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# Communications to the Editor

## Bis(31/31′){[Cys$^{31}$,Trp$^{32}$,Nva$^{34}$]NPY-(31−36)}: A Specific NPY Y-1 Receptor Antagonist

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Neuropeptide Y (NPY) is a 36-residue peptide amide abundantly distributed both in the central and peripheral nervous systems. NPY exhibits a wide spectrum of pharmacological effects mediated by Y-1, Y-2, and Y-3 receptor subtypes (see refs 1 and 2 for reviews). Most remarkable of these are effects on feeding, vasoconstriction, intestinal secretion, and cardiac contractility.[1] Moreover, tissue or plasma NPY levels have been reported to be altered under a number of pathophysiological conditions including obesity, anorexia, hypertension, and congestive heart failure. These observations suggest that NPY receptor antagonists may prove invaluable not only in delineating the physiological functions of NPY but also in developing new classes of therapeutic compounds.

A large number of antagonists with a variety of properties are required to elucidate the conformations needed to impart antagonism as well as selectivity. Toward this goal, a number of peptide- and non-peptide-based NPY receptor antagonists have been reported.[3−10] Although most of these compounds were either weak or nonselective, the two non-peptide antagonists reported recently appear to have promising properties.[9,10]

Recently, we reported that centrally truncated analogs of [D-Trp$^{32}$]NPY[4] such as Des-AA$^{7-24}$[D-Trp$^{5,32}$,Aoc$^{6}$]-NPY antagonized NPY-induced mobilization of intracellular calcium, $[Ca^{2+}]_i$, in HEL cells bearing Y-1 receptors.[11] Subsequent investigations led to the development of Des-Asn$^{29}$[D-Trp$^{28,32}$,Nva$^{34}$]NPY(27−36) and Des-Asn$^{29}$[ Trp$^{28,32}$,Nva$^{34}$]NPY(27−36).[12,13] On the basis of these findings, we have now developed specific NPY Y-1 receptor antagonists, and these results are described in this communication.

**Results and Discussion.** Centrally truncated analogs of NPY developed by Krstenansky et al.[14] have been shown to bind to Y-1 receptors with good affinity.[15] Since the hypothalamic NPY receptor antagonist, [D-Trp$^{32}$]NPY[4], did not bind to Y-1 receptors, we synthesized similar truncated analogs of [D-Trp$^{32}$]NPY and demonstrated that these analogs antagonized NPY effects on $[Ca^{2+}]_i$ in HEL cells.[11] One of these analogs, Des-AA$^{7-24}$[D-Ala$^{5}$,D-Trp$^{32}$,Aoc$^{6}$]NPY, was also shown to antagonize NPY-induced hypertension in rats.[11] Further structure−activity studies resulted in the development of Y-1 receptor antagonists, Des-Asn$^{29}$[D-Trp$^{28,32}$]-NPY(27−36) ($K_i$ for Y-1 > 10 000 nM) and Des-Asn$^{29}$[D-Trp$^{28,32}$,Nva$^{34}$]NPY(27−36) ($K_i$ for Y-1 = 328 ± 86 nM).[12,13] Although we speculated that the turn structures induced by both D-Trp$^{28}$ and D-Trp$^{32}$ may be crucial to imparting antagonistic properties, solution NMR studies revealed the existence of two quasihelical loop structures rather than $\beta$-turns.[12,13] Therefore, we synthesized and investigated the properties of Des-Asn$^{29}$[Trp$^{28,32}$,Nva$^{34}$]NPY(27−36). Although this peptide retained the antagonistic property, neither the affinity ($K_i$ for Y-1 = 359 ± 93 nM) nor the selectivity for Y-1 receptors increased compared to those of its D-Trp counterpart.[12,13]

2D NMR studies revealed that NPY exists as a dimer in solution with predominantly an α-helical structure in the C-terminal 11−36 region.[16−18] This observation and the presence of helical structures in our antagonists led us to speculate that dimerization of our antagonists may enhance the receptor affinity *via* the stabilization of the helical structures. A series of C-terminal NPY-(27−36) analogs dimerized *via* Cys$^{31}$ were therefore synthesized, and their properties were investigated using SK-N-MC and HEL (Y-1 receptors), and SK-N-BE2 (Y-2) cells. Position 31 was chosen for dimerization because previous studies have shown that residues 32−

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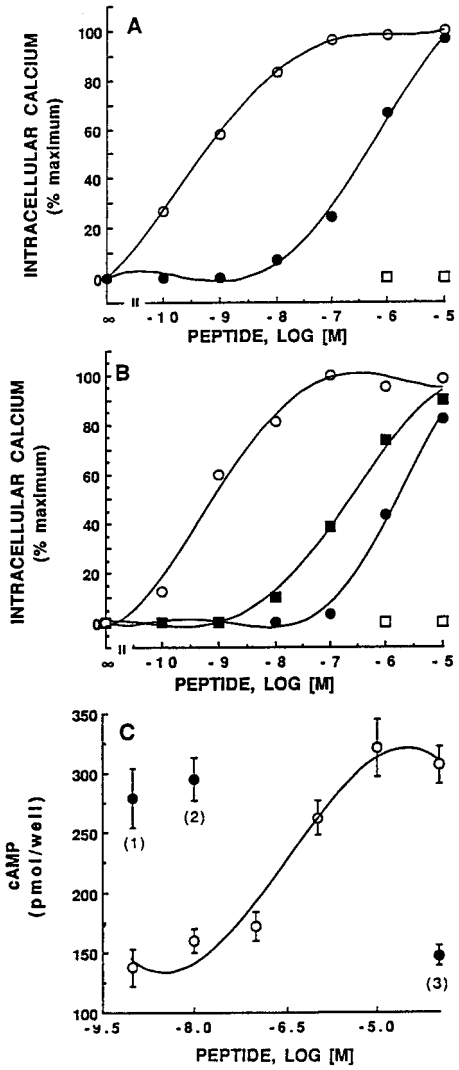


**Figure 1.** (A and B) Antagonism of NPY-induced intracellular calcium mobilization in HEL cells by (A) **4**, bis(31/31′)-{[Cys$^{31}$,Trp$^{32}$,Nva$^{34}$]NPY(27−36)}; (B) **8**, bis(31/31′){[Cys$^{31}$,Trp$^{32}$,-Nva$^{34}$]NPY(31−36). NPY alone (○), antagonist alone (□), NPY in the presence of 10 (●) and 1.0 (■) $\mu$M antagonist. Each point is the mean of of a typical experiment performed in triplicate. Similar results were obtained in at least two other experiments. (C) Antagonism of the inhibitory effect of NPY (10 nM) on isoproterenol (10 $\mu$M) stimulated cAMP synthesis by SK-N-MC cells by increasing concentrations of **8**, bis(31/31′)-{[Cys$^{31}$,Trp$^{32}$,Nva$^{34}$]NPY(31−36)} (○). Also shown are the effects on cAMP synthesis by 10 $\mu$M isoproterenol in the absence (1) and presence of 10 $\mu$M **8** (2) or 10 nM NPY (3). Each point is the mean ± SD of three experiments.

36 are important for binding and/or imparting antagonistic properties.[12,13] Since replacement of Gln$^{34}$ with Nva increases the hydrophobicity without losing the carbon backbone structure, [NHCH(CO)CH$_2$CH$_2$CONH$_2$ vs NHCH(CO)CH$_2$CH$_2$CH$_3$], and has been found to increase Y-1 receptor affinity 30-fold in monomers,[12,13] we synthesized bis(31/31′){[Cys$^{31}$,Nva$^{34}$]NPY(27−36)} (**2**, Table 1). Consistent with our expectations, this dimerization enhanced both the selectivity and afffinity to Y-1 receptors. Dimer **3**, deleting Asn$^{29}$, was then synthesized to shorten the peptide as well as to increase the proteolytic stability. It should be noted that the Asn$^{29}$−Leu$^{30}$ bond has been shown to be susceptible for proteolytic cleavage.[19] This peptide exhibited an affinity and selectivity comparable to that of **2** (Table 1), confirming our previous observations[12,13] that Asn$^{29}$ is not important for Y-1 binding. However, both **2** and **3** weakly mobilized [Ca$^{2+}$]$_i$ in HEL cells at 10 $\mu$M. As we have observed with our monomer antagonists, the Trp$^{32}$ substitution in **2** suppressed the residual agonist activity. This compound, **4**, retained the Y-1 receptor affinity and selectivity of **2** (Table 1) and antagonized the effects of NPY on [Ca$^{2+}$]$_i$ in HEL cells (Figure 1A).

Additional structure−activity studies were performed with **4** in an attempt to improve the antagonistic properties. However, deletion of Asn$^{29}$ as in **5** or both substitution of Ile$^{28}$ with Trp and deletion of Asn$^{29}$ as in **6** resulted in decreased Y-1 receptor affinity and selectivity (Table 1). Similar results were obtained with **7** containing D-Trp$^{28,32}$ subtitutions as in our monomers.[12,13] For these reasons, the properties of these dimers were not investigated in detail. Nevertheless, our preliminary investigations in HEL cells revealed that all these compounds, **5**, **6**, and **7**, retained Y-1 receptors antagonistic properties.

Since our goal is to develop short, potent, and selective antagonists, a truncated dimer, without residues 27−30, was also synthesized. This peptide, bis(31/31′)-{[Cys$^{31}$,Trp$^{32}$,Nva$^{34}$]NPY(31−36)} (**8**), bound almost exclusively to Y-1 receptors with affinity comparable to that of **4** (Table 1). Moreover, it antagonized NPY effects on [Ca$^{2+}$]$_i$ in HEL cells (Figure 1B). Also, the inhibitory effect of NPY on isoproterenol-stimulated cAMP synthesis by SK-N-MC cells was dose-dependently abolished by **8** with an IC$_{50}$ value of 266 ± 84 nM (Figure 1C). Bis(31/31′){[Cys$^{31}$]NPY(31−36)} exhibited neither agonist nor antagonistic properties in HEL cells (not shown) exemplifying the importance of Nva$^{34}$ and Trp$^{32}$ for imparting Y-1 receptor affinity and antagonism. Furthermore, the loss of Y-2 receptor affinity on going from **4** to **8** suggests that residues 27−30 may play a key role(s) in binding to Y-2 receptors. This observation is consistent with the determinations by Leban et al.[6] that the Tyr$^{27}$ and the Asn$^{29}$ turn structure in NPY(27−36) may be important for binding toY-2 receptors. Our findings suggest that the C-terminal hexapeptide moiety contains the structural features essential for binding to Y-1 receptors. This observation, and the development of nonapeptide Y-1 receptor antagonists by Leban et al.[6] and us,[12,13] alter the previously held notion that the both N- and C-termini are necessary for Y-1 receptor binding.[20] These findings will prove useful in the development of therapeutic compounds based on NPY.

We also tested the effects of intrahypothalamic administration of **4** and **8** on feeding in rats. Neither of these compounds exhibited agonist or antagonist effects, again confirming the high selectivity of these compounds for Y-1 receptors. It should be noted that feeding effects of NPY are now believed to be mediated by yet another undidentified receptor subtype.[4,21]

Lastly, while this manuscript was in preparation, Daniels et al.[22] reported the development of NPY Y-1 receptor antagonists based on C-terminal nonapeptide dimers. These dimers, unlike the antagonists described here, exhibit nearly equal affinity for both Y-1 and Y-2 receptors. Moreover, one of these compounds was also shown by Ihara et al.[23] to antagonize NPY-iduced feeding. These observations once again emphasize the importance of Trp$^{32}$ and Nva$^{34}$ substitutions for imparting selective antagonism at Y-1 receptors.

**Table 1.** Y-1 and Y-2 Receptor Affinities of NPY Receptor Antagonists

| peptides | mass found | receptor affinity ($K_i$, nM)[a] SK-N-MC (Y-1) | SK-N-BE2 (Y-2) |
|---|---|---|---|
| **1**, NPY | 4254.3 | 2.0 ± 0.66 | 0.29 ± 0.00 |
| **2**, bis(31/31′){[Cys$^{31}$,Nva$^{34}$]NPY(27−36)} Tyr−Ile−Asn−Leu−Cys−Thr−Arg−Nva−Arg−Tyr−$NH_2$ \| Tyr−Ile−Asn−Leu−Cys−Thr−Arg−Nva−Arg−Tyr−$NH_2$ | 2597.1 | 36 ± 8 | 2085 ± 29 |
| **3**, bis(31/31′){Des-Asn$^{29}$[Cys$^{31}$,Nva$^{34}$]NPY(27−36)} | 2369.5 | 49 ± 10 | 3980 ± 481 |
| **4**, bis(31/31′){[Cys$^{31}$,Trp$^{32}$,Nva$^{34}$]NPY(27−36)} | 2769.9 | 44 ± 12 | 3406 ± 895 |
| **5**, bis(31/31′){Des-Asn$^{29}$[Cys$^{31}$,Trp$^{32}$,Nva$^{34}$]NPY(27−36)} | 2539.0 | 95 ± 11 | 988 ± 79 |
| **6**, bis(31/31′){Des-Asn$^{29}$[Trp$^{28,32}$,Cys$^{31}$,Nva$^{34}$]NPY(27−36)} | 2685.0 | 93 ± 15 | 621 ± 29 |
| **7**, bis(31/31′){Des-Asn$^{29}$[D-Trp$^{28,32}$,Cys$^{31}$,Nva$^{34}$]NPY(27−36)} | 2685.8 | 143 ± 30 | 704 ± 210 |
| **8**, bis(31/31′){[Trp$^{32}$,Cys$^{31}$,Nva$^{34}$]NPY(31−36)} | 1759.8 | 46 ± 14 | >10,000 |
| **9**, [Leu$^{31}$,Pro$^{34}$]NPY | 4223.1 | 2.69 ± 0.60 | 215 ± 78 |

[a] Increasing concentrations of NPY analogs were used to displace [$^{125}$I]PYY (SK-N-MC) or [$^{125}$I]PYY(3−36) (SK-N-BE2) bound to membranes prepared from cells. The $IC_{50}$ values obtained from these experimentss were used to calculate the $K_i$ values. Data presented represents the mean ± SEM of at least three experiments performed in triplicate.

In summary, we have constructed a specific NPY Y-1 receptor antagonist based on NPY C-terminal hexapeptide. To our knowledge, this is the first peptide-based specific Y-1 receptor antagonist to be described. This antagonist has properties similar to the two non-peptide antagonists reported recently.[9,10] Further studies with these compounds should result in more potent and therapeutically useful NPY receptor antagonists.

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