British Journal of Pharmacology (1998) 125, 549–555 
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# Subtype selectivity of the novel nonpeptide neuropeptide Y Y1 receptor antagonist BIBO 3304 and its effect on feeding in rodents

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**1** The novel Y1-selective argininamide derivative BIBO 3304 ((R)-N-[[4-(aminocarbonylaminomethyl)-phenyl]methyl]-$N^2$-(diphenylacetyl)-argininamide trifluoroacetate) has been synthesized and was examined for its subtype selectivity, its *in vitro* antagonistic properties and its food intake inhibitory properties.

**2** BIBO 3304 displayed subnanomolar affinity for both the human and the rat Y1 receptor ($IC_{50}$ values $0.38 \pm 0.06$ nM and $0.72 \pm 0.42$ nM, respectively). The inactive enantiomer of BIBO 3304 (BIBO 3457) had low affinity for both the human and rat Y1 receptor subtype ($IC_{50} > 1000$ nM). BIBO 3304 showed low affinity for the human Y2 receptor, human and rat Y4 receptor as well as for the human and rat Y5 receptor ($IC_{50}$ values $> 1000$ nM).

**3** 30 $\mu$g BIBO 3304 administered into the paraventricular nucleus inhibited the feeding response induced by 1 $\mu$g NPY as well as the hyperphagia induced by a 24 h fast implying a role for Y1 receptors in NPY mediated feeding. The inactive enantiomer had no effect.

**4** BIBO 3304 inhibits neither the galanin nor the noradrenaline induced orexigenic response, but it blocked feeding behaviour elicited by both [Leu[31], Pro[34]]NPY and NPY (3–36) suggesting an interplay between different NPY receptor subtypes in feeding behavior.

**5** The present study reveals that BIBO 3304 is a subtype selective nonpeptide antagonist with subnanomolar affinity for the Y1 receptor subtype that significantly inhibits food intake induced by application of NPY or by fasting.

**Keywords:** BIBO 3304; NPY; Y1-receptor; food intake

## Introduction

Neuropeptide Y is a 36 amino acid polypeptide that is abundantly expressed within the hypothalamus (Tomaszuk *et al*., 1996). It is a neuronal and endocrine messenger involved in many physiological processes, most importantly it potently stimulates food intake (Bing *et al*., 1996; Stanley *et al*., 1992; Wilding, 1996) and elevates blood pressure (Edvinsson *et al*., 1987). So far, six NPY receptor subtypes have been identified (Y1–Y6) (Balasubramaniam, 1997; Michel *et al*., 1998). These NPY receptors are members of the G-protein coupled receptor superfamily and appear to be coupled to at least two second messenger systems, the intracellular $Ca^{2+}$ release and the inhibition of cAMP synthesis (Aakerlund *et al*., 1990).

The receptor subtypes can be characterized based on the rank order of potency of NPY and related peptides (Balasubramaniam, 1997; Michel *et al*., 1998). The Y1 subtype displays high affinity for the NPY analogue [Leu[31], Pro[34]]NPY, for the Y1 receptor antagonist BIBP3226 (Rudolf *et al*., 1994) and low affinity for the C-terminal fragments of NPY such as NPY (13–36) and NPY (3–36). In contrast, the Y2 receptor subtype exhibits high affinity for C-terminal fragments of NPY (Rose *et al*., 1995; Wieland *et al*., 1995a). The Y3 subtype displays low affinity for PYY whereas the Y4 receptor has very high affinity for pancreatic polypeptide (PP), somewhat lower affinity for PYY, and much lower affinity for NPY (Gregor *et al*., 1996). The Y5 subtype is characterized by high affinity for both [Leu[31], Pro[34]]NPY and NPY (3–36) (Gerald *et al*., 1996). The pharmacological profile of the rodent Y6 receptor subtype (Weinberg *et al*., 1996) appears to be similar to the Y5 receptor but its human orthologue binds neither iodinated NPY nor PYY due to a frameshift mutation occuring early in primate evolution (Matsumoto *et al*., 1996).

Whereas both Y1 and Y2 receptors seem to play an important role in the cardiovascular effect of NPY (Doods *et al*., 1995, Lundberg & Modin, 1995), the binding profile of the Y5 receptor most closely reflects the *in vivo* agonistic properties of NPY in food intake (Gerald *et al*., 1996). Until recently it has been discussed as 'the feeding receptor' but doubts were raised since a Y5 selective antagonist did not reduce food intake in animal studies (Fukuroda *et al*., 1997). Moreover, it has been published that the cyclic dimerized nonapeptide BW1229 (Daniels *et al*., 1995; Hedge *et al*., 1995) inhibits food intake by the blockade of Y1 receptors but not Y5 receptors (Kanatani *et al*., 1996, 1997). In addition, we have previously described a potent and selective non-peptide antagonist, BIBP3226 (Rudolf *et al*., 1994; Doods *et al*., 1995; Wieland *et al*., 1995b) for which food-intake inhibitory properties have been described (O'Shea *et al*., 1997). However, due to the CNS toxicity of this compound (Doods *et al*., 1996; O'Shea *et al*., 1997), it could not be unambiguously shown that the observed food intake inhibition is a result of selective inhibition of the Y1 receptor subtype.

In order to address the question whether the Y1 receptor subtype is involved in feeding, the efficacy of selective NPY agonists to induce feeding following application into the paraventricular nucleus (PVN) in rats were investigated. Moreover, it was the aim of the present study to characterize the receptor selectivity of a novel NPY antagonist out of our Y1 chemistry program, BIBO 3304, and to evaluate whether this compound is able to influence NPY mediated feeding behavior.

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## Methods

### *Rat hypothalamus*

The hypothalamus region has been homogenized in 50-fold volume in ice-cold sucrose (0.32 M) buffered with 5 mM HEPES (pH 7.4). The homogenate was centrifuged 5 min at 1000 × *g*, 4°C in a minifuge. The supernatant was then centrifuged 45 min, 10,000 × *g*, 4°C and the pellet resuspended in 100-fold volume of original organ weight in modified Krebs-Ringer buffer (assay buffer): 137.0 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, asorbic acid 0.1% w/v, glucose 1 mg $ml^{-1}$, buffered with (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) 20 mM, pH 7.4, 0.5% bovine serum albumin (BSA), 0.1% bacitracin and 50 μM phenylmethylsulfonyl fluoride (PMSF). 200 μl of the homogenate were incubated with 30 pM [$^{125}$I]NPY 45 min at 37°C and increasing concentrations of test compounds ($10^{-13}$–$10^{-4}$M). The incubation was stopped by centrifugation (5 min, 1500 × *g*), the pellet washed with 0.25 ml of incubation buffer, recentrifuged and membrane-bound radioactivity was counted. Non-specific binding was determined in presence of 100 nM NPY.

### *SK-N-MC (neuroblastoma) cells*

Cells were grown in MEM (MEM with Earl's salt, 10% FCS, 1 mM sodium pyruvate, 1% non-essential amino acids, 4 mM glutamine). Confluent cells were removed with 0.02% EDTA/PBS (phosphate buffered saline) and resuspended in 10 ml incubation buffer (MEM/25 mM HEPES + 0.5% BSA, 50 μM PMSF, 0.1% bacitracin, 3.75 mM $CaCl_2$). After 5 min centrifugation (150 × *g*) the pellet was resuspended in equal volume and after further centrifugation in incubation buffer. After counting the cells were diluted to give a concentration of 1.25 mio cells $ml^{-1}$. 200 μl of this cell suspension was incubated 3 h at room temperature (RT) with 30 pM [$^{125}$I]NPY solutions and increasing concentrations of test compounds in a total volume of 250 μl. The incubation was stopped as described for rat hypothalamus.

### *SMS-KAN (neuroblastoma) cells*

Membrane preparation: the cells were grown in 50% nutrient mixture Ham's F12/50% Dulbecco's modified Eagle medium with 15% fetal calf serum, 2 mM l-Glutamine, non-essential amino acids, 1% gentamycin at 37°C and 5% $CO_2$ until they are confluent. The medium was decanted. The cells were washed twice with 50 mmol Tris/HCl buffer, pH 7.5. Again Tris buffer with inhibitors (0.1% bacitracin and 50 μM Pefabloc SC, Merck, Darmstadt) was added and the cells were removed with a rubber policemen. The cell suspension was homogenized 10 s with an Ultraturrax, 15 times pottered with speed maximum. After centrifugation at 4°C, 10 min, 820 × *g* the supernatant was decanted and centrifuged at 4°C, 30 min, 37,000 × *g*. The supernatant was decanted and the pellet resuspended in 30 ml HEPES buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4) 0,1% bacitracin, 50 μM Pefabloc SC, homogenized with 15 strokes with a potter, recentrifuged at 4°C, 30 min, 37000 × *g* and the pellet was resuspended in 1 ml HEPES buffer per flask without protease inhibitors. An aliquot is used to determine the protein concentration and inhibitors were added accordingly. Membrane suspensions were resuspended five times with a syringe in incubation buffer (MEM/25 mM HEPES + 1% BSA, 50 μM PMSF, 0.1% bacitracin, 3.75 mM $CaCl_2$). 100 μl of the membrane suspension containing 10 μg protein were incubated with 30 pM [$^{125}$I]NPY in a total volume of 250 μl for 2 h at RT. The incubation was terminated as described for rat hypothalamus.

### *Human Y1 receptor stably expressed in baby hamster kidney (BHK) cells*

Cells were grown in DMEM with 4.5 g/l glucose, 10% fetal calf serum, 1% PENStrep, 1 mg $ml^{-1}$ G-418, 1 mg $ml^{-1}$ hygromycin B. 96 h before receptor binding assay 1 mM isopropylthiogalactoside (IPTG) was added in order to induce expression (Lac Switch Expression System from Stratagene). Confluent cells were removed with 0.06% EDTA/PBS (1 min incubation) and resuspended in 15 ml incubation buffer (MEM/25 mM HEPES + 1% bovine serum albumine, 50 μM PMSF, 0.1% bacitracin, 3.75 mM $CaCl_2$). After 10 min centrifugation at RT (150 × *g*), the pellet was resuspended in 50 ml incubation buffer, respun and resuspended in 30 ml incubation buffer. After counting, the cells were diluted to a final concentration of 2.5 × $10^5$ cells $ml^{-1}$. Two hundred microliters of this cell suspension was incubated 3 h at RT with 30 pM [$^{125}$I]NPY and increasing concentrations of test compounds ($10^{-13}$–$10^{-4}$M) in a total volume of 250 μl. The incubation was stopped by 10 min centrifugation, 3000 × *g* at 4°C. The pellet was resuspended with 0.25 ml PBS recentrifuged and the pellet measured in a γ-counter.

### *Rat Y1 receptor expressing human embryonic kidney (HEK) 293 cells*

Confluent cells were removed with 0.02% EDTA/PBS and resuspended in 10 ml incubation buffer (MEM/25 mM HEPES + 0.5% BSA, 50 μM PMSF, 0.1% bacitracin, 3.75 mM $CaCl_2$). After 5 min centrifugation (150 × *g*) the pellet was resuspended in equal volume and after further centrifugation in 10 ml incubation buffer. The cells were diluted to a concentration of one mio cells $ml^{-1}$. 100 μl of this cell suspension was incubated 3 h at RT with 30 pM [$^{125}$I]NPY solutions and increasing concentrations of test compounds in a total volume of 250 μl. The incubation was stopped as described for rat hypothalamus.

### *Human Y2 receptor stably expressed in BHK cells*

Assay was performed essentially as described for human Y1 expressing BHK cells, except that hygromycin and induction with IPTG was omitted.

### *Rat Y4 stably transfected in BHK*

Cultivation and receptor binding was performed as described for hY1/BHK with exception of incubation medium (25 mM HEPES, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) and final concentration of cell suspension 5 × $10^5$ cells $ml^{-1}$. Twenty-four hours before receptor binding assay 1.0 mM IPTG was added for induction of expression. Incubation time for binding assay: 2 h.

### *Human Y5 receptor stably transfected in HEK 293 cells*

Centrifuged cells were cultivated as described for BHK/Y1 cells except that a concentration of 0.7 mg $ml^{-1}$ G-418 was used, no hygromycin added and IPTG induction was not necessary. The incubation buffer cell cultivation and receptor binding was performed as described. Final concentration was

$1.5 \times 10^6$ cells $ml^{-1}$ and centrifugation stopped as described for Y1/BHK cells.

### *Transient transfection*

Transfection was performed using the lipofectamine method according to the manufacturer's protocol using expression plasmids encoding the rat Y5 or human Y4 receptor, respectively. Transiently transfected chinese hamster ovary (CHO) cells (human Y4 and rat Y5 receptor) were used for receptor binding studies.

### *cAMP assay*

SK-N-MC cells were washed twice with 20 ml cAMP buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES pH 7.4, supplemented with 0.5% BSA, 10 mM glucose, 37°C). Cells were removed by a rubber policeman and resuspended in 50 ml cAMP buffer (37°C), after 5 min at 150 × *g*, the pellet was washed again and resuspended in 10 ml cell medium (37°C) and the cell suspension was diluted to a final concentration of 1 million cells $ml^{-1}$. 1 ml of the cell suspension were preincubated 5 min at room temperature with 100 μM (final concentration) papaverine and buffer or different concentrations of the antagonist. 10 μl NPY solution of different concentrations and 10 μl forskolin (1.5 mM) were added and incubated 1 h at 37°C by shaking. The incubation was stopped by addition of 0.1 ml of 1 M HCl (15 min incubation) and centrifugation at 4°C, 15 min, 2000 × *g*. The supernatant was diluted with 0.05 M acetate buffer of the cAMP kit (Amersham, RPA 509) and assayed. The sample (100 μl) was incubated with 100 μl [$^{125}$I]-cAMP, 100 μl anti-cAMP antiserum 3 h, 4°C, by shaking. The second antibody was added, the solution mixed and incubated 10 min at RT (shaking). After 10 min centrifugation at 3200 × *g*, RT the supernatant was removed and the pellet counted in a gamma-counter.

### *Food-intake studies*

Adult male Chbb:Thom rats weighing between 300 and 340 g were individually housed and maintained on a 12 : 12 h light-dark cycle beginning at 06.00 h. Tap water and standard laboratory chow were available throughout except in the experiments where the animals were fasted for 24 h. After 1 week of habituation to their new housing conditions, the animals were anaesthetized with sodium pentobarbital (60 mg $kg^{-1}$, i.p) for the placement of stainless steel guide cannulae. Bilateral guide cannulae (26 gauge) were placed 1 mm above the paraventricular nucleus according to the stereotaxic coordinates (Paxinos & Watson, 1986): AP: −1.8, L:0.5, V:7.0. Guide cannulae were maintained in place on the skull with small metal screws and dental acrylic cement. Cannulae were closed with a stainless steel stylet when not in use. Rats were allowed to recover for at least 1 week and were adapted to the injection procedure. On the day of the experiments drugs were injected between 08.00 and 09.00 h. Injection cannulae (33 gauge) were inserted 1 mm beyond the tips of the guide cannulae. The injection cannulae were attached by polyethylene tubing to a Hamilton microsyringe mounted in an infusion pump. Injection volume was 0.5 μl infused with a rate of 0.0125 μl $s^{-1}$.

In the first set of experiments groups of 6–12 rats received increasing doses (0.5–32 μg, unilateral) of NPY receptor agonists into the PVN and food intake was monitored for at least 2 h. On the first treatment day the groups were randomly assigned to the various doses. Rats had a wash-out period of at least 3 days between injections, after which the groups were randomized again to test the next agonist. Not more than 5–6 injections were given in total. In the second set of experiments BIBO 3304 or its inactive enantiomer were given 10 min before the injection of different NPY receptor agonists, galanin or noradrenaline. All compounds were applied into the PVN and for each experiment 8–22 rats were used and for each dose a different group of rats were used. In the last series of experiments BIBO 3304 was given to animals which were fasted for 24 h. Five minutes after bilateral PVN injection of BIBO 3304 the rats ($n=12$) were given free access to food and food intake was monitored for another 24 h.

### *Analysis and statistics*

Competition binding experiments were analysed by a non-linear least square fitting method with a one or two binding site model, respectively (RS/1 software package, BBN Research Systems, Cambridge, MA, U.S.A.). Maximum of specific [$^{125}$I]NPY binding was set to 100%. All data ($n=3-9$) are expressed as mean ± s.e.mean.

Concentration-response relationships for the second messenger analysis were determined by fitting the experimental data to sigmoid functions. The $pK_b$ values were determined from dose-ratios calculated from the horizontal distances between ascending regions of the dose-response curves of NPY obtained in the absence and presence of antagonists ($n=3-4$).

Comparisons between the animal groups were made using Student's *t*-test. $P<0.05$ were considered statistically significant, values are presented as mean ± s.e.mean.

### *Materials*

Dulbecco's modified eagle medium (DMEM) was obtained from BioWhittaker (Boehringer Ingelheim Bioproducts Partnership, Verviers, Belgium); OPTI-MEM and Lipofectamine from GIBCO BRL (Eggenstein, Germany); fetal calf serum from BioWhittaker; HEPES from Fluka; IPTG, geneticin, hygromycin and noradrenaline were purchased from Sigma (Deisenhofen, Germany); Pefabloc SC, Merck, Darmstadt; [$^{125}$I]hNPY-Tyr$^{36}$ (specific activity: 2000 Ci $mmol^{-1}$) from Amersham and [$^{125}$I]-PP (specific activity: 2200 Ci $mmol^{-1}$) from NEN/Dupont (Bad Homburg, Germany); the rat Y5 cDNA was purchased from Dr. John Shine, Australia, and the human Y4 cDNA generously provided by NOVO Nordisk; BIBO 3304, BIBO 3457 were synthesized by Boehringer Ingelheim Pharma Deutschland. The chemical structure of BIBO 3304 (Engel *et al*., 1994) is shown in Figure 1. Peptides were purchased from Neosystème (Strasbourg, France) (pNPY, pPYY, hNPY(2–36)) and hPP and from Saxon Biochemicals (Hannover, Germany) (rGalanin, pNPY(13–36), hNPY(3–36) and [Leu$^{31}$, Pro$^{34}$]NPY.

## Results

### In vitro *NPY receptor profile of BIBO 3304*

BIBO 3304 displaced the radioligand completely in binding assays using cell lines expressing the Y1 receptor subtype. The Hill coefficient for the displacement curves was not significantly different from unity. BIBO 3304 displayed high affinity. ($IC_{50}=0.69\pm0.16$ nM) for the human Y1 receptor

stably expressed in BHK cells (Figure 2). The $IC_{50}$ value determined for SK-N-MC cells, a human neuroblastoma cell line endogenously expressing the Y1 receptor, was nearly identical ($IC_{50} = 0.38 \pm 0.06$ nM) (Table 1). Species selectivity between human and rat Y1 receptors was not detected which is reflected by the subnanomolar affinity of BIBO 3304 for cells stably transfected with the rat Y1 receptor cDNA. The (S-) enantiomer, BIBO 3457, exhibits low affinity for both the human and rat Y1 receptor subtype (>1000 nM). BIBO 3304 exhibits selective binding to the Y1 receptor subtype as shown by its more than 1000–10,000-fold lower affinity for the human Y2 receptor, for the human and rat Y4 receptor as well as for the human and rat Y5 receptor (Table 1, Figure 2). Furthermore, binding studies using more than seventy-five different receptor binding and enzyme systems were performed as described earlier (Rudolf *et al*., 1997) and no significant affinity was detected for BIBO 3304 (data not shown).

Two binding sites were found in the receptor binding assay using rat hypothalamic membranes (Table 1) with $32 \pm 6$% of total receptor number being composed of the high affinity site. The high affinity site corresponds to the Y1 receptor affinity ($IC_{50high} = 1.4 \pm 0.9$ nM). The low affinity site displays an $IC_{50low} > 100\,000$ nM.

BIBO 3304 was evaluated in terms of its properties to inhibit the NPY mediated signal transduction in SK-N-MC cells. No agonistic properties were found with 1 $\mu$M BIBO 3304 in the cAMP assay. The NPY induced inhibition of cAMP synthesis was antagonized by 100 nM BIBO 3304 with a $pK_b$ of $9.1 \pm 0.4$ ($n = 3-4$) (data not shown).

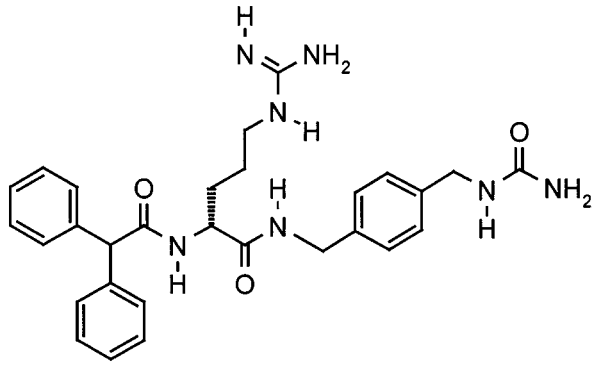

**Figure 1** Structure of BIBO 3304.

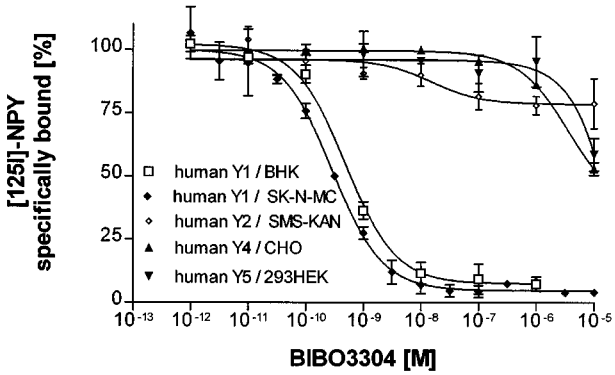


**Figure 2** Displacement of specifically bound [$^{125}$I]hNPY-Tyr$^{36}$ by BIBO 3304 ($n = 3-4$, mean$\pm$s.e.mean).

## *Food intake studies*

All NPY receptor agonists examined, irrespective of their receptor subtype selectivity, were able to increase feeding over 2 h in satiated rats. However, their potency varied as NPY(13–36) was approximately 30-fold less potent than NPY itself and PYY appeared to be the most potent agonist and produced the highest maximal response. NPY, NPY (2–36) and NPY (3–36) were almost equipotent. [Leu$^{31}$, Pro$^{34}$]NPY and hPP elicited feeding responses at low doses but were clearly less efficacious compared to NPY as depicted for [Leu$^{31}$, Pro$^{34}$]NPY in Figure 3. Except for the 2 $\mu$g of NPY (13–36)

**Table 1** $IC_{50}$ values of BIBO 3304 and the (S-) enantiomer BIBO 3457 obtained for the different receptor subtypes

| *Receptor cell line* | *BIBO 3304 (nM)* | *BIBO 3457 (nM)* |
|---|---|---|
| Human Y1/BHK | $0.69 \pm 0.16$ | >10000 |
| Human Y1/SK-N-MC | $0.38 \pm 0.06$ | $1300 \pm 70$ |
| Rat Y1/293 | $0.72 \pm 0.42$ | >1000 |
| Human Y2/SMS-KAN | >10000 | >10000 |
| Human Y2/BHK | >1000 | >10000 |
| Rat hippocampus (Y2) | >10000* | n.d. |
| Human Y4/CHO | $12300 \pm 5000$ | $24000 \pm 6500$ |
| Rat Y4/CHO | >10000 | >10000 |
| Human Y5/293 | >10000 | $28000 \pm 4000$ |
| Rat Y5/CHO | $21000 \pm 4500$ | $23000 \pm 2000$ |

| *BIBO 3304* | *$IC_{50high}$* | *(%)* | *$IC_{50low}$* | *(%)* |
|---|---|---|---|---|
| Rat hypothalamus | $1.4 \pm 0.9$ | $32 \pm 6$ | >100 000 | $68 \pm 6$ |

Data are presented as means$\pm$s.e.mean of the data obtained from three to nine different experiments; n.d., not determined, *(Wieland *et al*., Reg. Peptides in press).

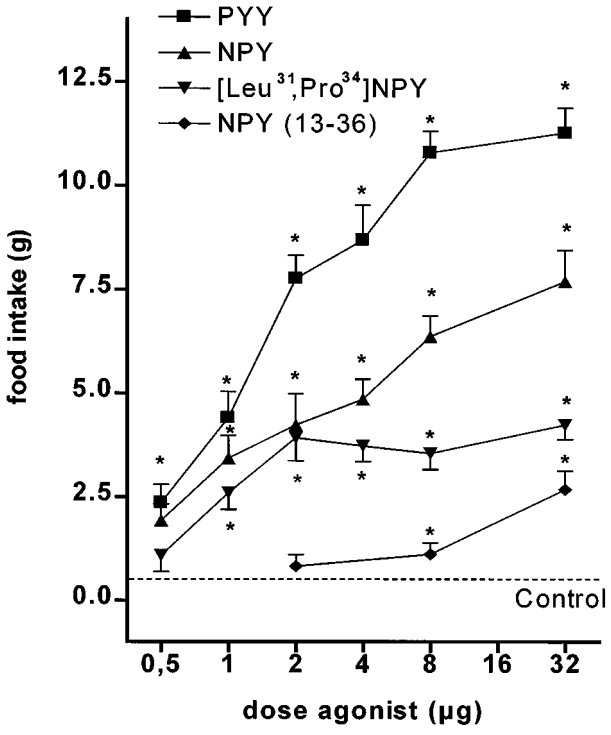


**Figure 3** Feeding (2 h response) induced by PYY, NPY, [Leu$^{31}$, Pro$^{34}$]NPY and NPY(13–36) following PVN administration in satiated rats ($n = 6-12$).

and 0.5 $\mu$g of [Leu$^{31}$, Pro$^{34}$]NPY all feeding response was significantly different from the control response. Animals that were injected with saline consumed 0.41 $\pm$ 0.2 g ($n$ = 24) food.

When satiated animals were given unilateral 1 $\mu$g of NPY into the PVN this resulted in an immediate feeding response with an intake of 3.58 $\pm$ 0.33 g at 2 h ($n$ = 25). Control animals consumed only 0.49 $\pm$ 0.29 g in the same 2 h period ($n$ = 10). BIBO 3304 dose-dependently inhibited the feeding reponse mediated by 1 $\mu$g NPY. A dose of 30 $\mu$g caused an approximately 50% inhibition (1.87 $\pm$ 0.3 g, $n$ = 18, Figure 4a). The inactive enantiomer BIBO 3457 in the same dose range had no effect on NPY induced feeding (Figure 4b). Noradrenaline (30 $\mu$g) and galanin (4 $\mu$g) both given into the PVN also stimulated feeding, their corresponding 2 h values being 3.5 $\pm$ 0.3 g ($n$ = 22) and 3.11 $\pm$ 0.18 g ($n$ = 21), respectively. BIBO 3304 (30 $\mu$g) had no effect on noradrenaline or galanin induced feeding. However, the feeding response mediated by NPY (2–36) (1 $\mu$g), NPY (3–36) (1 $\mu$g) and [Leu$^{31}$, Pro$^{34}$]NPY (2 $\mu$g) could be blocked by 30 $\mu$g BIBO 3304 (Figure 4c).

Bilateral application of 15 $\mu$g of BIBO 3304 into the PVN in rats, deprived from food for 24 h, attenuated the hyperphagia following fasting, especially during the first 2 h of refeeding (Figure 5). The inactive enantiomer had no effect in this model (data not shown).

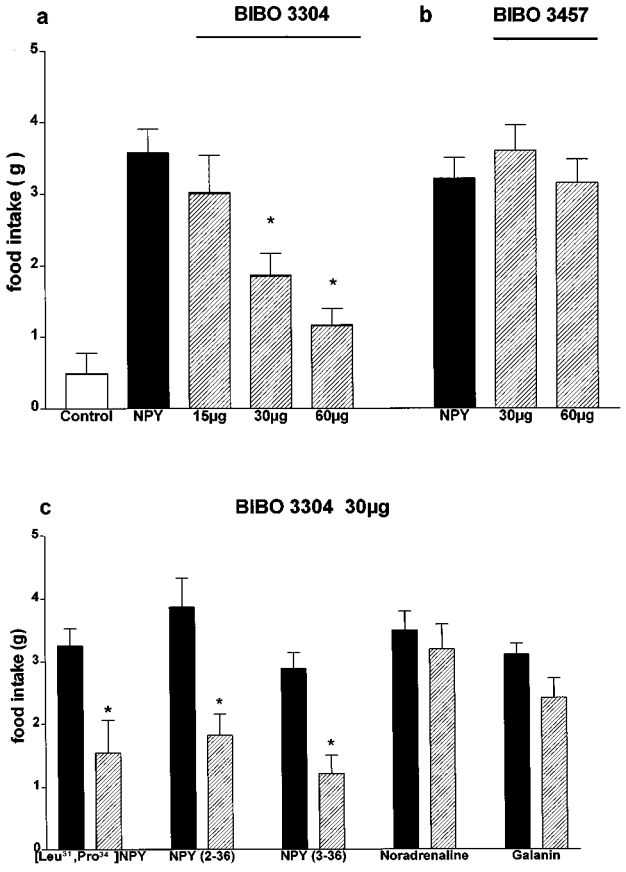


**Figure 4** (**a**) Inhibition of NPY (1 $\mu$g, $n$ = 25) induced feeding by BIBO 3304 (each dose $n$ = 18). (**b**) Lack of effect of the inactive enantiomer BIBO 3457 to inhibit NPY induced feeding (all three groups $n$ = 8). (**c**) Effect of BIBO 3304 (30 $\mu$g) on 2 h feeding induced by selective NPY agonists ([Leu$^{31}$, Pro$^{34}$]NPY, $n$ = 7; NPY(2–36), $n$ = 11; NPY(3–36), $n$ = 14), noradrenaline ($n$ = 22) and galanin ($n$ = 21). Hatched bars in presence of antagonist, solid bars in absence of antagonist. The number of control animals and those treated with antagonist were identical. Asterisk (*) indicates values statistically different from control values ($P$ < 0.01).

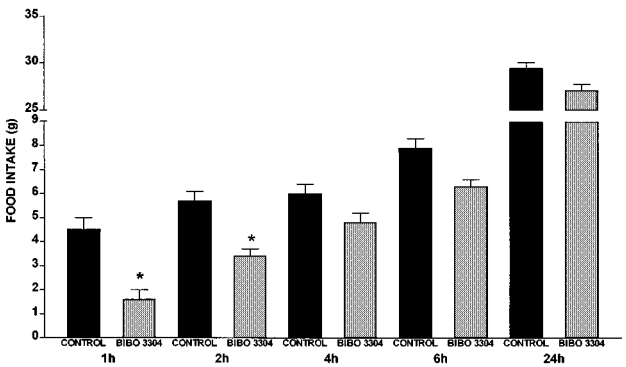


**Figure 5** Effects of 30 $\mu$g of BIBO 3304 (15 $\mu$g, bilateral) on food intake in 24 h fasted rats. Means $\pm$ s.e.mean, $n$ = 12 in each group. Asterisk (*) indicates values statistically different from control values ($P$ < 0.05).

## Discussion

Acute and chronic administration of NPY or PYY intracerebroventricularly or *via* the PVN induces feeding in various species such as mice, rats and monkeys (Kalra *et al*., 1991; Zarjevski *et al*., 1993, Larsen *et al*., 1997). The fact that [Leu$^{31}$, Pro$^{34}$]NPY (Kalra *et al*., 1991) and NPY(2–36) (Kalra *et al*., 1991; Stanley *et al*., 1992) but not NPY(13–36) (Kalra *et al*., 1991) display a robust orexigenic effect led to the conclusion that an atypical Y1 receptor mediates the feeding response. After identification of the Y5 receptor subtype (Gerald *et al*., 1996) this feeding pattern of agonists was found to correlate most closely to the rank order of agonist binding to the Y5 receptor subtype. Since in the latter publication only a single dose of agonist was used for food intake experiments, we characterized in our studies the so-called feeding receptor by examining a wide dose range (0.5-32 $\mu$g) of NPY receptor agonists.

The rank order of potency to elicit feeding (PYY $\sim$ NPY $\sim$ NPY(2–36) $\sim$ NPY(3–36) $\sim$ [Leu$^{31}$, Pro$^{34}$]NPY $\sim$ hPP > NPY(13–36)) corresponds to the *in vitro* potency or affinity described for the Y5 receptor (Gerald *et al*., 1996, Hu *et al*., 1996). However, in the feeding experiments, PYY elicited a higher efficacy compared to NPY. This pronounced effect compared to NPY has also been reported by Corp *et al*., (1990) investigating 90 min food intake following of NPY and PYY injected into the fourth ventricle by male Sprague-Dawley rats. In pilot experiments we also observed this phenomenon for other PYY analogues e.g. PYY(3–36) compared to NPY(3–36). It is unlikely that this is due to differences in receptor selectivity between NPY and PYY analogs but could be explained by differences in physico-chemical properties resulting in a different tissue penetration pattern. In contrast, [Leu$^{31}$, Pro$^{34}$]NPY exhibits reduced efficacy. The maximal feeding response that is reached is much lower when compared to NPY and remains constant even after increasing the dose by a factor of 20. This significant lower maximum effect has also been described for [Pro$^{34}$]NPY following i.c.v. administration (O'Shea *et al.,* 1997). It is difficult to explain this finding, however, the fact that the [Pro$^{34}$]analogues show a reasonable affinity (< 10 nM, own results, Bard *et al*., 1995, Gerald *et al*., 1996) for the Y4 receptor and that in our hands hPP, which exhibits high affinity for both Y4 and Y5 receptors, also shows the same reduced maximal response might indicate the involvement of Y4 receptors in the reduced efficacy of these

compounds. This could be in line with the localization of Y4 receptors in the hypothalamus (Bard *et al*., 1995; Trinh *et al*., 1996). The data obtained with hPP are in agreement with Clark *et al*., (1984) who observed that 10 $\mu$g hPP exhibited a 3-fold lower food intake response compared to 10 $\mu$g of NPY. In our study NPY (13–36) significantly stimulated the 2 h food intake at a dose of 32 $\mu$g which is in contrast to a recent publication by O'Shea *et al*., showing that NPY (13–36) does not induce food intake even at a dose of 50 nmol. Based on the results obtained with [Pro$^{34}$]NPY and NPY(13–36) these authors concluded that a novel receptor is involved in feeding. However the approximately 30-fold lower potency of NPY(13–36) compared to NPY to induce hyperphagia corresponds to the 25-fold lower affinity that NPY(13–36) displays for the Y5 receptor (own results, Gerald *et al*., 1996, Hu *et al*., 1996). NPY, NPY (2–36) and NPY (3–36) display equal potency to elicit a feeding response which also corresponds to the nanomolar affinity of these agonists for the Y5 receptor. Therefore, the Y5 receptor is the only receptor identified so far that is a likely candidate to be the so-called feeding receptor.

Nevertheless, it has been shown that the Y1 selective peptide BW1229 inhibits food intake in rats (Kanatani *et al*., 1996). This compound, a peptide NPY receptor antagonist, was found to be non-selective between the Y1 and Y4 receptor subtype: it exhibits highest affinity for the rat Y4 receptor ($IC_{50} = 0.062 \pm 0.017$ nM), subnanomolar affinity ($IC_{50} = 0.3 \pm 0.19$ nM) for both the human Y1 and human Y4 receptor subtype, approximately 1000-fold reduced affinity for the rat and human Y5 receptor and for the human Y2 receptor (own data, Kanatani *et al*., 1997). Since this compound displays high affinity for the Y1 receptor with antagonistic properties, the involvement of the Y1 receptor in feeding is indicated, although this compound also displays agonistic effects at the Y4 and Y5 receptor (Kanatani *et al*., 1997) which could interfere with food intake regulation. Consequently, the rather non-selective receptor subtype binding profile of BW1229 with its antagonistic effects (on the Y1 receptor) and agonistic effects on the Y2, Y4 and Y5 receptor subtypes (Kanatani *et al*., 1997) must be considered carefully when used for pharmacological studies.

Subnanomolar affinity of the novel compound BIBO 3304 was found for cells stably transfected with the human/rat Y1 receptor cDNA or cells endogenously expressing the Y1 receptor (SK-N-MC cells). BIBO 3304 displays 10–20-fold higher affinity than BIBP 3226 (Wieland *et al*., 1995b) for both the human and rat Y1 receptor subtype. The novel compound is selective for the Y1 subtype since it binds with more than 1000–10000-fold lower affinity to any of the other subtypes tested (Y2, Y4, Y5) both for human and rat species.

Both feeding elicited by endogeneous release of NPY through a 24 h fast as well as feeding induced by exogeneous NPY application was examined and BIBO 3304 was able to attenuate feeding under both circumstances. The BIBO 3304 mediated inhibition of food intake strongly indicates the involvement of the Y1 receptor in feeding and correlates with the expression of Y1 receptor transcripts in the hypothalamus (Jacques *et al*., 1996), a structure playing an important role in the control of ingestive responses (Tomaszuk *et al*., 1996) and also with the fact that membranes from rat hypothalamus revealed a high affinity binding site for BIBO 3304 which reflects the Y1 receptor. However, it contrasts to the observation that the Y1 selective compound BIBO 3304 does not only inhibit [Leu$^{31}$,Pro$^{34}$]NPY but also NPY(2–36) and NPY(3–36) induced feeding. This can be explained by an interplay between the Y1 receptor and e.g. the Y5-receptor. Our observations of approximately 70% non-Y1 receptors in the hypothalamus support this idea.

Since the Y1 receptor might be involved in an anxiolytic action (Wahlestedt *et al*., 1993; Kask *et al*., 1996) its antagonism could induce anxiogenic like side effects. In order to evaluate whether possible anxiogenic effects interfered with the BIBO 3304 mediated inhibition of feeding response the interaction between BIBO 3304 and the feeding response mediated by other stimulators, such as noradrenaline and galanin was examined. If anxiety plays a role in food intake inhibition mediated by BIBO 3304, noradrenaline and galanin induced food intake should also be inhibited. But since neither galanin nor noradrenaline induced food intake was inhibited a significant anxiogenic component in the food intake inhibitory properties of BIBO 3304 can be ruled out. It has been published earlier that BIBP 3226 mediated effects on feeding antagonism is not due to general side effects in the experimental setting used (O'Shea *et al*., 1997; Kask *et al*., personal communication). However, our own data using BIBP 3226 were not as conclusive (Doods *et al*., 1996). Consequently, the inactive enantiomer to BIBO 3304 was tested in order to show that the food intake inhibition was not due to a general toxicity of structural components of BIBO 3304. Indeed, the enantiomer did not affect the feeding response.

In the present study, the identification of BIBO 3304, a non-peptide compound that displays affinity in the subnanomolar range and selectivity for the Y1 receptor subtype is shown. We hypothesize that the Y1 receptor plays an important role in NPY induced feeding and BIBO 3304 is a novel tool to study both food intake and other central effects mediated *via* the Y1 receptor. Moreover, the data presented with BIBO 3304 as well as with the agonists, e.g. [Leu$^{31}$, Pro$^{34}$]NPY indicate a complicated interplay between the different NPY receptors in feeding behaviour.

The authors wish to thank S. Schacherl-Schmid, E. Schilling and B. Lämmle for skilled technical assistance and our colleagues from NOVO Nordisk for contributions to cDNAs and cell lines (S. Hjort, J.E. Jensen, H. Vissing, J.S. Rasmussen, P.B. Rasmussen, C.E. Stidsen).

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*(Received February 4, 1998*
*Revised June 22, 1998*
*Accepted June 25, 1998)*