Elsevier Editorial System(tm) for Peptides
Manuscript Draft

Manuscript Number: PEPTIDES-D-17-00263R1

Title: Peptide welding technology - a simple strategy for generating innovative ligands for G protein coupled receptors

Article Type: Review Article

Keywords: peptides, GPCR, PWT, nociceptin/orphanin FQ, opioids, neurokinins, neuropeptide S

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Abstract: Based on their high selectivity of action and low toxicity, naturally occurring peptides have great potential in terms of drug development. However, the pharmacokinetic properties of peptides, in particular their half life, are poor. Among different strategies developed for reducing susceptibility to peptidases, and thus increasing the duration of action of peptides, the generation of branched peptides has been described. However, the synthesis and purification of branched peptides is extremely complicated thus limiting their druggability. Here we present a novel and facile synthesis of tetrabranched peptides acting as GPCR ligands and their in vitro and vivo pharmacological characterization. Tetrabranched derivatives of nociceptin/orphanin FQ (N/OFQ), N/OFQ related peptides, opioid peptides, tachykinins, and neuropeptide S were generated with the strategy named peptide welding technology (PWT) and characterized by high yield and purity of the desired final product. In general, PWT derivatives displayed a pharmacological profile similar to that of the natural sequence in terms of affinity, pharmacological activity, potency, and selectivity of action in vitro. More importantly, in vivo studies demonstrated that PWT peptides are characterized by increased potency associated with long lasting duration of action. In conclusion, PWT derivatives of biologically active peptides can be viewed as innovative pharmacological tools for investigating those conditions and states in which selective and prolonged receptor stimulation promotes beneficial effects.

Response to Reviewers: see attached file

Dear Prof Herzig,

Thanks for your kind mail dated 28/07/2017 about our manuscript PEPTIDES-D-17-00263 GA Olson & RD Olson 2014/15 Prize review entitled "Peptide wilding technology – a simple strategy for generating innovative ligands for G protein coupled receptors".

We read with great attention all the comments and points raised by the reviewers and we did our best to cope with the reviewer requests. In the following lines you may find the comments/criticisms raised by the reviewers (in italic) about the original manuscript and our answers. The revised version of the manuscript has been generated using the track change mode of MS Word.

We hope that you and your referees will find the revised version of the manuscript suitable for publication in Peptides.

With best regards

Sincerely

Grolamo Calo'

Reviewer: #1

The authors discussed new technology, called peptide welding technology (PWT), which allows improvements of the peptide in terms of affinity, pharmacological properties. This manuscript is written well however, some concerns still exist. The manuscript will be much improved caring these suggestions.
We thank Reviewer #1 for his/her kind words (well written manuscript) and for his/her comments and suggestions which contributed to improve our manuscript.

Major comments:

(1) l52 cRGD is a good example, but D amino acid in small cyclic peptide is a general feature, since they never precede cyclization without D-amino acid. It would be better to refer another small cyclic peptide antagonist especially against GPCR, such as J Pharmacol Exp Ther. 1996 Nov;279(2):675-85. or something.
We do agree with Reviewer #1 on this point and the suggested reference has been included in the revised version of the manuscript.

(2) One main subject you mentioned in the abstract is stability of the peptide. Because many readers are curious about this and to this end, PEGylation is known as robust approach. The authors should address little bit more about pegylation.
As suggested by Reviewer #1 a new sentence and reference about PEGylation has been included in the revised version of the manuscript.

(3) For example, although it is said to be 4 fold potent in EC50, if 4 times the molar number as monomer is contained in one molecule, it is substantially the same. The author should clarify this point.
As requested by Reviewer #1 this point has been clarified in the revised version of the manuscript.

(4) p10 l1 The authors used Schild plot, however, it is a kind of analysis based on pharmacological effects. Exactly Scatchard plot is required to discuss if the molecules are competitive or not.
We do not agree with Reviewer 1 on this specific point; in fact the Schild analysis is the correct approach to the evaluation of receptor antagonism in pharmacological studies. Details about this issue can be found in the following article: Neubig RR, Spedding M, Kenakin T and Christopoulos A (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev* **55**:597-606.

(5) p24 B Especially about core structure, the authors should depict the structure with more clear. Three conjugation manners are possible with Lys, alpha, epsilon and C. And also how about the linkers? The authors used bifunctional cross linkers to introduced maleimide, right?
We are not sure to fully understand the point raised by the Reviewer. However the structure of PWT cores is shown in figure 2 and the general structure of PWT peptides is depicted in figure 1B. Moreover as clearly stated at the end of section 3, details about the experimental conditions and procedures for the synthesis of PWT cores and PWT peptides are reported in Guerrini et al., 2014.

Minor comments:

p4 l41&l58: N- (italic)
p4 l51, 52: why CH3 instead methylation at l51 and in l52 as Me instead CH3. I recommend them consistently.
p5 l23 need "-" GLP-
p7 l20 et al. italic
p7 l12 Michael instead Michel and need "," and also it is not so general to use "thiol-Michael reaction". Simply "Michael reaction with thiol"
p10 l31 in vivo (italic)
All corrections and small changes suggested by Reviewer 1 have been included in the revised version of the manuscript.

Have you investigated dissociation rate from the receptors? It would be a good information for the readers.
We fully agree with Reviewer #1 on this point and actually we are starting to investigate this aspect in the frame of a collaborative study. However at present we do not have results.

Reviewer #2:

The authors review different strategies of prolonging in vivo life of peptide ligands of GPCRs, specifically focusing on the approach they call peptide welding technology that generates tetra-branched peptides. Overall, the review is useful. However, excessively detailed section 4 should be greatly compressed and merged with section 5 (the latter summarizes salient points of section 4). In addition, some editing is needed: p. 4, line 16, and p. 18, lines 9-10, and Table 1 "amino acid residues" should be either "amino acids" or "residues"; throughout "terminal" should be "terminus"; etc.

We thank Reviewer #2 regarding his/her positive comments about the usefulness of our review.

To cope with the Reviewer request we tried to shorten section 4 that summarizes published results (subsections 1, 2, 5, and 6) and describes novel findings (subsections 3 and 4). However we were unable to substantially shorten this section because we consider the information reported here not only useful but also necessary for the reader to fully appreciate the critical analysis of the overall results presented in section 5. Finally we rechecked the manuscript in order to correct typos and eventually errors, as suggested by this Reviewer.

***Highlights (for review)**

- the PWT strategy allows the facile synthesis of tetrabranched peptides
- in vitro PWT derivatives maintain the pharmacological features of parent peptides
- in vivo PWT derivatives displayed high potency and particularly long lasting action

Peptide welding technology – a simple strategy for generating innovative ligands for G protein coupled receptors

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Abstract

Based on their high selectivity of action and low toxicity, naturally occurring peptides have great potential in terms of drug development. However, the pharmacokinetic properties of peptides, in particular their half life, are poor. Among different strategies developed for reducing susceptibility to peptidases, and thus increasing the duration of action of peptides, the generation of branched peptides has been described. However, the synthesis and purification of branched peptides is extremely complicated thus limiting their druggability. Here we present a novel and facile synthesis of tetrabranched peptides acting as GPCR ligands and their *in vitro* and vivo pharmacological characterization. Tetrabranched derivatives of nociceptin/orphanin FQ (N/OFQ), N/OFQ related peptides, opioid peptides, tachykinins, and neuropeptide S were generated with the strategy named peptide welding technology (PWT) and characterized by high yield and purity of the desired final product. In general, PWT derivatives displayed a pharmacological profile similar to that of the natural sequence in terms of affinity, pharmacological activity, potency, and selectivity of action *in vitro*. More importantly, *in vivo* studies demonstrated that PWT peptides are characterized by increased potency associated with long lasting duration of action. In conclusion, PWT derivatives of biologically active peptides can be viewed as innovative pharmacological tools for investigating those conditions and states in which selective and prolonged receptor stimulation promotes beneficial effects.

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Contents

1. Introduction

Peptides play important roles in controlling several different biological functions and can be viewed as valuable models for the development of innovative drugs. The advantage of peptides as drugs are the very high selectivity of action associated to a virtual absence of toxicity. However, the poor pharmacokinetic properties of peptides, particularly their short half life, represent an important limit in terms of druggability. To display drug like pharmacokinetic properties, a new molecule should be compliant with the Lipinski's rules [1] i.e. a molecular weight less than 500 Da, no more than 5 H-bond donors, and no more than 10 H-bond acceptors atoms. These rules are matched only by very short peptides e.g. 3-4 amino acids ~~residues~~. More importantly, peptides are in general very rapidly metabolized by peptidases with a consequent short half life and duration of action. Despite their poor pharmacokinetic properties, more recently, a growing number of peptide drugs have been successfully developed [2] [3]. In most of the cases, this has been made possible by the identification of chemical strategies improving peptide resistance to peptidases. In mammals, peptides and proteins exclusively contain amino acids with relative configuration L while in other classes i.e. amphibians, the presence of D amino acids in peptides is not infrequent [4]. For instance, the selective delta opioid receptor ligand deltorphins (endogenous heptapeptides isolated from frogs of the genus *Phyllomedusa*) are characterized by the presence in position 2 of a D amino acid (D-Met for Deltorphin A and D-Ala for Deltorphin I and II) [5]. This chemical feature provides a reduction in protease susceptibility prolonging deltorphin half life in rat plasma to more than 2 hours [6]. Several examples of peptides containing D amino acids acting as GPCR ligands can be found in literature e.g. [7]. ~~The use o~~f D amino acids has been ~~extensively~~ also ~~applied~~ used for generating peptide drugs such as agonists (i.e. goserelin, buserelin, leuprolide) and ~~as well as~~ antagonists (i.e. cetrorelix, ganirelix) of the gonadotropin releasing hormone receptor [8]. Another chemical modification useful for improving peptide druggability is the N-methylation of the peptide bond [9]. This simple chemical modification consists of the replacement of the hydrogen atom of the peptide bond with a methyl group. The substitution of N-H with N-CH_3 produces changes in peptide conformation due to steric hindrance and modification of the intra- and inter-molecular hydrogen bond networking. This peptide bond modification has been used for generating angiotensin receptor ligands such as saralasin [10] and the β-arrestin biased agonist TRV120027 [11]. N-CH_3 has been also employed for the design of the cyclic RGD peptide analogue cilengitide (cyclo[Arg-Gly-Asp-DPhe-(~~NMe~~N-CH_3)Val]; [12]), a molecule currently under development as novel anticancer drug. This molecule was identified by manipulating the recognition sequence Arg-Gly-Asp (RGD) of integrins by combining an N-methyl amino acid, a D-amino acid and the head to

tail cyclization of the peptide sequence. This last chemical modification works by limiting the peptide flexibility and increasing the steric hindrance, preventing entrance into the catalytic pocket of proteases and, consequently, increases the duration of action of the molecule. Another example of a cyclic natural peptide containing non proteinogenic amino acids is that of the immunosuppressant cyclosporine. Cyclosporine is one of the few examples of a drug with a peptide structure that can be orally administered [13]. Cyclization of peptides can be achieved by using different chemical moieties that leads to the formation of a lactam or a disulfide bridge. In all cases, peptide cyclization reduces the conformational freedom of the molecule in comparison with its linear form. Recently, the design of bicyclic peptide derivatives produced molecules highly resistant to peptidases [14] and able to cross cell membranes [15]. The improvement of the pharmacokinetic properties of bicyclic peptides could lead in the near future to the identification of orally available compounds [16]. Another chemical strategy for reducing peptide degradation has been described by researchers of Zealand Pharma and named SIP (structure inducing probe) technology: it functions by adding a hexalysine sequence to the active peptide. Via SIP technology, the GLP-1 receptor agonist lixisenatide [17] has been identified; this molecule is now on the market for the treatment of type 2 diabetes mellitus. Another example of SIP based drug is the NOP receptor selective agonist ZP 120 [18]. Other approaches for improving the pharmacokinetic features of peptides include peptide functionalization with cell penetrating peptide sequences [19], peptide pegylation [20] and technologies focusing on peptide drugs formulation [21]. It is worthy of mention that several pegylated products has been approved as drugs (see table 1 in [22]).

2. Multimeric peptides

The first example of multimeric molecules of a peptide nature was developed by Tam and co-workers [23] as an "octopus immunogen" for generating antibodies against the $alpha_o$ subunit of G protein. The multimeric peptides used for the immunization protocol were named multiple antigen peptide. These structures were later used for obtaining antibodies against cobra-toxins, for generating innovative antimicrobial agents, or as carriers for tumor targeting [24]. In addition, this same chemical strategy has been used for generating tetrabranched derivatives of neuropeptides such as enkephalins, neurotensin and nociceptin/orphanin FQ (N/OFQ). These molecules were able to bind their respective receptors with high affinity and displayed higher stability in human plasma and rat brain membranes compared to the linear peptides [25]. Collectively, these findings support the proposal that branched peptide molecules may have potential as innovative therapeutics.

However, the bigger the final multimeric macromolecules are, the more complex their synthesis becomes, as does their purification and analytical characterization, and this strongly limits the pharmaceutical feasibility of such a class of molecules and their development as drugs.

3. The peptide welding technology (PWT)

Multimeric peptides can be prepared by using either divergent or convergent approaches (see Fig. 1 for a general scheme). With the divergent approach (Fig. 1A), a variable number of peptide arms are assembled by stepwise solid phase peptide synthesis methods on a branched core (in most cases polylysine). However, the purification of compounds prepared with this procedure is extremely difficult due to the contamination of the desired product by tens of deleted sequences (lacking one or more amino acids) with chemical physical properties (molecular weight, charge, polarity, hydrophilicity, etc.) very similar to the desired product. Even using the most efficient purification techniques available, the overall yield of this process is very low and the purity levels required for pharmaceutical purposes cannot be achieved [26] [27]. With the convergent approach (Fig. 1B) the desired product is produced by using a two-step procedure: i) the peptide sequences and the core are separately synthesized and purified; ii) peptides and the core are linked through mutually reactive functional groups. The crucial issue of the convergent approach is the chemoselectivity of the reaction used to link together the peptide sequences and the core. In fact, the presence of several reactive functionalities in the amino acid side-chains makes the use of an extremely chemoselective reaction between the core and the attachment point of the peptide sequence mandatory [28] [29] [30] [31]. The thiol-Michael reaction used for the PWT strategy perfectly meets this requirement. The reaction occurs between a thiol (i.e. the side chain of Cys) and an α,β-unsaturated carbonyl group (which is not present in the side chain of proteinogenic amino acids) to yield a thioether addition product (Fig. 1B). It is worthy of mentioning that the thiol-Michael reaction generates a novel chiral centre; this center may eventually contribute to the overall architecture of the macromolecule however it is relatively far from the peptide backbone and thus a possible influence on the bioactive conformation of the peptide sequence is extremely unlikely.

To investigate the possible architecture-activity relationship of PWT molecules, three different cores have been investigated (Fig. 2). PWT1 is based on the classical Lys branched moiety originally employed by Tam [23], PWT2 is a cyclam-based scaffold, and PWT3 is constituted by a symmetrical branched ethylendiamine scaffold. Four maleimide moieties are linked to each of the cores. As already underlined, the PWT strategy requires the presence of a thiol function in the peptide sequence; the nucleophilic character of the Cys SH moiety promotes the addition of the

peptide on the maleimide α,β-unsatured carbonyl generating a thioether bond that links peptide and core (Fig. 1B). The mandatory role of Cys for the synthesis of PWT derivatives implies the fact that the peptide sequence should not contain this residue or, more precisely, the Cys residue should not be present in (or near to) the biologically active portion (pharmacophore) of the peptide. Moreover, as nicely reviewed [32], for the design of bivalent or multivalent ligands the choice of attachment point is crucial. In the design of PWT peptides, we inserted the Cys residue as far as possible from the peptide pharmacophore. For instance, in N/OFQ and its related peptides, opioids, and NPS sequences the pharmacophoric portion is located at the N ~~terminal~~terminus and consequently Cys was added at the C ~~terminal~~terminus. Conversely, for tachykinins whose pharmacophore is located at the C ~~terminal~~terminus, Cys was introduced at the N ~~terminal~~terminus (Table 1). For the thiol-Michael reaction mild conditions (room temperature, pH around 8, H_2O/CH_3CN as reaction solvent) are required making the PWT strategy a powerful method for the synthesis of tetrabranched peptides. In addition, the reaction reaches completion in a few minutes with a practical 100% yield. Details about experimental conditions and procedures for the synthesis of PWT cores and PWT peptides are reported in Guerrini *et al*. [33]. In our hands, all the peptide sequences investigated up to now (Table 1), showed a superimposable solubility profile as linear or branched derivatives suggesting a negligible contribution of the core to the hydrophobic/hydrophilic balance of the molecule.

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4. Pharmacological profile of PWT peptides

In the following section we will briefly summarize the pharmacological profile of PWT tetrabranched peptides that have been previously published including N/OFQ [33-35], [Dmt^1]N/OFQ(1-13)-NH_2 [36], tachykinins [37], and neuropeptide S (NPS) [38] . Moreover, original findings regarding the pharmacological features of PWT2-dermorphin and PWT2-UFP-101 are described.

4.1. PWT-N/OFQ

The peptide N/OFQ (see Table 1 for primary structure) is the endogenous ligand of a previously orphan GPCR now named N/OFQ peptide (NOP) receptor [39]. Via selective activation of the NOP receptor, N/OFQ modulates several biological functions including pain transmission, learning and memory, emotional states, locomotor activity, food intake, drug abuse, cardiovascular and gastrointestinal functions, and the cough and micturition reflexes [40]. N/OFQ and synthetic NOP receptor agonists have been shown to produce beneficial effects in different animal models of

pathology [41] and, currently, some of these molecules are in clinical development including cebranopadol [42] as innovative analgesic [43], SER100 (alias ZP-120 [18]) for systolic hypertension [44] [45] and REC 0438 (alias UFP-112, [46]) for urinary incontinence due to overactive bladder [47].

The N/OFQ sequence has been used to generate the first series of PTW peptides using as cores the structures displayed in Fig. 2. In receptor binding experiments, PWT1-N/OFQ, PWT2-N/OFQ, and PWT3-N/OFQ displayed 3 fold higher NOP affinity than N/OFQ and similar selectivity over classical opioid receptors [34]. In the electrically stimulated mouse vas deferens, PWT derivatives mimicked the inhibitory action of the natural peptide showing similar maximal effects and approximately 3 fold higher potencies. Interestingly the action of PWT derivatives was, when compared to N/OFQ, slower to develop and more resistant to washing. In this preparation, the NOP selectivity of PWT derivatives of N/OFQ was demonstrated in receptor antagonist (SB-612111) and knockout (tissues taken from NOP receptor gene knockout mice (NOP(-/-)) studies [33, 34]. The NOP full agonist activity, the high potency and selectivity of action of tetrabranched derivatives of N/OFQ were confirmed in [^{35}S]GTPγS binding and calcium mobilization studies [34]. PWT2-N/OFQ was also investigated in a BRET assay for its ability to promote NOP/G protein and NOP/β-arrestin 2 interaction. Interestingly, PWT2-N/OFQ compared to N/OFQ displayed a significant bias toward G protein [48].

The promising pharmacological profile displayed by PWT derivatives of N/OFQ *in vitro* prompted us to investigate the compounds *in vivo*. N/OFQ PWT derivatives mimicked the inhibitory effects exerted by the natural peptide on mouse locomotor activity showing 40-fold higher potency. Interestingly the onset of action of PWT peptides was slower compared to N/OFQ and their duration of action was longer lasting. In fact, the inhibitory effects of N/OFQ lasted for less than one hour while those exerted by equieffective doses of PWT2-N/OFQ were still statistically significant after one day post injection. The exclusive involvement of the NOP receptor in this action of PWT2-N/OFQ was demonstrated by its lack of effect in NOP(−/−) mice [34]. Moreover, after supraspinal administration in mice PWT2-N/OFQ stimulated food intake mimicking the action of N/OFQ; compared to the natural peptide PWT2-N/OFQ was 40 fold more potent and elicited larger effects [33]. In a separate study [35] the tail withdrawal assay in mice and monkeys was used as a nociceptive pain model and mechanical threshold in mice subjected to chronic constriction injury was used as a neuropathic pain model for assessing the antinociceptive effects of spinally administered N/OFQ and PWT2-N/OFQ. PWT2-N/OFQ mimicked the spinal antinociceptive effects of N/OFQ both in nociceptive and neuropathic pain models in mice as well as in non-human

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primates displaying 40-fold higher potency and a markedly prolonged duration of action. The effects of N/OFQ and PWT2-N/OFQ were sensitive to SB-612111, but not to naltrexone thus demonstrating that both molecules act as selective NOP agonists.

4.2. PWT2-[Dmt^1]N/OFQ(1-13)

Several converging studies recently reviewed in [39, 49] suggest that mixed NOP and opioid receptor agonists may represent an innovative and promising class of analgesics. The peptide [Dmt^1]N/OFQ(1-13)-NH_2 (see Table 1 for primary structure) displays the above mentioned pharmacological profile and elicits a robust and dose-dependent antinociceptive action after spinal administration in non human primates [50]. Thus, the PWT2 derivative of [Dmt^1]N/OFQ(1-13)-NH_2 (PWT2-[Dmt^1]N/OFQ(1-13)) was generated and pharmacologically characterized *in vitro* and *in vivo*. In receptor binding studies, PWT2-[Dmt^1]N/OFQ(1-13) displayed approximately 10 fold lower affinity than the parent peptide, but maintained a similar profile of selectivity i.e. NOP = mu = kappa > delta. In functional studies performed with different assays, PWT2-[Dmt^1]N/OFQ(1-13) always behaved, similarly to [Dmt^1]N/OFQ(1-13)-NH_2, as a full agonist. Interestingly, in the previously described NOP [48] and mu [51] BRET assays, PWT2-[Dmt^1]N/OFQ(1-13) displayed similar potency and efficacy as [Dmt^1]N/OFQ(1-13)-NH_2, at both NOP and mu receptors in receptor/G protein experiments while in receptor/β-arrestin studies it displayed reduced potency (particularly at mu) associated with reduced efficacy (particularly at NOP). Thus, PWT2-[Dmt^1] behaved as a G protein biased agonist both at NOP and mu receptors. After spinal administration in monkeys PWT2-[Dmt^1]N/OFQ(1-13) elicited antinociceptive effects, was at least 10 fold more potent than [Dmt^1]N/OFQ(1-13)-NH_2, and produced longer lasting effects.

4.3. PWT2-UFP-101

UFP-101 is a potent and selective NOP receptor antagonist; since its identification [52] this peptide has been used in a large number of *in vitro* and *in vivo* studies (reviewed in [39, 53]) contributing to increase our knowledge on the control exerted by the N/OFQ system on several biological functions and on the possible therapeutic indications of NOP selective antagonists particularly as innovative antidepressant agents [54] and as novel drugs to treat Parkinson disease [55]. Thus, the PWT chemical strategy has been applied to the peptide sequence of UFP-101 (see Table 1) to generate the tetrabranched derivative PWT2-UFP-101. In the BRET based NOP/G protein assay, PWT2-UFP-101 competitively antagonized N/OFQ stimulatory effects showing a value of potency (pA_2 8.58) similar to that displayed in parallel experiments by UFP-101 (pA_2 8.32) (Fig. 3A). Thus, the PWT

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chemical modification did not modify the NOP antagonist activity and the potency of UFP-101. This finding, obtained for the first time with a peptide antagonist, is in line with previous results obtained by applying the PWT to various peptide sequences with agonist activity. In addition the PWT modification did not affect the competitive nature of UFP-101 antagonism as demonstrated by Schild analysis (Fig. 3B). This suggests that the N-~~terminal~~terminus tetrapeptide (Nphe^{1}-Gly^{2}-Gly^{3}-Phe^{4}) of both UFP-101 and of its PWT derivative interacts with the binding pocket of the NOP receptor in a very similar manner. The atomic details of this interaction have been elucidated by docking UFP-101 to the crystal structure of the inactive state of the NOP receptor (see [56] and Figure 2 of [53]). The electrically stimulated mouse vas deferens has been identified as a N/OFQ-sensitive pharmacological preparation soon after the discovery of the peptide [57] [58]. Since then, this preparation has proven to be extremely useful as a bioassay for evaluating the pharmacological profile of novel NOP ligands at native NOP receptors (see Tables 2 and 3 in [39]). In this preparation, UFP-101 antagonized N/OFQ inhibitory effects with a potency value (7.02) in line with previous findings (7.29, [52]). The Schild analysis of the action of PWT2-UFP-101 confirmed the NOP antagonist activity and competitive behavior of the interaction with N/OFQ and yielded a potency value of 7.59 (Fig. 3C and D). Importantly, when challenged against the delta receptor selective agonist DPDPE, PWT2-UFP-101 displayed a value of potency more than 30 fold lower (data not shown). Of note, previous studies with UFP-101 demonstrated very high NOP selectivity in bioassay studies [52]. Thus the present results suggest that the PWT modification does promote a certain reduction of the UFP-101 selectivity of action.

PWT2-UFP-101 was then tested *in vivo* in the mouse forced swimming test. In this assay the i.c.v. injection of peptide NOP antagonists as well as the systemic injection of brain penetrant non peptide NOP antagonists elicits antidepressant like effects (reviewed in [54]). These findings were corroborated by knockout studies that demonstrated that both NOP(-/-) mice [59] and rats [60] displayed an antidepressant phenotype in the forced swimming assay. In addition, the recently identified NOP selective antagonist LY2940094 promoted antidepressant effects in rodents and, more importantly, displayed antidepressant efficacy in patients with major depressive disorder [61]. PWT2-UFP-101 displayed slow developing effects in the forced swimming test since its effects were evident after 60 but not 5 min from injection. This finding is in line with previous studies demonstrating that PWT derivatives of N/OFQ and substance P displayed slow developing effects [34] [37]. PWT2-UFP-101 promoted a dose dependent antidepressant like action (Fig. 3E) eliciting statistically significant effects starting from the dose 0.1 nmol. Thus compared with UFP-101 [59] its tetrabranched derivative was approximately 10 fold more potent. A separate series of

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experiments were performed for investigating the duration of action of PWT2-UFP-101. In these studies, the effect of equieffective doses of UFP-101 and PWT2-UFP-101, i.e. 10 and 1 nmol, respectively, were compared at different pretreatment times. The compounds elicited statistically significant effects when injected 1 and 2 h before the assay, but not when the injection was performed 3 h before the assay. These results demonstrated that there are no major difference in terms of duration of action between UFP-101 and its tetrabranched derivative. This result contrasts with previous findings demonstrating long lasting actions of PWT derivatives which has been interpreted as due to lower susceptibility to peptidases [25]. Previous studies demonstrated that aminopeptidase recognizes N/OFQ as a substrate [62] generating [desPhe^{1}]N/OFQ a peptide lacking affinity for the NOP receptor [63]. Eventually the presence of the unnatural amino acid ~~residue~~ Nphe at position 1 of UFP-101 may confer to this compound some resistance to peptidases that is not further increased by the PWT chemical modification.

False positive results may be obtained in behavioral assays with drugs that affect locomotion [64]. Thus the possible effects of UFP-101 and PWT2-UFP-101 on mouse spontaneous locomotor activity were evaluated in the open field test. In line with previous findings [52] [59], UFP-101 did not modify the animal motor behavior. On the contrary, PWT2-UFP-101 produced a robust inhibition of horizontal and vertical motor activity in the open field test (Figure 3G and H). It is unlikely that this effect may bias the interpretation of the results obtained with PWT2-UFP-101 in the forced swimming assay since the compound promoted a reduction of immobility time i.e. promoted swimming behavior; eventually the inhibitory action of PWT2-UFP-101 on locomotor activity may cause un underestimation of its antidepressant like effect in the forced swimming assay. However, the inhibitory effect elicited by PWT2-UFP-101 on locomotion may clearly limits the usefulness of this compound as pharmacological tool for *in vivo* investigations.

In summary, *in vitro* PWT2-UFP-101 maintains the antagonist activity, competitive behavior, and potency of the linear peptide. *In vivo* in the mouse forced swimming test, PWT2-UFP-101 mimicked the antidepressant like effects of UFP-101 being 10 fold more potent. Taken together, these results suggest that the PWT strategy can be applied to peptide antagonists to increase their *in vivo* potency. This proposal needs to be experimentally validated by designing, synthesizing and pharmacologically evaluating different PWT derivatives of peptides acting as receptor antagonists.

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4.4. PWT2-dermorphin

Dermorphin (Table 1) is a potent opioid peptide isolated from amphibian skin by the Espamer group in the early 80' [65]. Dermorphin displayed potent inhibitory effects in the guinea pig

bioassay and long lasting, and naloxone sensitive, antinociceptive activity in rodents [66]. The dermorphin peptide sequence has been the subject to a large number of structure activity studies that increased our knowledge about the chemical requirement for mu opioid receptor binding and activation [67] [68]. We synthesised and pharmacologically characterised *in vitro* PWT2-dermorphin, the tetrabranched PWT derivative of the mu opioid peptide dermorphin.

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PWT2-dermorphin was assayed in calcium mobilization studies performed on cells coexpressing the human opioid receptors and chimeric G proteins. This assay has been previously validated [69] [70] and then used for investigating novel compounds. On mu receptor expressing cells, dermorphin produced stimulatory effects in line with literature data. PWT2-dermorphin mimicked the stimulatory effects of dermorphin, showing slightly lower efficacy and being 3 fold less potent (Table 2). In cells expressing kappa, delta, or NOP receptors the standard agonists dynorphin A, DPDPE, N/OFQ stimulated the release of intracellular calcium with maximal effects and potency values in line with previous findings [69] [70]. In these cells, dermorphin and its PWT derivative were either inactive (kappa and NOP) or produced a modest stimulation at micromolar concentrations (delta) (Table 2). Therefore, the high selectivity displayed by the natural peptide dermorphin on the mu receptor is maintained by its PWT derivative. The ability of dermorphin and PWT2-dermorphin to promote mu/G protein and mu/β-arrestin 2 interaction has been assessed using a BRET assay [51, 71]. Dermorphin and PWT2-dermorphin promoted receptor interaction with G protein and β-arrestin 2 with similar efficacy and potency values (Fig. 4A and B). Thus PWT2-dermorphin behaves as an unbiased mu agonist. This result is somewhat different from those previously obtained with PWT2-N/OFQ [48] and PWT2-[Dmt^{1}]N/OFQ(1-13) [36] where the PWT chemical modification confers to the molecules G protein biased agonism.

In order to investigate the pharmacological features of PWT2-dermorphin at native animal receptors experiments were performed in the electrically stimulated guinea pig ileum bioassay. In this preparation dermorphin inhibited the electrically induced contractions (Fig. 4C) with efficacy and potency in line with literature data [50]. PWT2-dermorphin mimicked the inhibitory effect of the natural peptide showing similar efficacy but approximately 10 fold lower potency (Fig. 4D). Interestingly, similar to previous reports on PWT2-N/OFQ [33], the kinetic of action of PWT2-dermorphin was slower than that of dermorphin and its inhibitory effects were only partially reversible by washing. The opioid receptor antagonist naloxone produced a rightward shift of the concentration response curve to dermorphin and PWT2-dermorphin without modifying the maximal effects induced by the agonists; pA_2 values of 9.07 and 8.82 were obtained for naloxone against dermorphin and PWT2-dermorphin, respectively. The high pA_2 value of naloxone suggests that the

biological effects of both dermorphin and PWT2-dermorphin in this preparation are solely due to their ability to activate the mu opioid receptor.

In summary, PWT2-dermorphin displays *in vitro* a profile, in terms of pharmacological activity, unbiased agonism, potency and selectivity of action, similar if not superimposable to that of the parent peptide. This result further corroborates the proposal that the application of the PWT strategy does not affect the *in vitro* pharmacological features of biologically active peptides. Further studies are under way to investigate the *in vivo* actions of PWT2-dermorphin.

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4.5. PWT2-tachykinins

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) share a common C-~~terminal~~ terminus sequence (see Table 1) that is crucial for their ability to bind to and activate three different GPCRs: the NK1, NK2 and NK3 receptors [72]. Tachykinins are neurotransmitters widely distributed in the central and peripheral nervous systems where they control several biological functions including pain transmission, nausea and vomiting, mood and anxiety, drug abuse and inflammatory conditions of the gastrointestinal tract [73]. PWT2 derivatives of SP, NKA and NKB were prepared using the N terminus~~al~~ of the peptide sequence as an attachment point for the Cys residue crucial for the PWT reaction. PWT tachykinins were assayed *in vitro* in calcium mobilization studies performed on cells expressing the human recombinant NK receptors and in bioassays studies in tissues expressing the native animal NK receptors. In calcium mobilization studies, PWT tachykinin derivatives behaved as full agonists at NK receptors with a selectivity profile (NK1: PWT2-SP > PWT2-NKA > PWT2-NKB; NK2: PWT2-NKA > PWT2-SP = PWT2-NKB; NK3: PWT2-NKB > PWT2-NKA > PWT2-SP) similar to that of the natural peptides. NK receptor antagonists (aprepitant, GR159897, and SB222200 for NK1, NK2, and NK3 receptors, respectively) display similar potency values when tested against PWT2 derivatives and natural tachykinin peptides [37]. In bioassay experiments, PWT2-SP mimicked the effects of SP with similar potency, maximal effects and sensitivity to aprepitant. Interestingly and in line to what reported for PWT derivatives of N/OFQ and dermorphin, the effect of PWT2-SP in the guinea pig ileum and, particularly, the rat urinary bladder tissues were relatively resistant to washing [37].

The intrathecal injection of SP in mice elicits a typical nociceptive behavior consisting of scratching (S), biting (B) and licking (L) [74] [75]. Thus the SBL test was used to investigate the *in vivo* effects of PWT2-SP. After spinal administration in mice, PWT2-SP mimicked the nociceptive effects of SP, but with higher potency and a longer-lasting action. Similar to what ~~has been~~

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previously reported for SP [75], aprepitant counteracted the effects of PWT2-SP *in vivo* thus demonstrating the involvement of the NK1 receptor in the pronociceptive effect of PWT2-SP [37]. In summary, the above mentioned results demonstrated that the PWT chemical strategy can be successfully applied to the peptide sequence of tachykinins to generate tetrabranched derivatives with a pharmacological profile similar to the native peptides *in vitro*. PWT2-SP, compared with SP, displayed *in vivo* higher potency and a marked prolongation of action.

4.6. PWT1-NPS

NPS (see Table 1 for primary structure) has been identified as the endogenous ligand of a previously orphan GPCR which was accordingly named NPS receptor (NPSR) [76]. In NPSR expressing cells, NPS stimulates calcium mobilization and cAMP accumulation suggesting Gq and Gs coupling. Evidence coming from pharmacological studies performed with peptides [77] and non peptides NPSR ligands [78] as well as from knockout studies (NPSR(-/-) [79] and ppNPS(-/-) [80] mice) demonstrates that NPS via selective stimulation of NPRS controls several biological functions including stress and anxiety, locomotor activity, wakefulness, learning and memory, drug abuse, food intake and gastrointestinal functions.

With the PWT approach, the NPS tetrabranched derivative PWT1-NPS has been synthesized and pharmacologically characterized. In calcium mobilization studies performed on cells expressing the murine NPSR, PWT1-NPS behaved as a full agonist displaying three fold higher potency than NPS. The selective NPSR antagonists [tBu-D-Gly^5]NPS and SHA 68 displayed similar potency values against NPS and PWT1-NPS [38]. *In vivo*, PWT1-NPS mimicked the stimulatory effect of NPS on locomotor activity in mice, however, it was 10 fold more potent. In the righting reflex assay, NPS was able to reduce the percentage of mice losing the righting reflex after diazepam administration and their sleep time 5 min after injection, but it was totally inactive 2 h after the injection. On the contrary, PWT1-NPS injected 2 h before diazepam, displayed statistically significant wake-promoting effects. This PWT1-NPS stimulant effect was no longer evident in NPSR(-/-) mice [38]. In summary, the application of the PWT strategy to the peptide sequence of NPS generated a NPSR ligand displaying an *in vitro* pharmacological profile similar to NPS but showing higher potency and long-lasting action *in vivo*.

5. General pharmacological features of PWT peptides

Table 3 summarizes the *in vitro* pharmacological features of the PWT peptides presented in section 4. As far as ligand efficacy is concerned, all the PWT molecules maintained the same efficacy as

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their parent peptides. In fact, the PWT derivatives of N/OFQ and [Dmt^1]N/OFQ(1-13)-NH_2, dermorphin, tachykinins, and NPS displayed in the various assays maximal effects similar to the parent peptide thus behaving as full agonists. Similarly PWT-UFP-101 maintained the antagonist feature of UFP-101. Thus we can propose that in general the PWT chemical modification does not affect ligand efficacy. For a subset of compounds, including PWT2-N/OFQ, PWT2-[Dmt^1]N/OFQ(1-13), and PWT2-dermorphin, data are available regarding the ligand ability to promote the interaction of the receptor with both G protein and β-arrestin 2. Compared to N/OFQ, PWT2-N/OFQ behaved as a NOP biased agonist toward the G protein [48]. Similar findings were obtained with PWT2-[Dmt^1]N/OFQ(1-13) that displayed G protein biased agonism both at NOP and at mu opioid receptors [36]. These results are rather unexpected and difficult to interpret. In fact the N-~~terminal~~terminus-pharmacophoric peptide sequences are identical in the linear peptides and in their PWT derivatives. Eventually the reduced flexibility of the peptide C ~~terminal~~ terminus linked to the core of the PWT molecule may affect the ability of the N-~~terminal~~terminus pharmacophoric sequences to adopt some conformational states that are more important for promoting the interaction of the receptor with β-arrestin than with G protein. However, the ability to promote G protein biased agonism of the PWT chemical modification is not a general phenomenon since PWT2-dermorphin maintained the unbiased behavior of the natural peptide at mu opioid receptors. Thus further studies are needed to investigate the possible relationship between the PWT chemical modification and biased agonism.

As far as ligand potency is concerned, no major differences (always less than 10 fold) were measured between the potency of the parent peptides and that of their PWT derivatives. It should be also underlined that PWT peptides contain 4 peptide sequences thus, on molar basis, a 4 fold increase in potency should be the expected result. Of note, in most cases, PWT derivatives displayed lower potency in the calcium mobilization assay than in the other tests. As previously discussed in details [34], this phenomenon is probably due to the non-equilibrium conditions that characterize the calcium assay associated to the "slow associating ligand" [81] features of PWT peptides. The latter characteristic of PWT tetrabranched peptides is suggested by bioassay studies performed in the mouse vas deferens and rat urinary bladder where PWT derivatives of N/OFQ and SP displayed slow developing effects compared to parent peptides. Thus, the relatively long time needed to obtain full activation of the receptor by PWT peptides might be not compatible with the rapid and transient nature of the calcium response. Therefore, the calcium assay tends to underestimate the potency of PWT peptides.

Together with efficacy and potency, selectivity of action is another crucial feature of a receptor ligand. The PWT modification did not modify the selectivity of action over opioid receptors of N/OFQ, [Dmt^{1}]N/OFQ(1-13)-NH_2 or dermorphin. Similar results were obtained by comparing SP, NKA, and NKB and their PWT derivatives over the three NK receptors. However, the crucial test for receptor selectivity is that with knockout tissue lacking the gene of the receptor of interest. This kind of results, that are only available for N/OFQ and the NOP receptor, demonstrated that the PWT modification reduced N/OFQ selectivity for the NOP receptor. In particular, the extremely high selectivity of N/OFQ for the NOP receptor demonstrated by the lack of effect of the peptide in NOP(-/-) tissues is reduced to approximately 100-fold for PWT2-N/OFQ and PWT3-N/OFQ and to only 20 fold for PWT1-N/OFQ. This suggests that the PWT2 and PWT3 cores are superior to PWT1 in maintaining the selectivity of action of the peptide natural sequence. However, the reduction of selectivity displayed by PWT derivatives in the mouse vas deferens should not be overemphasized. In fact the PWT derivatives of N/OFQ are still more NOP selective that all the available NOP non peptide ligands [82]. More importantly, the *in vivo* effects of PWT2-N/OFQ on locomotor activity were no longer evident in NOP(-/-) mice [34] and its antinociceptive effects in mice were sensitive to the NOP selective antagonist SB-612111 [35].

Table 4 summarizes the *in vivo* pharmacological features of the PWT peptides presented in section 4. The most important differences between the *in vivo* effects of PWT derivatives and their parent peptides are the increased potency and the longer lasting duration of action. The increased *in vivo* potency is the most consistent feature of PWT peptides since all PWT derivatives were from 3 to 30 fold more potent than their parent peptides. As far as longer lasting action is concerned, this feature was demonstrated for all the PWT compounds with the only exception of PWT2-UFP-101. However the amount of the difference in duration of action was very dissimilar depending on various factors including i) peptide sequence: a large increase in duration of action was observed with N/OFQ (up to 50 fold), a moderate increase with SP (approximately 5 fold), and a very small difference with NPS (2 fold); ii) route of administration: in mice PWT2-N/OFQ displayed longer lasting effects when the compound was given supraspinally (50 fold) than spinally (3 fold); iii) animal species: when given spinally PWT2-N/OFQ displayed longer lasting effects in monkeys (20 fold) than in mice (3 fold). The reasons underlying the increase in potency and duration of action of PWT derivatives are not completely understood. It has been previously reported that multibranched peptides display reduced susceptibility to cleavage by peptidases [25] and a structure-based hypothesis of branched peptide resistance to proteolysis has been proposed [83]. Peptide

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metabolism is likely to be more relevant *in vivo* than *in vitro* therefore it is reasonable to propose that the increase in agonist potency and duration of action displayed by PWT peptides *in vivo* is at least in part due to their higher metabolic stability. An additional *in vitro* characteristic common to PWT derivatives of N/OFQ, dermorphin and SP is reduced sensitivity to washing in organ bath experiments. This feature suggests longer-lasting binding to the receptor compared with native peptides. Several mechanisms including receptor clustering, cooperative binding, rebinding and subsite binding [84] have been proposed to explain the mode of action of multivalent ligands such as PWT peptides. Longer lasting receptor binding may contribute to the prolonged *in vivo* drug action [85]. Finally, as already mentioned the PWT modification seems to promote G protein biased agonism. Enhanced and prolonged morphine-induced antinociception has been reported in β-arrestin 2 knockout mice [86]. Thus, G protein biased agonism of PWT peptides is another factor that could contribute to their *in vivo* persistent effects. Clearly further studies, particularly with the use of knockout mice lacking the β-arrestin 2 gene, are needed to validate this possibility. Collectively, the combination of the different mechanisms mentioned above may explain the high potency and long-lasting effects displayed by PWT derivatives *in vivo*.

Regarding the *in vivo* selectivity of action of PWT derivatives, as mentioned before *in vitro* studies with knockout tissues demonstrated a certain degree of loss of NOP selectivity for N/OFQ. Certainly, further studies are needed to better investigate if and how much the PWT modification affects receptor selectivity. However, most of the *in vitro* results demonstrated no major changes of receptor selectivity by PWT peptides and this is in line with *in vivo* findings. In fact, studies performed with PWT derivatives of N/OFQ and NPS demonstrated that their actions are no longer evident in mice knockout for the NOP [34] and NPSR [38] receptor, respectively. Moreover selective antagonists such as aprepitant for NK1 and SB-612111 for NOP similarly prevented the *in vivo* actions of SP [75] and N/OFQ [87] as well as of their tetrabranched derivatives [37] [35].

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6. Conclusions

In conclusion, the review of the results obtained with the first generation of PWT peptides suggests the following general statements: i) in terms of medicinal chemistry, the PWT technique is an innovative chemical strategy that allows the facile synthesis of tetrabranched derivative of peptides with very consistent and unprecedented high purity and yield; ii) in terms of *in vitro* pharmacology, PWT derivatives of biologically active peptides maintain the pharmacological activity, affinity, and potency of their parent linear sequences. This profile might be associated with a certain loss of selectivity that is, in most cases, ~~negligible~~unimportant; iii) in terms of *in vivo* pharmacology, PWT

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derivatives are characterized by higher potency and particularly longer lasting action compared to parent peptides. The amount of increase in potency and duration of action appears to be dependent on different factors including peptide sequence, route of administration and animal species. Collectively, PWT derivatives of biologically active peptide are certainly useful pharmacological tools for investigating *in vitro* and particularly *in vivo* the consequences of the selective activation of a given receptor; this is particularly true when beneficial actions are associated to a prolonged receptor activation (or blockage).

In terms of perspectives, we intend in the near future to further investigate the following aspects related to the PWT approach: i) optimal distance between the core and the pharmacophoric peptide sequence. The available results were obtained with peptide sequences in the range of 7 - 20 amino acids ~~residue~~. Studies are now under way to compare the biological activity of PWT derivatives in which spacers of different length are inserted between the core and the pharmacophoric sequence; ii) position of the mandatory Cys residue. The available examples of PWT peptides were generated by placing the Cys residue either at the N or C terminus of the peptide sequence. PWT derivatives containing the Cys residue in different positions of the peptide sequence can be generated in order to assess the role of the attachment point to the overall conformation and biological activity of the PWT peptides; iii) heteromeric PWT derivatives. The PWT chemical approach allows for the generation of homomeric tetrabranched derivatives. However it would be extremely interesting to generate heteromeric tetrabranched derivatives containing 2 pairs of distinct peptide sequences. This would allow for the easy generation of receptor ligands able to simultaneously activate (or block) two distinct receptors.

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Conflict of interest

G. C., S. S. and R. G. are inventors of the patent application (EP13162532.9) focused on PWT and are founders of the University of Ferrara spin off company UFPeptides s.r.l., the assignee of such patent application.

Acknowledgments

We would like to thanks Mark Bird (University of Leicester, UK) for proofreading this article.

References

[1] Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced drug delivery reviews. 2001;46:3-26.

[2] Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug discovery today. 2010;15:40-56.

[3] Rafferty J, Nagaraj H, McCloskey AP, Huwaitat R, Porter S, Albadr A, et al. Peptide Therapeutics and the Pharmaceutical Industry: Barriers Encountered Translating from the Laboratory to Patients. Current medicinal chemistry. 2016;23:4231-59.

[4] Kreil G. D-amino acids in animal peptides. Annual review of biochemistry. 1997;66:337-45.

[5] Erspamer V, Melchiorri P, Falconieri-Erspamer G, Negri L, Corsi R, Severini C, et al. Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for delta opioid binding sites. Proceedings of the National Academy of Sciences of the United States of America. 1989;86:5188-92.

[6] Marastoni M, Tomatis R, Balboni G, Salvadori S, Lazarus LH. On the degradation of the deltorphin peptides by plasma and brain homogenate. Farmaco. 1991;46:1273-9.

[7] Masuda Y, Sugo T, Kikuchi T, Kawata A, Satoh M, Fujisawa Y, et al. Receptor binding and antagonist properties of a novel endothelin receptor antagonist, TAK-044 [cyclo[D-alpha-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]-L-alanyl-L-alpha-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl]disodium salt], in human endothelinA and endothelinB receptors. J Pharmacol Exp Ther. 1996;279:675-85.

[8] Engel JB, Schally AV. Drug Insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone. Nature clinical practice Endocrinology & metabolism. 2007;3:157-67.

[9] Chatterjee J, Rechenmacher F, Kessler H. N-methylation of peptides and proteins: an important element for modulating biological functions. Angewandte Chemie. 2013;52:254-69.

[10] Solomon TA, Buckley JP. Inhibitory effects of central hypertensive activity of angiotensin I and II by 1-sar-8-ala-angiotensin II (saralasin acetate). Journal of pharmaceutical sciences. 1974;63:1109-13.

[11] Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. J Pharmacol Exp Ther. 2010;335:572-9.

[12] Dechantsreiter MA, Planker E, Matha B, Lohof E, Holzemann G, Jonczyk A, et al. N-Methylated cyclic RGD peptides as highly active and selective alpha(V)beta(3) integrin antagonists. Journal of medicinal chemistry. 1999;42:3033-40.

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[13] Aguirre TA, Teijeiro-Osorio D, Rosa M, Coulter IS, Alonso MJ, Brayden DJ. Current status of selected oral peptide technologies in advanced preclinical development and in clinical trials. Advanced drug delivery reviews. 2016;106:223-41.

[14] Diderich P, Heinis C. Directed evolution of bicyclic peptides for therapeutic application. Chimia. 2013;67:910-5.

[15] Lian W, Jiang B, Qian Z, Pei D. Cell-permeable bicyclic peptide inhibitors against intracellular proteins. Journal of the American Chemical Society. 2014;136:9830-3.

[16] Baeriswyl V, Heinis C. Polycyclic peptide therapeutics. ChemMedChem. 2013;8:377-84.

[17] Thorkildsen C, Neve S, Larsen BD, Meier E, Petersen JS. Glucagon-like peptide 1 receptor agonist ZP10A increases insulin mRNA expression and prevents diabetic progression in db/db mice. J Pharmacol Exp Ther. 2003;307:490-6.

[18] Rizzi A, Rizzi D, Marzola G, Regoli D, Larsen BD, Petersen JS, et al. Pharmacological characterization of the novel nociceptin/orphanin FQ receptor ligand, ZP120: in vitro and in vivo studies in mice. British journal of pharmacology. 2002;137:369-74.

[19] Kristensen M, Birch D, Morck Nielsen H. Applications and Challenges for Use of Cell-Penetrating Peptides as Delivery Vectors for Peptide and Protein Cargos. International journal of molecular sciences. 2016;17.

[20] Zhang X, Wang H, Ma Z, Wu B. Effects of pharmaceutical PEGylation on drug metabolism and its clinical concerns. Expert opinion on drug metabolism & toxicology. 2014;10:1691-702.

[21] Ismail R, Csoka I. Novel strategies in the oral delivery of antidiabetic peptide drugs - Insulin, GLP 1 and its analogs. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2017;115:257-67.

[22] Pasut G, Veronese FM. State of the art in PEGylation: the great versatility achieved after forty years of research. Journal of controlled release : official journal of the Controlled Release Society. 2012;161:461-72.

[23] Chang KJ, Pugh W, Blanchard SG, McDermed J, Tam JP. Antibody specific to the alpha subunit of the guanine nucleotide-binding regulatory protein Go: developmental appearance and immunocytochemical localization in brain. Proceedings of the National Academy of Sciences of the United States of America. 1988;85:4929-33.

[24] Pini A, Falciani C, Bracci L. Branched peptides as therapeutics. Current protein & peptide science. 2008;9:468-77.

[25] Bracci L, Falciani C, Lelli B, Lozzi L, Runci Y, Pini A, et al. Synthetic peptides in the form of dendrimers become resistant to protease activity. J Biol Chem. 2003;278:46590-5.

[26] Niederhafner P, Sebestik J, Jezek J. Peptide dendrimers. Journal of peptide science : an official publication of the European Peptide Society. 2005;11:757-88.

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[27] Veprek P, Jezek J, Velek J, Tallima H, Montash M, El Ridi R. Peptides and multiple antigen peptides from Schistosoma mansoni glyceraldehyde 3-phosphate dehydrogenase: preparation, immunogenicity and immunoprotective capacity in C57BL/6 mice. Journal of peptide science : an official publication of the European Peptide Society. 2004;10:350-62.

[28] Brabez N, Lynch RM, Xu L, Gillies RJ, Chassaing G, Lavielle S, et al. Design, synthesis, and biological studies of efficient multivalent melanotropin ligands: tools toward melanoma diagnosis and treatment. Journal of medicinal chemistry. 2011;54:7375-84.

[29] Wang LX, Ni J, Singh S. Carbohydrate-centered maleimide cluster as a new type of templates for multivalent peptide assembling. synthesis of multivalent HIV-1 gp41 peptides. Bioorg Med Chem. 2003;11:159-66.

[30] Li S, McGuire MJ, Lin M, Liu YH, Oyama T, Sun X, et al. Synthesis and characterization of a high-affinity {alpha}v{beta}6-specific ligand for in vitro and in vivo applications. Molecular cancer therapeutics. 2009;8:1239-49.

[31] Pessi A, Langella A, Capito E, Ghezzi S, Vicenzi E, Poli G, et al. A general strategy to endow natural fusion-protein-derived peptides with potent antiviral activity. PloS one. 2012;7:e36833.

[32] Shonberg J, Scammells PJ, Capuano B. Design strategies for bivalent ligands targeting GPCRs. ChemMedChem. 2011;6:963-74.

[33] Guerrini R, Marzola E, Trapella C, Pela M, Molinari S, Cerlesi MC, et al. A novel and facile synthesis of tetra branched derivatives of nociceptin/orphanin FQ. Bioorg Med Chem. 2014;22:3703-12.

[34] Rizzi A, Malfacini D, Cerlesi MC, Ruzza C, Marzola E, Bird MF, et al. In vitro and in vivo pharmacological characterization of nociceptin/orphanin FQ tetrabranched derivatives. British journal of pharmacology. 2014;171:4138-53.

[35] Rizzi A, Sukhtankar DD, Ding H, Hayashida K, Ruzza C, Guerrini R, et al. Spinal antinociceptive effects of the novel NOP receptor agonist PWT2-nociceptin/orphanin FQ in mice and monkeys. British journal of pharmacology. 2015;172:3661-70.

[36] Cerlesi MC, Ding H, Bird MF, Kiguchi N, Ferrari F, Malfacini D, et al. Pharmacological studies on the NOP and opioid receptor agonist PWT2-[Dmt1]N/OFQ(1-13). Eur J Pharmacol. 2017;794:115-26.

[37] Ruzza C, Rizzi A, Malfacini D, Cerlesi MC, Ferrari F, Marzola E, et al. Pharmacological characterization of tachykinin tetrabranched derivatives. British journal of pharmacology. 2014;171:4125-37.

[38] Ruzza C, Rizzi A, Malfacini D, Pulga A, Pacifico S, Salvadori S, et al. In vitro and in vivo pharmacological characterization of a neuropeptide S tetrabranched derivative. Pharmacology research & perspectives. 2015;3:e00108.

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[39] Toll L, Bruchas MR, Calo G, Cox BM, Zaveri NT. Nociceptin/Orphanin FQ Receptor Structure, Signaling, Ligands, Functions, and Interactions with Opioid Systems. Pharmacological reviews. 2016;68:419-57.

[40] Lambert DG. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. Nat Rev Drug Discov. 2008;7:694-710.

[41] Zaveri NT. Nociceptin Opioid Receptor (NOP) as a Therapeutic Target: Progress in Translation from Preclinical Research to Clinical Utility. Journal of medicinal chemistry. 2016.

[42] Linz K, Christoph T, Tzschentke TM, Koch T, Schiene K, Gautrois M, et al. Cebranopadol: a novel potent analgesic nociceptin/orphanin FQ peptide and opioid receptor agonist. J Pharmacol Exp Ther. 2014;349:535-48.

[43] Lambert DG, Bird MF, Rowbotham DJ. Cebranopadol: a first in-class example of a nociceptin/orphanin FQ receptor and opioid receptor agonist. British journal of anaesthesia. 2015;114:364-6.

[44] Villar IC, Bubb KJ, Moyes AJ, Steiness E, Gulbrandsen T, Levy FO, et al. Functional pharmacological characterization of SER100 in cardiovascular health and disease. British journal of pharmacology. 2016.

[45] Kantola I, Scheinin M, Gulbrandsen T, Meland N, Smerud KT. Safety, Tolerability, and Antihypertensive Effect of SER100, an Opiate Receptor-Like 1 (ORL-1) Partial Agonist, in Patients With Isolated Systolic Hypertension. Clinical pharmacology in drug development. 2016.

[46] Rizzi A, Spagnolo B, Wainford RD, Fischetti C, Guerrini R, Marzola G, et al. In vitro and in vivo studies on UFP-112, a novel potent and long lasting agonist selective for the nociceptin/orphanin FQ receptor. Peptides. 2007;28:1240-51.

[47] Lazzeri M, Calo G, Spinelli M, Malaguti S, Guerrini R, Salvadori S, et al. Daily intravesical instillation of 1 mg nociceptin/orphanin FQ for the control of neurogenic detrusor overactivity: a multicenter, placebo controlled, randomized exploratory study. The Journal of urology. 2006;176:2098-102.

[48] Malfacini D, Ambrosio C, Gro MC, Sbraccia M, Trapella C, Guerrini R, et al. Pharmacological Profile of Nociceptin/Orphanin FQ Receptors Interacting with G-Proteins and beta-Arrestins 2. PloS one. 2015;10:e0132865.

[49] Gunther T, Dasgupta P, Mann A, Miess E, Kliewer A, Fritzwanker S, et al. Targeting multiple opioid receptors - improved analgesics with reduced side effects? British journal of pharmacology. 2017.

[50] Molinari S, Camarda V, Rizzi A, Marzola G, Salvadori S, Marzola E, et al. [Dmt1]N/OFQ(1-13)-NH2: a potent nociceptin/orphanin FQ and opioid receptor universal agonist. British journal of pharmacology. 2013;168:151-62.

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[51] Molinari P, Vezzi V, Sbraccia M, Gro C, Riitano D, Ambrosio C, et al. Morphine-like opiates selectively antagonize receptor-arrestin interactions. J Biol Chem. 2010;285:12522-35.

[52] Calo G, Rizzi A, Rizzi D, Bigoni R, Guerrini R, Marzola G, et al. [Nphe1,Arg14,Lys15]nociceptin-NH2, a novel potent and selective antagonist of the nociceptin/orphanin FQ receptor. British journal of pharmacology. 2002;136:303-11.

[53] Calo G, Guerrini R. Medicinal chemistry, pharmacology, and biological actions of peptide ligands selective for teh nociceptin/orphanin FQ receptor. In: Ko MC, Husbands SM, editors. Research and development of opioid-related ligands. Washington, DC: Oxford University Press; 2013. p. 275-325.

[54] Gavioli EC, Calo G. Nociceptin/orphanin FQ receptor antagonists as innovative antidepressant drugs. Pharmacol Ther. 2013;140:10-25.

[55] Arcuri L, Mercatelli D, Morari M. Parkinson's disease: no NOP, new hope. Oncotarget. 2017;8:8995-6.

[56] Thompson AA, Liu W, Chun E, Katritch V, Wu H, Vardy E, et al. Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. Nature. 2012;485:395-9.

[57] Calo G, Rizzi A, Bogoni G, Neugebauer V, Salvadori S, Guerrini R, et al. The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. Eur J Pharmacol. 1996;311:R3-5.

[58] Berzetei-Gurske IP, Schwartz RW, Toll L. Determination of activity for nociceptin in the mouse vas deferens. Eur J Pharmacol. 1996;302:R1-2.

[59] Gavioli EC, Marzola G, Guerrini R, Bertorelli R, Zucchini S, De Lima TC, et al. Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: pharmacological and genetic evidences from the mouse forced swimming test. Eur J Neurosci. 2003;17:1987-90.

[60] Rizzi A, Molinari S, Marti M, Marzola G, Calo G. Nociceptin/orphanin FQ receptor knockout rats: in vitro and in vivo studies. Neuropharmacology. 2011;60:572-9.

[61] Post A, Smart TS, Krikke-Workel J, Dawson GR, Harmer CJ, Browning M, et al. A Selective Nociceptin Receptor Antagonist to Treat Depression: Evidence from Preclinical and Clinical Studies. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology. 2016;41:1803-12.

[62] Noble F, Roques BP. Association of aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by nociceptin/orphanin FQ in mice. FEBS Lett. 1997;401:227-9.

[63] Dooley CT, Houghten RA. Orphanin FQ: receptor binding and analog structure activity relationships in rat brain. Life Sci. 1996;59:PL23-9.

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[64] Bourin M, Fiocco AJ, Clenet F. How valuable are animal models in defining antidepressant activity? Hum Psychopharmacol. 2001;16:9-21.

[65] Montecucchi PC, de Castiglione R, Piani S, Gozzini L, Erspamer V. Amino acid composition and sequence of dermorphin, a novel opiate-like peptide from the skin of Phyllomedusa sauvagei. International journal of peptide and protein research. 1981;17:275-83.

[66] Broccardo M, Erspamer V, Falconieri Erspamer G, Improta G, Linari G, Melchiorri P, et al. Pharmacological data on dermorphins, a new class of potent opioid peptides from amphibian skin. British journal of pharmacology. 1981;73:625-31.

[67] Mizoguchi H, Bagetta G, Sakurada T, Sakurada S. Dermorphin tetrapeptide analogs as potent and long-lasting analgesics with pharmacological profiles distinct from morphine. Peptides. 2011;32:421-7.

[68] Marastoni M, Salvadori S, Balboni G, Borea PA, Marzola G, Tomatis R. Synthesis and activity profiles of new dermorphin-(1-4) peptide analogues. Journal of medicinal chemistry. 1987;30:1538-42.

[69] Camarda V, Calo G. Chimeric g proteins in fluorimetric calcium assays: experience with opioid receptors. Methods Mol Biol. 2013;937:293-306.

[70] Camarda V, Fischetti C, Anzellotti N, Molinari P, Ambrosio C, Kostenis E, et al. Pharmacological profile of NOP receptors coupled with calcium signaling via the chimeric protein G alpha qi5. Naunyn Schmiedebergs Arch Pharmacol. 2009;379:599-607.

[71] Rizzi A, Cerlesi MC, Ruzza C, Malfacini D, Ferrari F, Bianco S, et al. Pharmacological characterization of cebranopadol a novel analgesic acting as mixed nociceptin/orphanin FQ and opioid receptor agonist. Pharma Res Per. 2016;in press.

[72] Regoli D, Boudon A, Fauchere JL. Receptors and antagonists for substance P and related peptides. Pharmacol Rev. 1994;46:551-99.

[73] Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiological reviews. 2014;94:265-301.

[74] Hylden JL, Wilcox GL. Intrathecal substance P elicits a caudally-directed biting and scratching behavior in mice. Brain Res. 1981;217:212-5.

[75] Rizzi A, Campi B, Camarda V, Molinari S, Cantoreggi S, Regoli D, et al. In vitro and in vivo pharmacological characterization of the novel NK(1) receptor selective antagonist Netupitant. Peptides. 2012;37:86-97.

[76] Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, et al. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. Neuron. 2004;43:487-97.

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[77] Guerrini R, Salvadori S, Rizzi A, Regoli D, Calo G. Neurobiology, pharmacology, and medicinal chemistry of neuropeptide S and its receptor. Med Res Rev. 2010;30:751-77.

[78] Ruzza C, Calo G, Di Maro S, Pacifico S, Trapella C, Salvadori S, et al. Neuropeptide S receptor ligands: a patent review (2005-2016). Expert opinion on therapeutic patents. 2016:1-16.

[79] Ruzza C, Pulga A, Rizzi A, Marzola G, Guerrini R, Calo G. Behavioural phenotypic characterization of CD-1 mice lacking the neuropeptide S receptor. Neuropharmacology. 2012;62:1999-2009.

[80] Liu X, Si W, Garau C, Jungling K, Pape HC, Schulz S, et al. Neuropeptide S precursor knockout mice display memory and arousal deficits. Eur J Neurosci. 2017.

[81] Charlton SJ, Vauquelin G. Elusive equilibrium: the challenge of interpreting receptor pharmacology using calcium assays. British journal of pharmacology. 2010;161:1250-65.

[82] Ferrari F, Malfacini D, Journigan BV, Bird MF, Trapella C, Guerrini R, et al. In vitro pharmacological characterization of a novel unbiased NOP receptor-selective non peptide agonist AT-403. Pharmacology research & perspectives. 2017;5:e00333.

[83] Falciani C, Lozzi L, Pini A, Corti F, Fabbrini M, Bernini A, et al. Molecular basis of branched peptides resistance to enzyme proteolysis. Chem Biol Drug Des. 2007;69:216-21.

[84] Gestwicki JE, Cairo CW, Strong LE, Oetjen KA, Kiessling LL. Influencing receptor-ligand binding mechanisms with multivalent ligand architecture. Journal of the American Chemical Society. 2002;124:14922-33.

[85] Vauquelin G, Charlton SJ. Long-lasting target binding and rebinding as mechanisms to prolong in vivo drug action. British journal of pharmacology. 2010;161:488-508.

[86] Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science. 1999;286:2495-8.

[87] Rizzi A, Gavioli EC, Marzola G, Spagnolo B, Zucchini S, Ciccocioppo R, et al. Pharmacological Characterization of the Nociceptin/Orphanin FQ Receptor Antagonist SB-612111 [(-)-cis-1-Methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9 -tetrahydro-5H-benzocyclohepten-5-ol]: In Vivo Studies. J Pharmacol Exp Ther. 2007;321:968-74.

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Table 1. peptide sequences used for generating PWT derivatives

Peptide	PWT peptide sequence
N/OFQ	FGGFTGARKSARKLANQ**C**
[Dmt^{1}]N/OFQ(1-13)	[Dmt]GGFTGARKSARK**C**
UFP-101	N(Bz)GGGFTGARKSARKRKNQ**C**
NPS	SFRNGVGTGMKKTSFQRAKS**C**
SP	**C**RPKPQQFFGLM-NH_2
NKA	**C**HKTDSFVGLM-NH_2
NKB	**C**DMHDFFVGLM-NH_2
Dermorphin	YaFGYPS**C**

The amino acids ~~residues~~ crucial for biological activity are indicated in gray. The Cys residue needed for generating PWT derivatives is indicated in bold.

Table 2. Effects of dermorphin and PWT2-dermorphin in CHO cells expressing the human recombinant receptors and chimeric G proteins in the calcium mobilization assay.

	mu		kappa		delta		NOP	
	pEC_{50} ($CL_{95\%}$)	E_{max}±sem	pEC_{50} ($CL_{95\%}$)	E_{max}±sem	pEC_{50} ($CL_{95\%}$)	E_{max}±sem	pEC_{50} ($CL_{95\%}$)	E_{max}±sem
dermorphin	8.19 (7.80-8.58)	345±14%	inactive		5.98 (5.76-6.19)	180±31%	inactive	
PWT2-dermorphin	7.66 (7.26-8.06)	293±12%	inactive		inactive		inactive	

Table 3. *In vitro* biological activity of PWT tetrabranched peptides.

PWT peptide	Test	Potency	Efficacy	References
PWT1-N/OFQ	receptor binding	+		[34]
	[^{35}S]GTPγS binding	+	=	[34]
	Ca^{2+} mobilization	-	=	[34]
	mVD	+	=	[33]
PWT2-N/OFQ	receptor binding	+		[34]
	[^{35}S]GTPγS binding	+	=	[34]
	Ca^{2+} mobilization	-	=	[34]
	BRET G protein	+	=	[48]
	BRET β-arrestin 2	-	=	[48]
	mVD	+	=	[33]
PWT3-N/OFQ	receptor binding	+		[34]
	[^{35}S]GTPγS binding	+	=	[34]
	Ca^{2+} mobilization	-	=	[34]
	mVD	+	=	[33]
PWT1-NPS	Ca^{2+} mobilization	+	=	[38]
PWT2-SP	Ca^{2+} mobilization	-	=	[37]
	BRET G protein	=	=	[37]
	gpI	=	=	[37]
	rUB	=	=	[37]
PWT2-NKA	Ca^{2+} mobilization	-	=	[37]
PWT2-NKB	Ca^{2+} mobilization	=	=	[37]
PWT2-[Dmt^{1}]N/OFQ(1-13)	receptor binding	-		[36]
	[^{35}S]GTPγS binding	=	=	[36]
	Ca^{2+} mobilization	-	=	[36]
	BRET G protein	=	=	[36]
	BRET β-arrestin 2	-	-	[36]
PWT2-UFP-101	BRET G protein	=	=	present article
	mVD	-	=	present article
PWT2-dermorphin	Ca^{2+} mobilization	-	=	present article
	BRET G protein	-	=	present article
	BRET β-arrestin 2	-	=	present article
	gpI	-	=	present article

+, -, = higher, lower, or similar compared to the linear peptide; mVD, mouse vase deferens; gpI, guinea pig ileum; rUB, rat urinary bladder; BRET, bioluminescence resonance energy transfer

Table 4. *In vivo* biological activity of PWT tetrabranched peptides.

	Species	Test	Potency	Duration of action	Reference
PWT1-N/OFQ	mouse	locomotor activity	++	+++	[34]
PWT2-N/OFQ	mouse	tail-withdrawal assay	++	+	[35]
	mouse	rotaroad	++	n.d.	[35]
	mouse	allodynia in CCI	++	+	[35]
	mouse	locomotor activity	++	+++	[34]
	mouse	food intake	++	n.d.	[33]
	rhesus monkey	tail-withdrawal assay	++	+++	[35]
PWT3-N/OFQ	mouse	locomotor activity	++	+++	[34]
PWT1-NPS	mouse	locomotor activity	+	=	[38]
	mouse	recovery of RR	+	+	[38]
PWT2-SP	mouse	SBL test	+	+	[37]
PWT2-[Dmt[1]]N/OFQ(1-13)	rhesus monkey	tail-withdrawal assay	+	+	[36]
PWT2-UFP-101[a]	mouse	forced swimming test	+	=	present article

[a]PWT2-UFP-101 but not UFP-101 reduced mouse locomotor activity.
+, = higher or similar to the linear peptide. CCI, chronic constriction injury; RR, righting reflex, SBL, scratching, biting, licking.

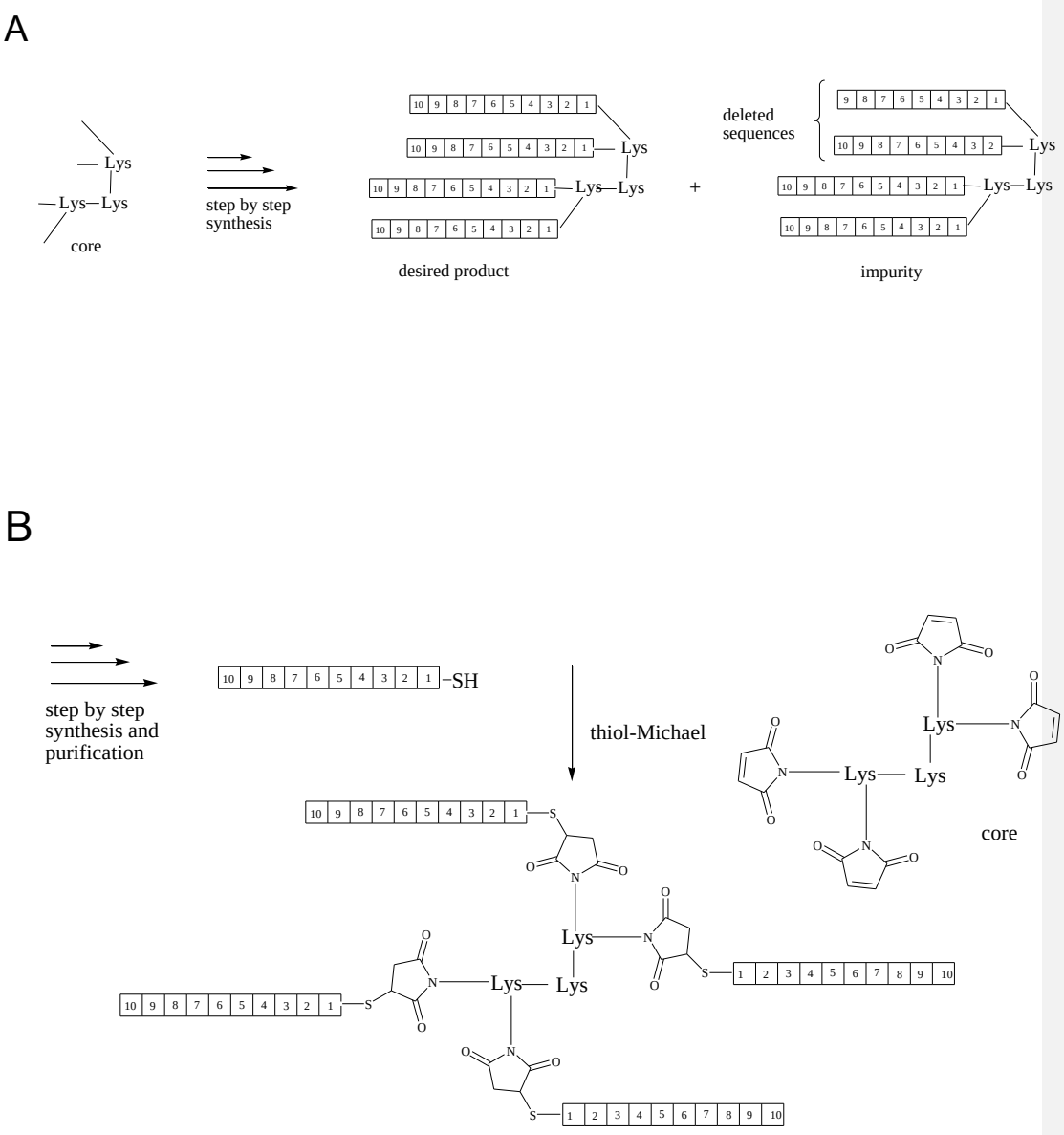

Figure 1. Scheme representing the divergent (panel A) and convergent (panel B) approaches for the synthesis of branched peptides.

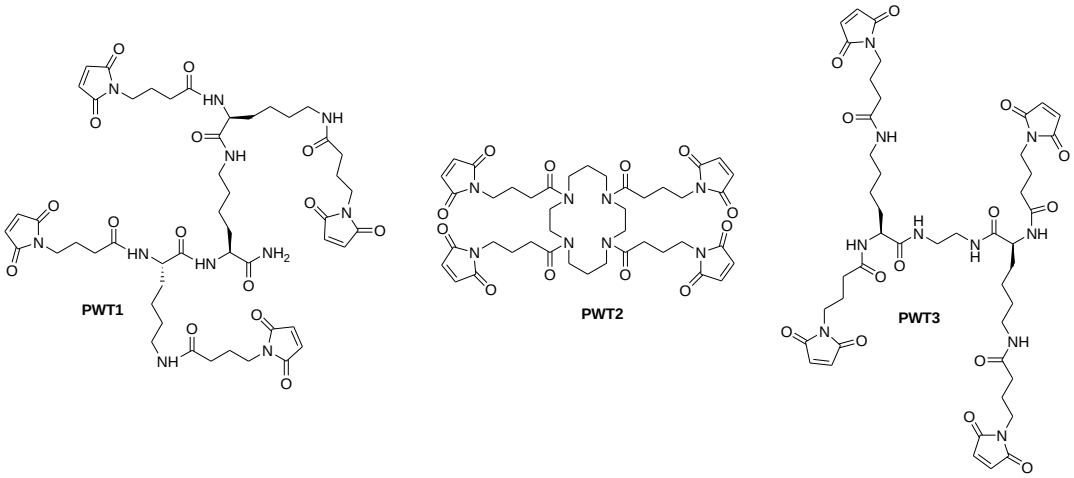

Figure 2. Chemical structures of the cores used for generating PWT peptides.

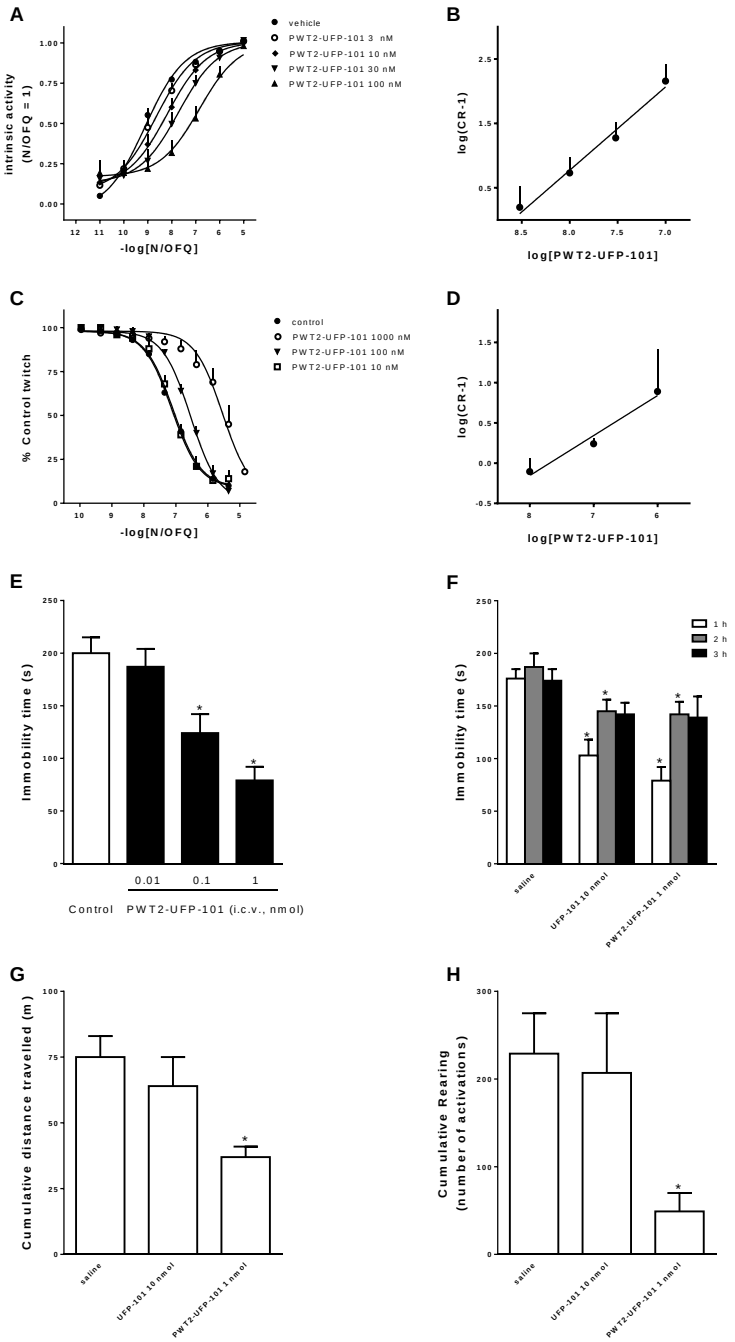

Figure 3. Pharmacological profile of PWT2-UFP-101. Concentration response curves to N/OFQ in absence and presence of increasing concentrations of PWT2-UFP-101 and the respective Schild plots in the BRET G protein assay (panel A and B) and in the electrically stimulated mouse vas

deferens (panel C and D). Dose response curve to PWT2-UFP-101 (panel E) and effects of equieffective doses of UFP-101 and PWT2-UFP-101 at different pretreatment times (panel F) in the mouse forced swimming test. Effects of 10 nmol UFP-101 and 1 nmol PWT2-UFP-101 on mouse locomotor activity (panel G and H).*$p<0.05$ vs saline according to one-way ANOVA followed by the Dunnett's post hoc test.

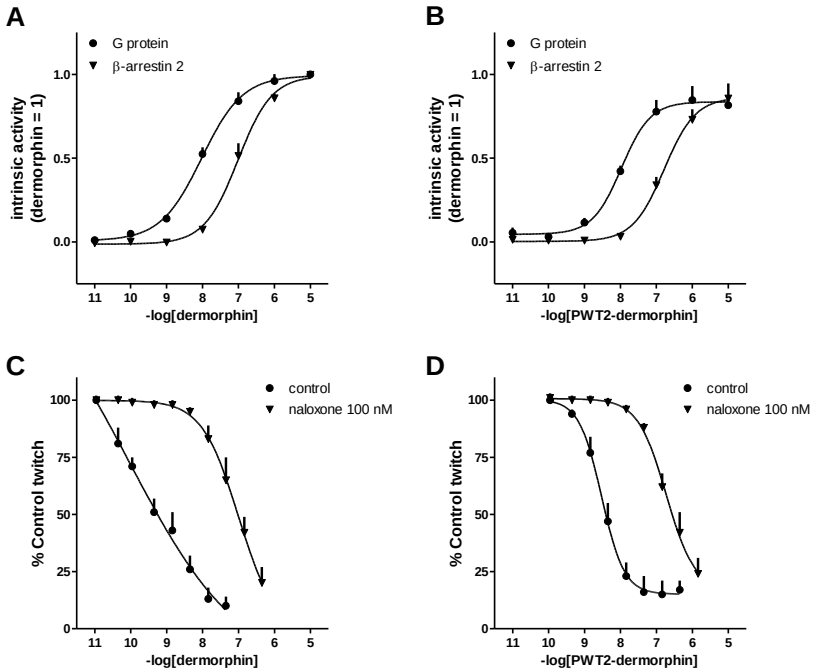

Figure 4. Pharmacological profile of PWT2-dermorphin. Concentration response curves to dermorphin (panel A) and PWT2-dermorphin (panel B) in the BRET assay measuring mu/G protein and mu/β-arrestin 2 interaction. Concentration response curve to dermorphin (panel C) and PWT2-dermorphin (panel D) obtained in the absence (control) and presence of naloxone 100 nM in the electrically stimulated guinea pig ileum.