Modified Peptides

Peptide Modifications Differentially Alter G Protein-Coupled Receptor Internalization and Signaling Bias**

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Abstract: *Although G protein-coupled receptors (GPCRs) are targeted by more clinically used drugs than any other type of protein, their ligand development is particularly challenging. Humans have four neuropeptide Y receptors: hY_1R and hY_5R are orexigenic, while hY_2R and hY_4R are anorexigenic, and represent important anti-obesity drug targets. We show for the first time that PEGylation and lipidation, chemical modifications that prolong the plasma half-lives of peptides, confer additional benefits. Both modifications enhance pancreatic polypeptide preference for hY_2R/hY_4R over hY_1R/hY_5R. Lipidation biases the ligand towards arrestin recruitment and internalization, whereas PEGylation confers the opposite bias. These effects were independent of the cell system and modified residue. We thus provide novel insights into the mode of action of peptide modifications and open innovative venues for generating peptide agonists with extended therapeutic potential.*

The neuropeptide Y (NPY) hormone family signals through cell-surface receptors belonging to class A GPCRs.[1] With four Y receptors expressed in humans (hY_1R, hY_2R, hY_4R, hY_5R) and the three endogenous agonists NPY, peptide YY, and pancreatic polypeptide (PP),[2] this system is involved in many physiological processes, such as food intake.[3] Both hY_1R and hY_5R mediate orexigenic effects, whereas activa-tion of hY_2R and hY_4R produce anorexigenic signals. Clearly, selective stimulation or blocking of the differentially acting Y receptors are favorable approaches for anti-obesity thera-peutics.[4] Such a potential anti-obesity drug is Obinepitide (7TM Pharma), which is the human PP-derived ligand [Q^{34}]hPP with a dual specific agonism for hY_4R and hY_2R.[5] Although Obinepitide was active in vivo, it was hampered by a poor pharmacokinetic profile because of its rapid clearance and degradation.[6]

Here, we investigated the impact of two commonly used chemical modifications—methoxy polyethylene glycol (PEGylation) and fatty acids (lipidation)[7]—on Obinepitide stability and, for the first time, on receptor signaling and endocytosis. The peptide core sequence [Q^{34}]hPP was modi-fied with palmitic acid (Pam) and diverse PEGs (5 kDa (PEG5) and 22 kDa (PEG22)) at the N^ε groups of lysine-22, along with N-terminal labeling with fluorescent 6-carboxy-tetramethylrhodamine (TAMRA) (Figure 1). The high purity of all the peptides and maintenance of the α-helical character were confirmed, while in vitro studies using human blood plasma verified the increased metabolic stability of the PEGylated and palmitoylated analogues (see Tables S1 and S2 as well as Figure S1 in the Supporting Information).

The lead peptide and all the analogues were potent agonists at the hY_4R (see Figure S2 in the Supporting

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a)

hPP   H-APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH$_2$
[K^{22},Q^{34}]hPP   H-APLEPVYPGDNATPEQMAQYA**K**DLRRYINMLTR**Q**RY-NH$_2$

b)

pancreatic polypeptide → modification
C
lipidation
PEGylation
N
fluorescence label

c)

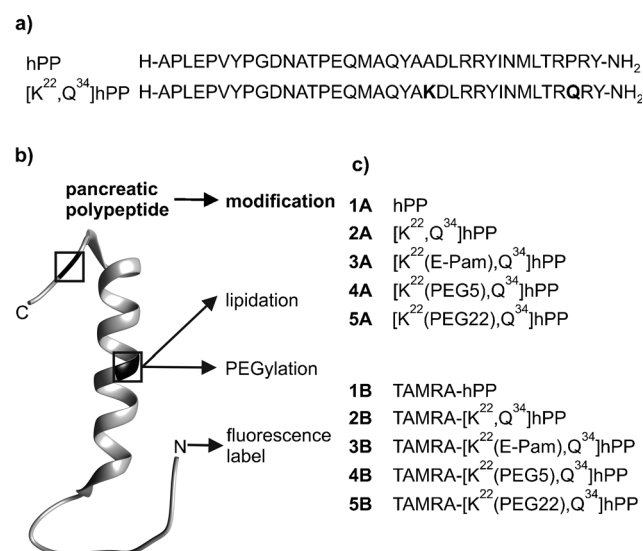|	
1A	hPP
2A	[K^{22},Q^{34}]hPP
3A	[K^{22}(E-Pam),Q^{34}]hPP
4A	[K^{22}(PEG5),Q^{34}]hPP
5A	[K^{22}(PEG22),Q^{34}]hPP
1B	TAMRA-hPP
2B	TAMRA-[K^{22},Q^{34}]hPP
3B	TAMRA-[K^{22}(E-Pam),Q^{34}]hPP
4B	TAMRA-[K^{22}(PEG5),Q^{34}]hPP
5B	TAMRA-[K^{22}(PEG22),Q^{34}]hPP

Figure 1. a) Sequences and b) ribbon model (PDB ID: 1LJV) with highlighted positions of modifications. c) hPP analogues modified by palmitoylation, PEGylation, and TAMRA.

Table 1: Signal transduction determined by the inositol phosphate accumulation assay, for all peptide–receptor combinations.

compound	EC_{50}/nM ($pEC_{50} \pm$ S.E.M.)			
	hY_1R	hY_2R	hY_4R	hY_5R
pNPY	3.3	0.3	44.7	4.7
	(8.5 ± 0.1)	(9.6 ± 0.1)	(7.4 ± 0.3)	(8.3 ± 0.1)
1A	76.0	341.4	1.3	11.4
	(7.1 ± 0.1)	(6.5 ± 0.1)	(8.9 ± 0.1)	(7.9 ± 0.1)
2A	51.6	0.4	1.0	6.5
	(7.3 ± 0.1)	(9.4 ± 0.2)	(9.0 ± 0.2)	(8.2 ± 0.2)
3A	83.5	2.8	2.1	6.7
	(7.1 ± 0.0)	(8.6 ± 0.2)	(8.7 ± 0.1)	(8.2 ± 0.3)
4A	>1000	11.7	2.9	195.3
	(5.2 ± 0.1)	(7.9 ± 0.1)	(8.5 ± 0.1)	(6.7 ± 0.2)
5A	>1000	32.4	13.8	>1000
	(4.7 ± 0.1)	(7.5 ± 0.1)	(7.9 ± 0.1)	(5.9 ± 0.1)

Information), as indicated by the pEC_{50} values being comparable to those of hPP, and are also relatively well-tolerated by the hY_2R, similar to its native ligand NPY (Table 1). Importantly, the same analogues showed low activities at hY_1R. Nanomolar potencies are mostly observed at hY_5R. However, as hY_5R is predominantly expressed in the brain, it is not necessarily accessible to peripherally administered peptide drugs. Radioligand displacement experiments showed that the palmitoylated ligand maintained nanomolar affinity for hY_4R (Figure 2; IC_{50} values: **1A**: 0.7, **2A**: 0.5, **3A**: 1.2 nM). However, the addition of a PEG moiety with an increased size significantly reduced the hY_4R affinity (IC_{50}: **4A**: 36.8, **5A**: 94.2 nM). As reported earlier, restricted binding[6,8] of PEGylated peptides might result from steric hindrance of agonist–receptor interactions.
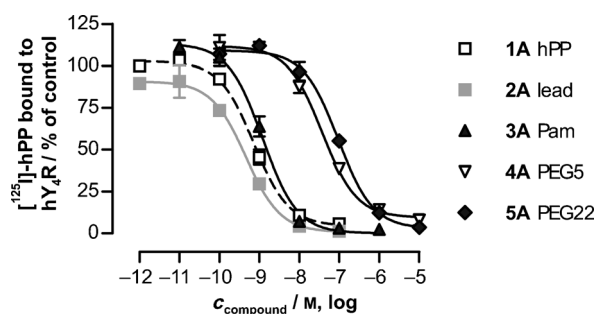

Figure 2. Mean [^{125}I]-hPP displacement curves at the hY_4R.

Fluorescence microscopy was used to characterize the uptake of the TAMRA-labeled hPP variants by receptor-mediated endocytosis in HEK293 cells stably expressing the hY_4R–EYFP fusion protein (Figures 3a and 4a, see also Figure S3 in the Supporting Information). In response to ligand stimulation, intracellular accumulation of TAMRA fluorescence was seen for hPP (**1B**) and lead peptide (**2B**), while palmitoylation (**3B**: ca. 150%) facilitated internalization significantly. Surprisingly, PEGylated variants (**4B**, **5B**#: <2%) showed markedly decreased endocytosis with no detectable peptide uptake. Specific [^{125}I]-hPP binding to
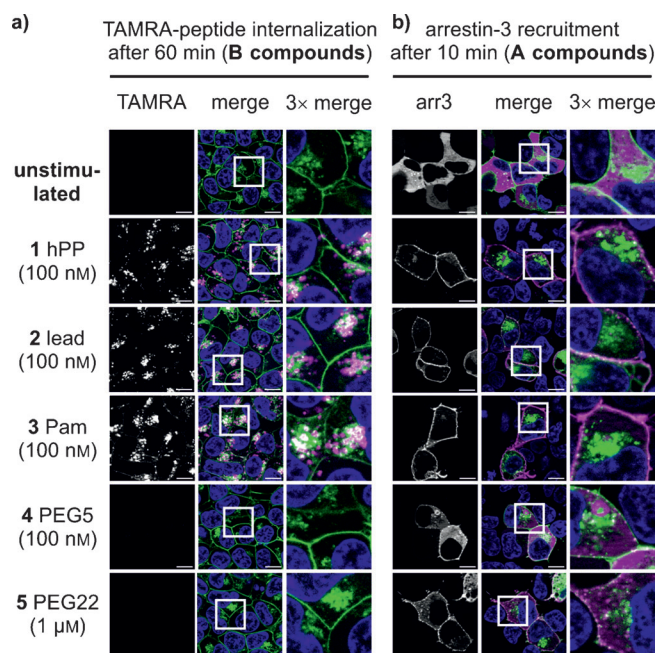

Figure 3. a) hY_4R-mediated uptake of TAMRA-modified peptide analogues and b) arr3 recruitment. White vesicles in the overlayed channel indicate co-localization of hY_4R (green) and peptide or arr3 (magenta). Scale bar: 10 µm.
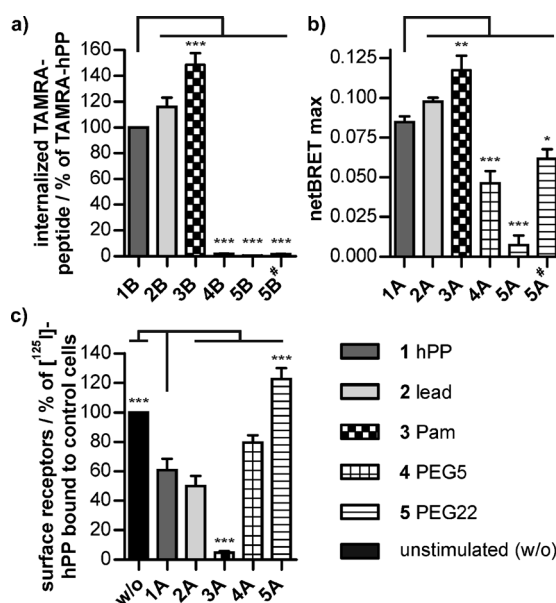

Figure 4. a) Internalization of TAMRA-labeled peptides after 60 min stimulation. b) Agonist-induced arr3 association after 10 min measured by BRET. c) Cell-surface receptors before (w/o) and after 60 min exposure to the agonist. The stimulation concentration was 100 nM for all compounds except for **5**# (1 µM).

cell-surface receptors after 1 h stimulation (Figure 4c) confirmed that the palmitoylated variant strongly increased internalization of the ligand–receptor complex, thereby eliminating virtually all the [^{125}I]-hPP binding sites from the cell surface (**3A**: <5%). PEGylated peptides did not induce internalization or loss of cell-surface receptor binding (**4A**:

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80%, **5A**: 120%). Importantly, these effects were specific because they required covalent attachment of the modification to the peptide and its binding and activation of the cognate receptor (see Figure S4 in the Supporting Information).

Arrestin recruitment was visualized using HEK293 cells transiently expressing EYFP-tagged hY_4R and mCherry-fused arrestin-3 (arr3; Figure 3b). Rapid arr3 redistribution was seen after stimulation by all the analogues, which is in full agreement with previous demonstrations of arr3 involvement[9] in Y_4R internalization.[10] Only the PEGylated variants (**4A**, **5A**[#]) showed reduced arr3 recruitment. Arr3–receptor interactions were further quantified by bioluminescence resonance energy transfer (BRET; Figure 4b). The high maximal netBRET (see Figure S5 in the Supporting Information) showed robust arr3 recruitment by hPP (**1A**) and lead peptide (**2A**). Stimulation with the palmitoylated analogue (**3A**) increased arr3 recruitment, whereas PEGylated peptides (**4A**, **5A**) showed considerably reduced $BRET_{max}$ values in a concentration-dependent manner. Thus, ligand palmitoylation enhanced internalization and arr3 recruitment to the receptor without showing increased potency and affinity. This is in contrast to other receptors, where lipidation of the ligand led to enhanced receptor activity.[11]

However, the effects of lipidation critically depend on the length of the fatty acid chain, as peptides modified with shorter fatty acids (see Figure S6 in the Supporting Information) did not enhance arr3 recruitment or internalization (see Figure S7 in the Supporting Information).

PEGylated peptides, in contrast, showed diminished arr3 recruitment and no receptor internalization. PEGylation of various molecules has resulted either in enhanced[12] or hindered internalization,[13] but the exact sizes of the attached PEG were inconsistent.[14] In the present study, a low-molecular-weight PEG moiety (PEG5) attached to the peptide resulted in high activity, despite impaired uptake, arr3 recruitment, and receptor internalization. Clearly, the receptor conformation is biased towards the G protein for lower molecular weight PEG moieties.

To investigate the behavior of the peptide analogues in a more translatable system, the antisecretory responses (I_{sc})[15] were measured in Col-24 epithelial layers, where the lead peptide showed a rapid and transient activity with maximum effects at about 5 min (Figure 5a), as observed previously for hPP.(15) In contrast, the response onset to palmitoylated (**3A**) and PEGylated (**4A**) hPP variants was delayed, and both analogues showed prolonged reductions in I_{sc}, with **3A** exhibiting a higher maximum than the parental peptide (**2A**). Interestingly, **5A** did not alter the I_{sc} value during 45 min exposure. Analogous time courses were obtained in human colon mucosa (which expresses hY_2R and hY_4R; see Figure S8a in the Supporting Information).[16]

Concentration–response curves in Col-24 cells revealed good activity and increased efficacy for **3A**, and one order of magnitude enhanced potency (but unaltered efficacy) for **4A** (see Figure S8b in the Supporting Information). Prestimulation with **2A** or **3A**, but not with **4A**, resulted in a concentration-dependent desensitization of hY_4R (Figure 5b–d).
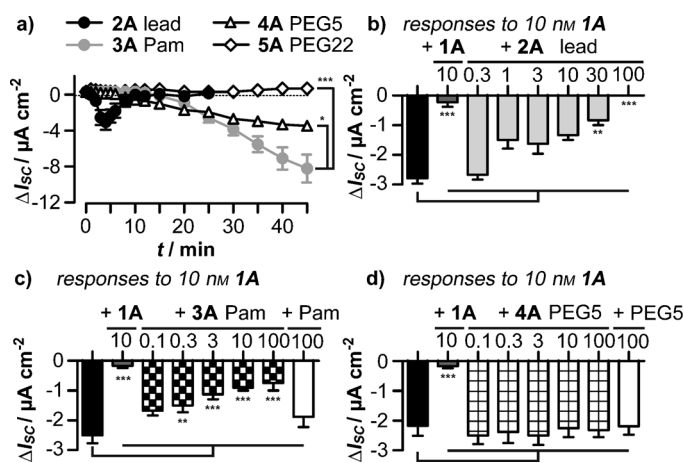

Figure 5. Responses of human Col-24 epithelial layers after treatment with a) 100 nM **2A**, **3A**, **4A**, or **5A**. Desensitization of responses to 10 nM hPP after the indicated concentrations of **1A** (b–d), or **2A** (b), **3A** (c), or **4A** (d) in nM. The black bars show responses to hPP without prestimulation. The white bars in (c,d) reflect hPP responses after the addition of 100 nM unconjugated Pam or PEG5.

In Col-24 cells, additionally transfected with hY_4R-EYFP, we also observed rapid internalization of native (**1B**), lead (**2B**), and palmitoylated (**3B**) peptides, whereas the PEGylated derivative (**5B**) showed impaired cellular uptake (Figure 6a,b).

The impact of peptide modification on hY_4R activation and internalization were also transferable to hY_2R, despite displaying a distinct binding mode with respect to hY_4R (see Figure S9 in the Supporting Information). Moreover, we investigated a different set of chemically modified peptides, namely [K^{13},Q^{34}]hPP conjugates, which we had fully characterized previously,[6] bearing Pam as well as PEGs of different sizes at position 13 and another fluorophore (5(6)-carboxy-fluorescein (CF)) at the N-terminus (Figure 6c). These derivatives were tested for internalization and arr3 recruitment in HEK293 cells expressing hY_2R (Figure 6d–f). Increased receptor-mediated uptake of the peptide upon palmitoylation (**3D**) and significantly reduced internalization of PEGylated derivatives (**4D**, **5D**[#]) was confirmed by live-cell microscopy. Enhanced arr3 recruitment for **3C** compared to porcine NPY (**1C**) and the lead peptide (**2C**) was observed, while minimal arr3 recruitment was detected with **4C** or **5C**. These results are in good agreement with the data obtained for [K^{22},Q^{34}]hPP peptides, thus showing that the impact of different modifications depends only on their nature and is transferable to different receptor and peptide combinations.

In conclusion, our data show that different chemical modifications, used to improve the metabolic stability of ligands, can diametrically bias peptide agonists towards either G proteins or arrestins (Figure 7). Long fatty acids bias the agonist towards arrestins, which results in enhanced internalization of the receptor. In contrast, PEGylation allows G-protein activation but disrupts arr3 recruitment, thereby blocking internalization and receptor desensitization. These new findings provide powerful tools to control ligand uptake and receptor response. Accordingly, the type of modification
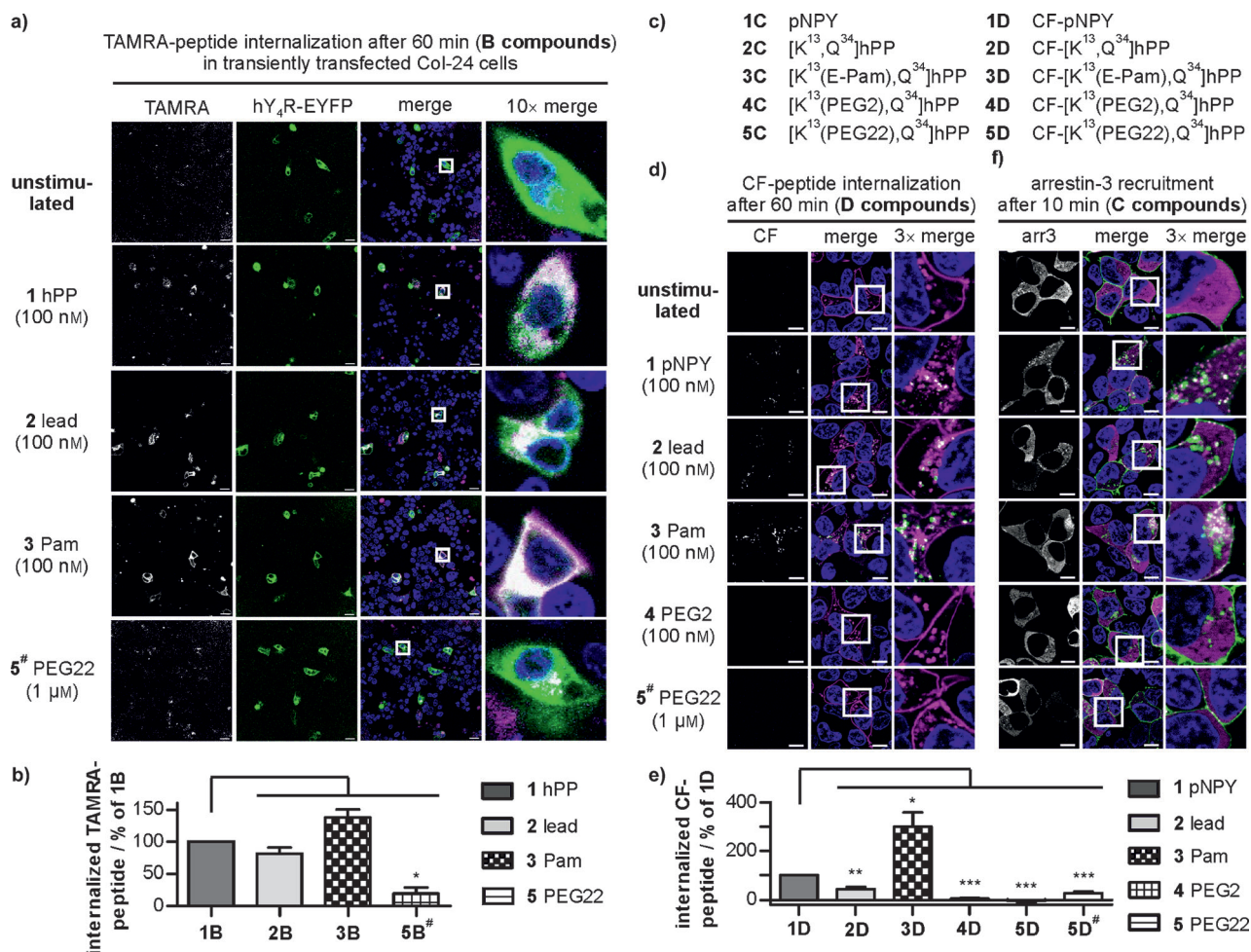
a)

TAMRA-peptide internalization after 60 min (**B compounds**)
in transiently transfected Col-24 cells

	TAMRA	hY$_4$R-EYFP	merge	10× merge
unstimu-lated				
1 hPP (100 nM)				
2 lead (100 nM)				
3 Pam (100 nM)				
5$^{\#}$ PEG22 (1 µM)				

c)

1C	pNPY	**1D**	CF-pNPY
2C	[K^{13},Q^{34}]hPP	**2D**	CF-[K^{13},Q^{34}]hPP
3C	[K^{13}(E-Pam),Q^{34}]hPP	**3D**	CF-[K^{13}(E-Pam),Q^{34}]hPP
4C	[K^{13}(PEG2),Q^{34}]hPP	**4D**	CF-[K^{13}(PEG2),Q^{34}]hPP
5C	[K^{13}(PEG22),Q^{34}]hPP	**5D**	CF-[K^{13}(PEG22),Q^{34}]hPP

d)

CF-peptide internalization
after 60 min (**D compounds**)

f)

arrestin-3 recruitment
after 10 min (**C compounds**)

	CF	merge	3× merge		arr3	merge	3× merge
unstimu-lated							
1 pNPY (100 nM)							
2 lead (100 nM)							
3 Pam (100 nM)							
4 PEG2 (100 nM)							
5$^{\#}$ PEG22 (1 µM)							

b)

internalized TAMRA-peptide / % of 1B

- **1** hPP
- **2** lead
- **3** Pam
- **5** PEG22

(bars: 1B, 2B, 3B, 5B$^{\#}$)

e)

internalized CF-peptide / % of 1D

- **1** pNPY
- **2** lead
- **3** Pam
- **4** PEG2
- **5** PEG22

(bars: 1D, 2D, 3D, 4D, 5D, 5D$^{\#}$)

Figure 6. a) Live-cell images and b) quantification of ligand uptake (magenta) after 60 min exposure in Col-24 cells transiently transfected with hY$_4$R (green). Scale bar: 20 µm. c) Porcine NPY (pNPY) and modified [K^{13},Q^{34}]hPP analogues. Imaging (d) and quantification (e) of the hY$_2$R-mediated (magenta) internalization of CF compounds (green) after 60 min stimulation. f) Arr3 distribution (magenta) after 10 min stimulation of hY$_2$R (green) in transiently transfected HEK293 cells. Scale bar: 10 µm. Peptide concentration: 100 nM, except for **5 B$^{\#}$** and **5 D$^{\#}$** (1 µM).

lipidation   **PEGylation**

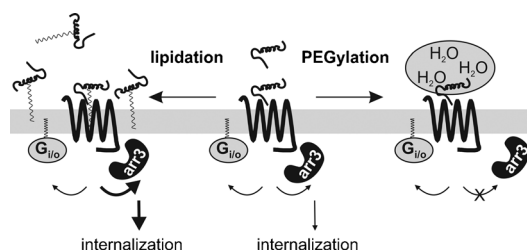internalization   internalization

Figure 7. Impact of peptide modifications on agonist bias.

to improve the half-life of peptide drugs deserves careful consideration in the future.

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