Müller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G.; Buschauer, A. Synthesis and neuropeptide Y Y_1 receptor antagonistic activity of N,N-disubstituted omega-guanidino- and omega-aminoalkanoic acid amides. *Arch. Pharm.* **1997**, *330*, 333-342.

(39) Cheng, Y.-C.; Prusoff, W. H. Relation between the inhibition constant (K_i) and the concentration of inhibitor which causes fifty per cent inhibition (IC_{50}) of an enzymic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.

TOC Graphic



Radioligand	n	K_d (Y_1R)	Residence time ($1/k_{off}$)
[^{3}H]**4**	4	2.0 nM	4.8 min
[^{3}H]**38**	2	0.044 nM	135 min

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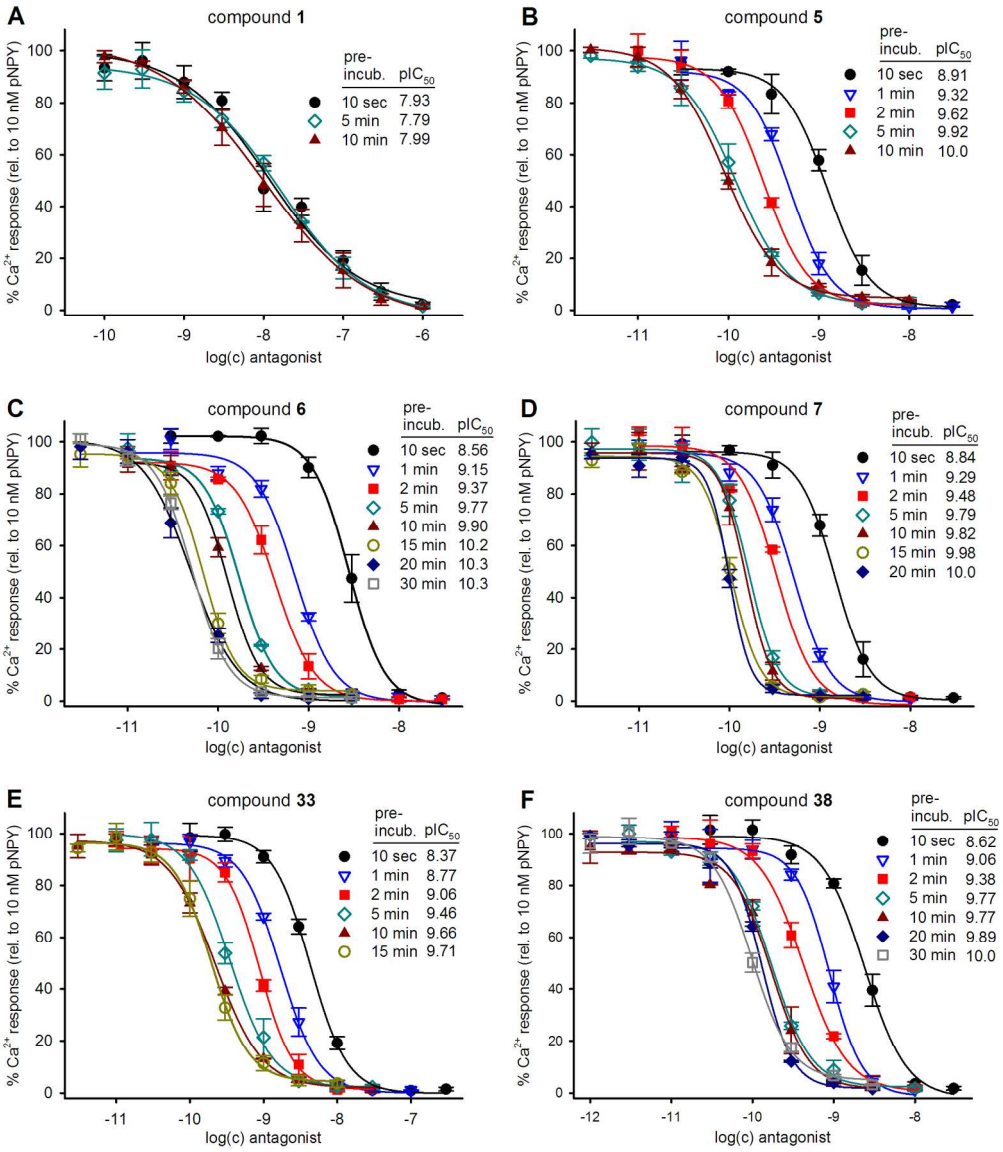

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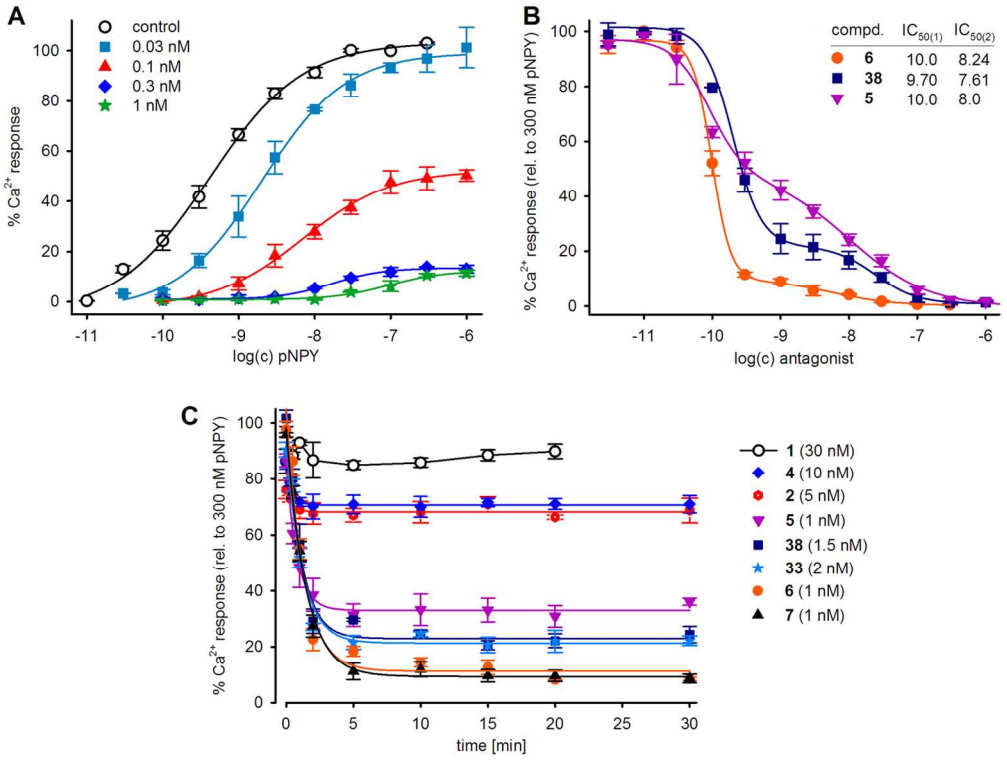

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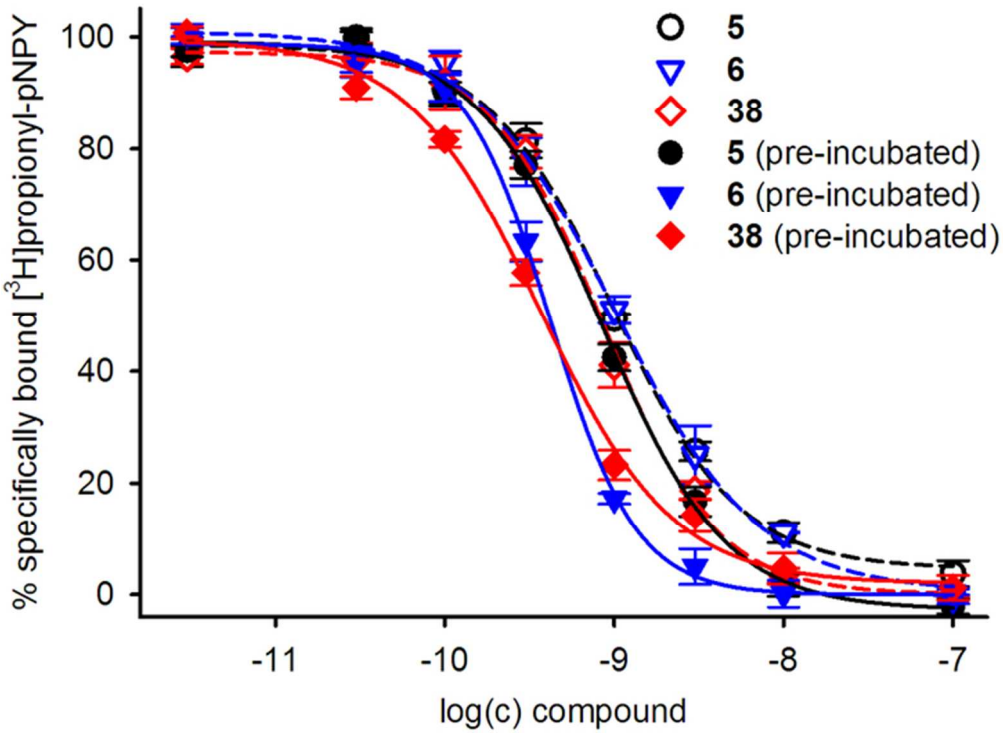

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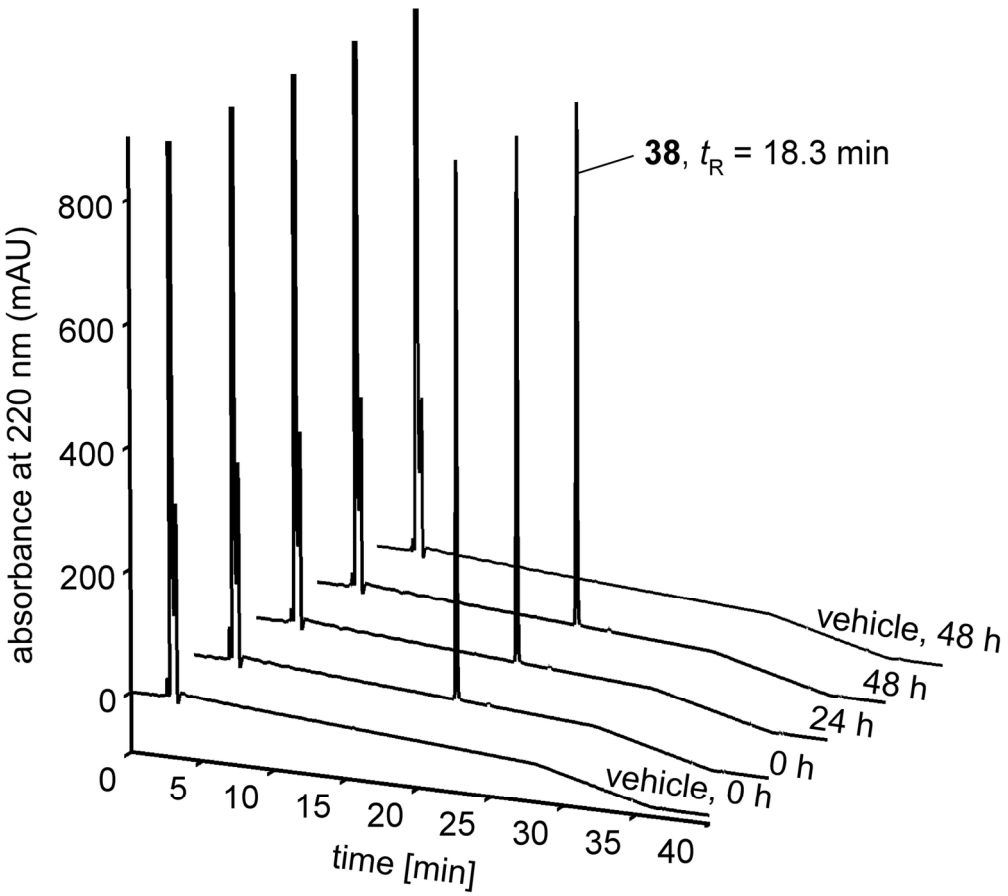

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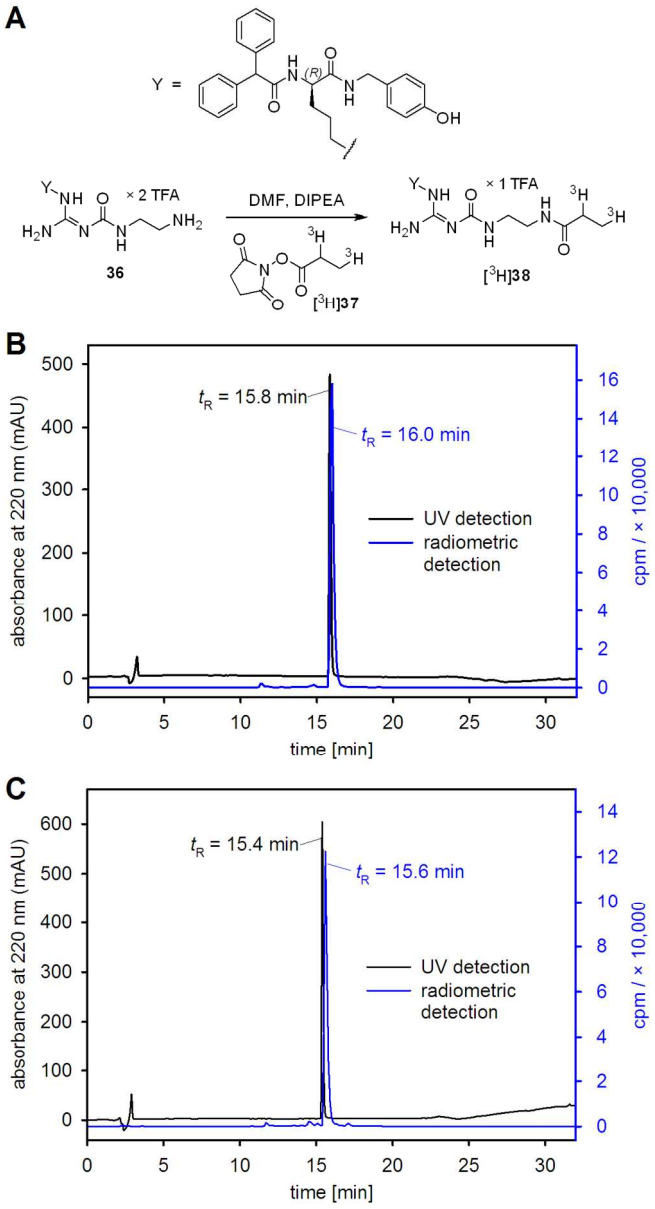

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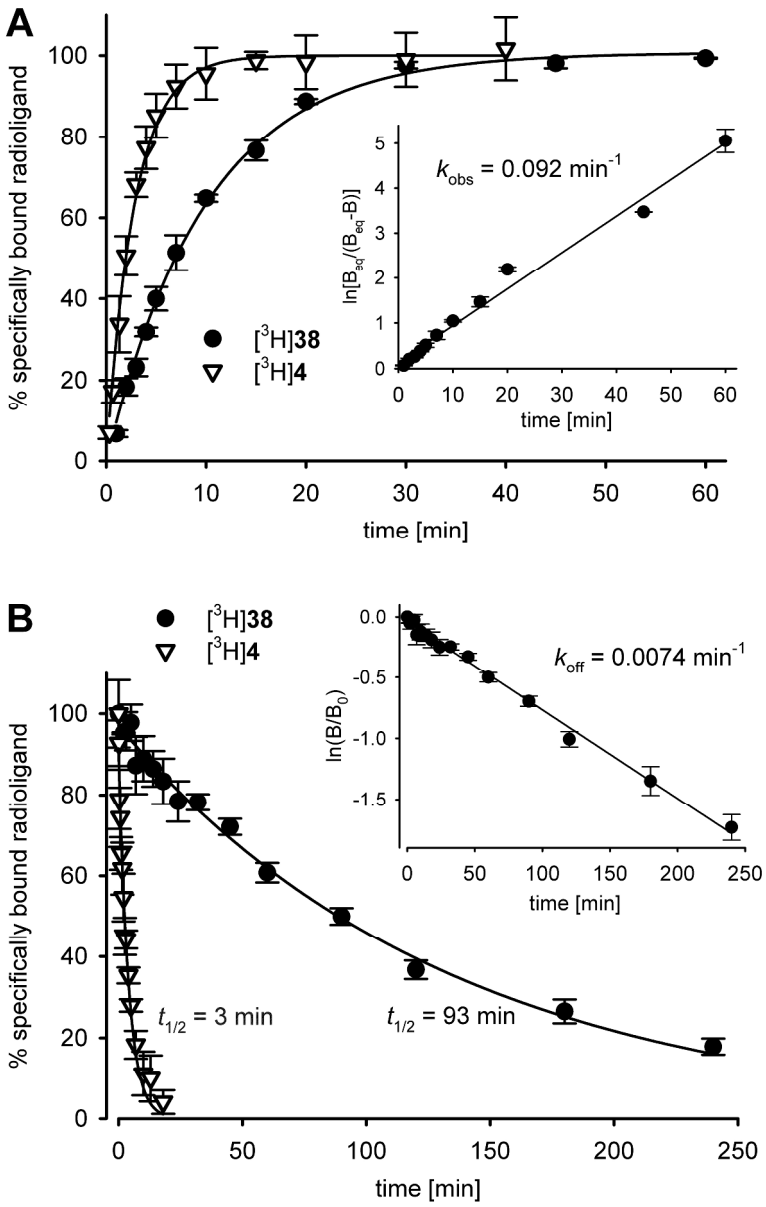

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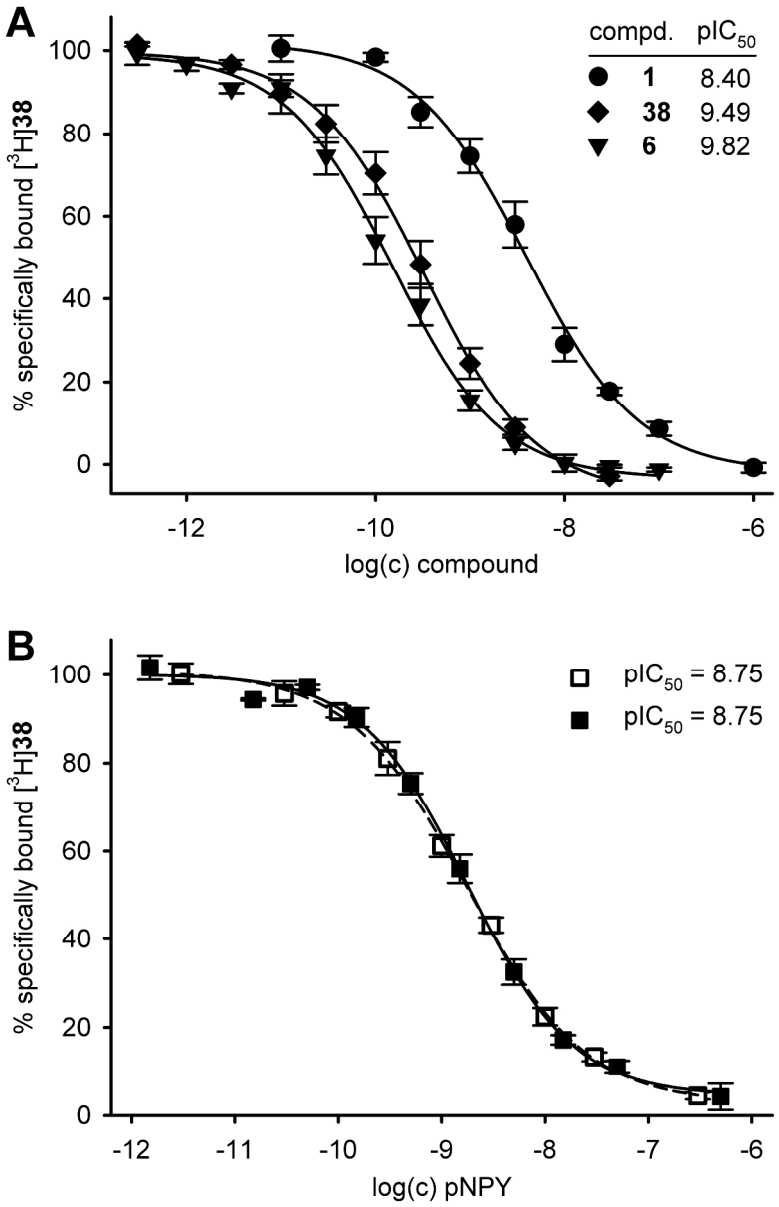

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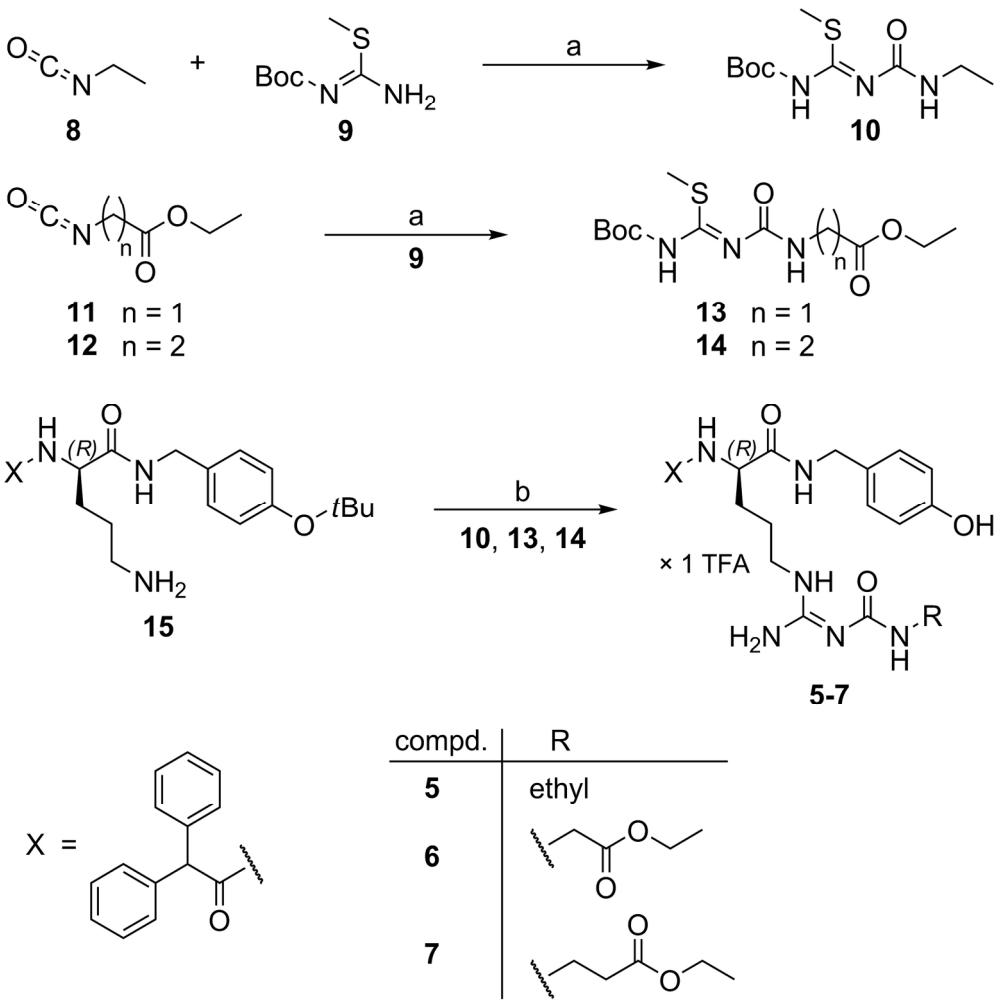

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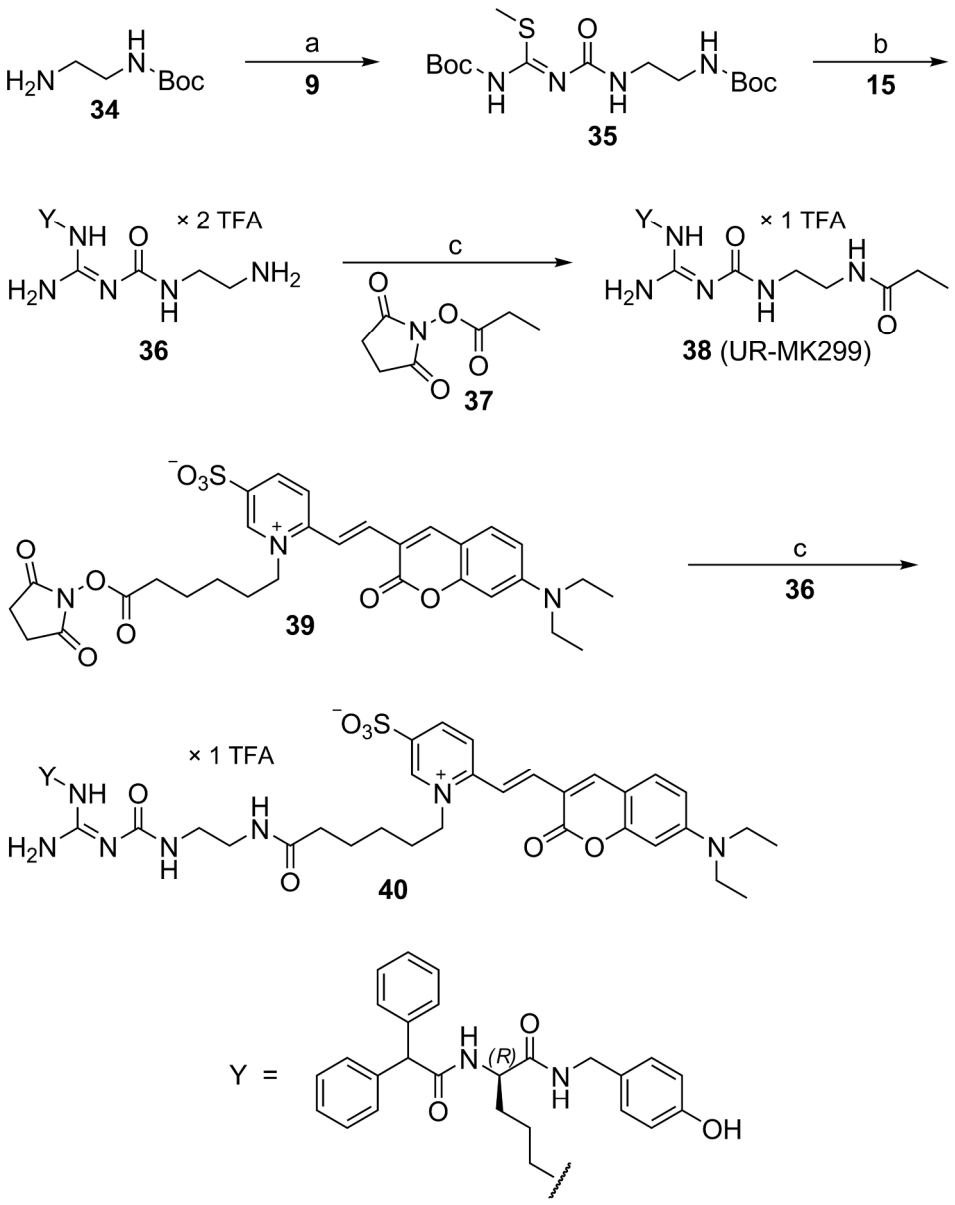

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