IBMS BoneKEy. 2009 February;6(2):71-85
http://www.bonekey-ibms.org/cgi/content/full/ibmske;6/2/71
doi: 10.1138/20090364

PERSPECTIVES

Mimetic Ligands for the PTHR1: Approaches, Developments, and Considerations

Thomas J. Gardella

Massachusetts General Hospital, Boston, Massachusetts, USA

Abstract

The peptide ligands PTH and PTHrP can robustly stimulate new bone formation via agonist actions on the PTH/PTHrP receptor (PTHR1). Thus there is considerable interest in finding orally active small-molecule ligands for this target receptor, but so far no potent mimetic agonist has been described. Several small-molecule ligands that show either antagonistic or weak agonistic activities on the PTHR1 have recently appeared in the literature. These compounds represent potential leads for further chemical development. Advances made in our understanding of the molecular processes by which PTH and PTHrP peptide ligands and their analogs bind to and activate the PTHR1 are likely to be relevant to the mechanisms of action used by any PTHR1 mimetic ligand. This article aims to place these recent findings in a context that helps illuminate the challenges posed by the PTH/PTHrP receptor, and perhaps suggest new paths to take for the eventual development of potent and orally active mimetic agonists for this medically and biologically important G protein-coupled receptor. *IBMS BoneKEy*. 2009 February;6(2):71-85.

The PTH/PTHrP receptor (PTHR1) has long been recognized as an important target for drug discovery efforts aimed at finding new treatments for osteoporosis, as well as several other diseases relating to disturbances in calcium and bone metabolism. A potent and orally active agonist would be particularly useful, but so far, no small-molecule ligand that potently activates the PTHR1 has been described.

The PTHR1 is a family B, G protein-coupled receptor (GPCR) that mediates the actions of two vital endogenous polypeptide ligands, PTH and PTHrP. The fully active, synthetic peptides, PTH(1-34) and PTHrP(1-36), bind to the PTHR1 via a two-site process that involves docking interactions between the ligand's C-terminal domain and the receptor's amino-terminal extracellular (N) domain, and signaling interactions between the ligand's N-terminal portion and the receptor's juxtamembrane (J) domain containing the seven transmembrane helices and connecting loops (Fig. 1). Modified N-terminal PTH peptide fragment analogs have been developed that behave as potent agonists at the PTHR1, and these interact only with the receptor's J domain. It should thus be possible to similarly activate the PTHR1 via the binding of a small-molecule compound that interacts appropriately with the J domain. This raises the question as to why such potent mimetic PTHR1 agonists have not been reported, and what kinds of approaches might be taken for their eventual discovery.

Several recent reports in the literature describe small-molecule compounds that function either as antagonists or weak-potency agonists for the PTHR1. These studies provide some of the first clues that the PTHR1 is indeed an accessible target for small-molecule development. More information is needed concerning how these compounds interact with the PTHR1, but the reported molecules can be viewed as at least candidate scaffolds for further medicinal chemistry work aimed at developing fully potent agonists. An alternative, and similarly challenging approach, not yet implemented for the PTHR1, is to use rationale design strategies to transform the peptide ligand's pharmacophoric domain into a non-

IBMS BoneKEy. 2009 February;6(2):71-85
http://www.bonekey-ibms.org/cgi/content/full/ibmske;6/2/71
doi: 10.1138/20090364

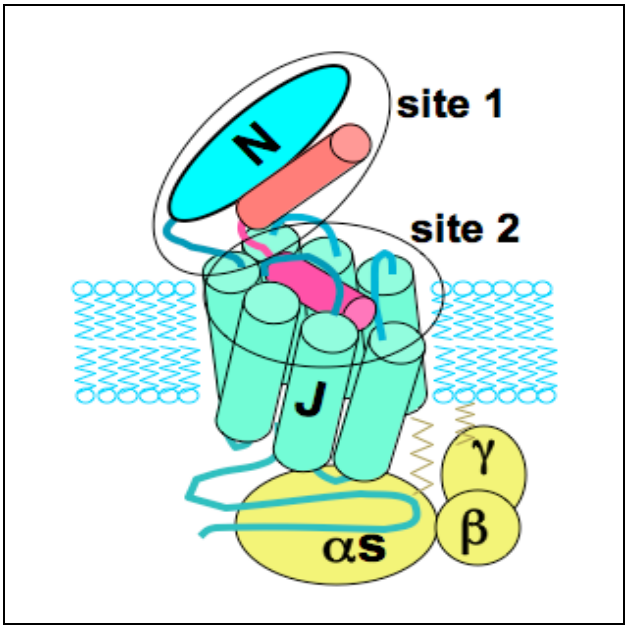

Fig. 1. Two-site binding model for the PTHR1. According to the model, the C- and N-terminal helices of PTH (red and magenta, respectively) interact with the receptor's extracellular amino-terminal domain (N) and its juxtamembrane region (J), respectively. The N interaction provides binding energy, whereas the J interaction produces the conformational changes involved in transmembrane signaling and coupling to heterotrimeric G proteins (αs,β/γ).

peptidomimetic. This *Perspective* summarizes the current state of peptidomimetic ligand development for the PTHR1, focusing on the properties of the small-molecule ligands that have recently emerged, and on the advances made towards defining and minimizing the N-terminal pharmacophoric signaling domain of the native peptide ligands. Such information, taken together, should help to frame, and eventually surmount, the difficulties involved in obtaining the elusive class of orally active mimetic ligands for the PTHR1.

Therapeutic Development of PTH Peptides

Osteoporosis is a debilitating disease associated with the aging process, and is a health issue of worldwide concern. Current therapies for osteoporosis include the so-called anti-resorptive class of drugs, as represented by the bisphosphonates, and the peptidic ligand, calcitonin, which achieve their efficacy by impairing osteoclast-mediated bone resorption. Peptide agonist ligands for PTHR1, such as PTH(1-34), offer an alternative treatment modality, in that they can promote the formation of new bone, which they do via direct actions on osteoblasts (1). Indeed, a large multi-center clinical trial, reported in 2001, proved that PTH(1-34) significantly increases bone mineral density (BMD) and decreases the risk of new bone fracture in osteoporotic women (2), and the peptide, produced as recombinant human (rh)PTH(1-34), is now among the leading osteoporosis therapies in use. The current treatment modality for PTH(1-34) involves a once-daily, subcutaneous injection of 20 μg of peptide (~300 ng peptide/kg body weight). The parenteral delivery is required to avoid peptide degradation in the gut. The daily dose regimen is designed to achieve an alternating pattern of ligand exposure and ligand absence, which is known to be critical for achieving a net bone formation effect with PTH (3). More prolonged or continuous administration of the ligand typically results in an increase in rates of bone resorption, via indirect activation of osteoclasts. This potential bone catabolic action of PTH introduces the risk of hypercalcemia, one of the main dose-limiting adverse events

associated with PTH therapy (4). Full-length human PTH(1-84) is also effective in stimulating bone formation, and is an approved osteoporosis therapy in Europe (5-7).

The potent capacity of PTH and PTHrP peptide ligands to stimulate bone formation validates the PTHR1 as a viable target for drug discovery effort. The driving force behind such an effort is the need to develop new PTHR1-based therapeutic agents that are: 1) optimized to produce a good bone anabolic effect with a minimal risk of hypercalcemia, and 2) orally active. Several PTH peptide analogs have been suggested to have a wider therapeutic margin, as compared to PTH(1-34), in terms of their capacity to produce a bone anabolic response relative to the hypercalcemic risk. These include PTHrP(1-36) (8;9), ostabolin C (ZT-031), a PTH(1-31) analog with a helix-stabilizing lactam modification introduced between the side chains of Glu^{22} and Lys^{26} that is under development (10), and BA058 (BIM-44058), a PTHrP(1-34) analog containing a number of C-terminal side chain substitutions and now in a phase II clinical study (11;12). While these possibilities remain to be more firmly established in larger-scale clinical testing, they nevertheless serve to emphasize the need to improve upon current PTH therapy modalities. Recent studies by the MGH group (13-15) suggest that certain PTH analogs, and to a limited extent, PTH(1-34), but not PTHrP(1-36), can form highly stable complexes with the PTHR1 that remain active for many minutes, if not hours, and thereby produce markedly prolonged cAMP responses in cells, and prolonged hypercalcemic and hypophosphatemic responses in animals (15). The signaling mechanisms that govern the extent to which PTHR1 agonists promote bone anabolic effects versus bone catabolic effects are complex and not fully understood, but prolonged ligand exposure clearly favors the catabolic response (1;3). PTHR1 agonists, peptidic or mimetic, with limited residency times on the receptor, and hence limited signaling duration times in target cells, might thus be worth exploring as future PTHR1-based therapeutics for osteoporosis.

In addition to osteoporosis, an improved PTHR1 agonist ligand could have utility in the treatment of cases of hypoparathyroidism, particularly those involving life-long and chronic perturbations in calcium homeostasis. Such cases are typically treated with calcium and vitamin D supplements, although PTH(1-34) has been shown to be effective (16). In such cases, a long-acting PTHR1 agonist could offer considerable advantage over PTH(1-34). At the other end of the pharmacological spectrum, antagonist ligands for the PTHR1 could be useful in treating the hypercalcemia that often develops in late-stage malignancies due to tumor overproduction of PTHrP (17). Finally, it is worth considering that mimetic ligands for the PTHR1 could serve as powerful new tools with which to dissect further the molecular mechanisms of ligand binding and activation at the PTHR1. Aspects of the PTHR1 binding and activation mechanisms relevant to the drug discovery problem are outlined in the following section.

Mechanistic Aspects of the PTHR1

There is currently no crystal structure available for the PTHR1, or for any family B GPCR, at least for the heptahelical, J domain region, which is arguably the most relevant receptor domain to consider for drug development purposes. The crystal structure of the unattached, PTHR1 N domain in complex with the PTH(16-34) binding region was recently determined (18). This result represents a major breakthrough in the field of PTHR1 structure-activity relationship studies, but its potential impact on mimetic development for the PTHR1 is less evident, given that the N domain is generally not predicted to be directly involved in PTHR activation. Structural views of the PTHR1 J domain are currently limited to computer models that can be generated using structure co-ordinates from one of the several class A GPCRs that have now been crystallized (*e.g.*, rhodopsin or the β2-adrenergic receptor) (19). A nearly complete lack of amino acid sequence homology between the family A GPCRs and the PTHR1 most likely

limits the interpretative value of any such model for the PTHR1.

Although the two domain model of the PTHR1-PTH interaction process implies that the N and J domain components function somewhat autonomously, the possibility that the N domain closes in on, and/or functionally integrates with the J domain component is not firmly excluded, and indeed several observations suggest this possibility. First, certain modifications in the 17-26 binding region of PTH affect, albeit modestly, the capacities of the ligand to interact with PTHR-delNT, a PTHR1 construct that lacks nearly the entire N domain (20). Second, a PTH(1-34) analog containing a photo-reactive *para*benzoyl-L-phenylalanine (Bpa) modification at position 18 cross-links to a receptor segment extending from the extracellular end of transmembrane (TM) 1 to the extracellular end of TM 3 (21). Perhaps most interestingly, in the related secretin receptor, short peptides (3-5 amino acids) derived from an exposed loop segment of that receptor's N domain can induce a weak but measurable cAMP response in cells expressing the intact secretin receptor; moreover, the same peptides derivatized with Bpa cross-link to the extracellular end of TM 6 (22). The biological and pharmacological significance of these findings, which were extended to two other family B receptors – the vasoactive intestinal polypeptide type 1 and calcitonin receptors – remains to be established, but they hint at a potentially novel aspect of the family B receptor mechanism that could potentially be exploited for mimetic development.

The capacity of modified N-terminal PTH fragments, such as M-PTH(1-14) (in the "M" PTH analogs referred to herein, M indicates the substitutions of Ser^{1}→Ala, Ser^{3}→Aib {α-amino-isobutyric acid}, Asn^{10}→Gln, Leu^{11}→homoarginine, Gly^{12}→Ala, and His^{14}→Trp, depending on fragment length) and M-PTH(1-11) to induce cAMP and IP_3 responses via PTHR1-delNT with the same potency as they do via the intact PTHR1 indicates that the PTHR1 N domain is not essential for the ligand-induced receptor activation. These data also suggest that the N domain does not occlude access to the receptor's activation pocket within the J domain, at least for the N-terminal PTH peptides. Thus there does not seem to be any architectural feature of the PTHR1 that would prevent activation by any small-molecule mimetic ligand that happens to be targeted to that J domain activation pocket. Any such mimetic ligand for the PTHR1, if found, would be of value not only as a potential lead towards new PTHR1-based therapeutics, but also as a tool with which to further probe molecular mechanisms of action at the receptor.

New Small-Molecule Ligands

AH3960

Several recent reports in the scientific and patent literature confirm that considerable pharmaceutical effort has indeed been applied to the PTHR1 drug discovery problem. In 2006, a compound, AH3960 (dibutyl-diaminomethylene-pyrimidine-2,4,6-trione) was reported that behaves as a weak agonist for the PTHR1 (23). This compound was identified in a high throughput screen (HTS) of a chemical compound library; the read-out for the screen used was not specified, but it was likely based on either a cAMP response element-luciferase (CRE-LUC) response assay, or a fluorescent imaging plate reader (FLIPR) assay that measures intracellular calcium responses, as both assay formats, established in HEK293 cells stably transfected with the hPTHR1, were used to characterize the activity of the compound. AH3960 fully stimulated both the CRE-LUC and FLIPR responses to the same maximal extent as did PTH(1-34); however, the potencies (EC_{50}s) of the two signaling responses induced by the compound (1.5 μM and 3.2 μM, respectively), were several thousand-fold weaker than those observed for PTH(1-34) (0.2 nM and 1 nM, respectively; Fig. 2). The cAMP-stimulating capacity of AH3960 was confirmed by direct RIA measurement of intracellular cAMP in the HEK293 cell system, as well as in the rat osteoblastic cell line ROS 17/2.8, although by the RIA format, only incomplete dose-response curves were obtained for the compound, in which the

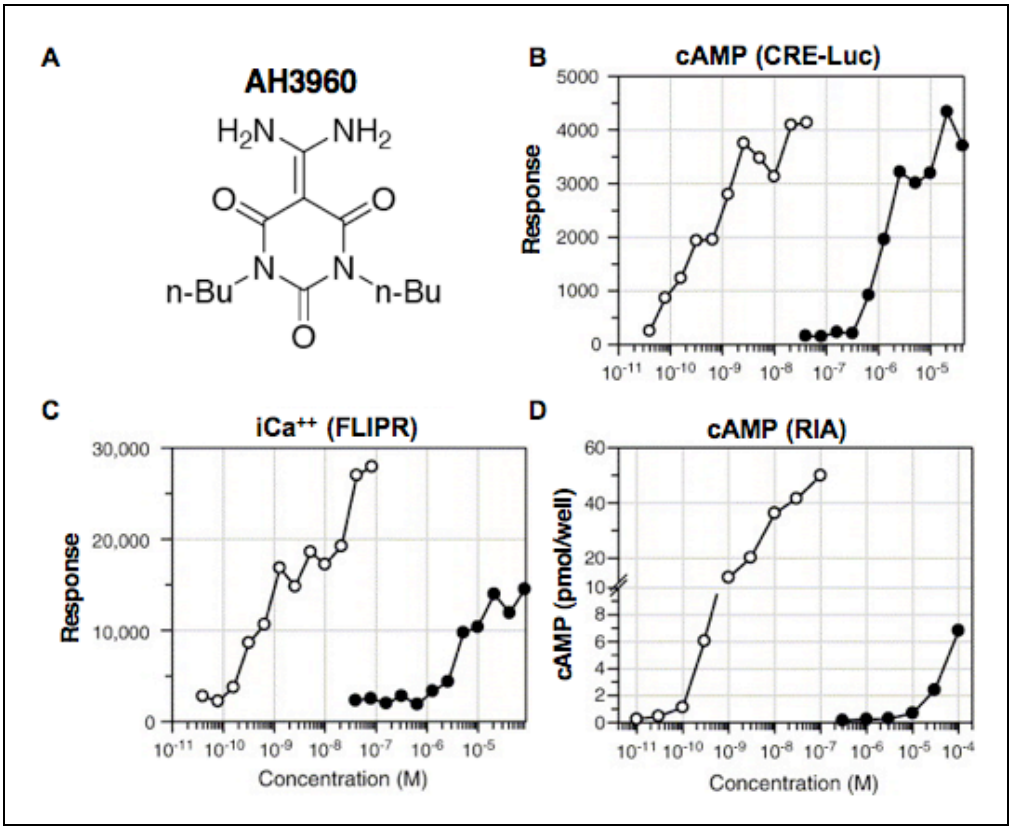

Fig. 2. A small-molecule agonist for the PTHR1. In 2006 a compound with agonist actions at the PTHR1 was reported (23). The structure of the compound (A), and its agonist activities in HEK293 cells stably transfected with the hPTHR (B-D) are shown (graph symbols: PTH(1-34) open circles, AH3960 filled circles). Reprinted from *Bone*, Vol. 39, issue 6, Rickard *et al*. Intermittent treatment with parathyroid hormone (PTH) as well as a non-peptide small molecule agonist of the PTH1 receptor inhibits adipocyte differentiation in human bone marrow stromal cells, pp. 1361-72, Copyright (2006), with permision from Elsevier.

highest concentration tested, 100 μM, induced only ~ half of the maximal response observed for PTH(1-34), which itself displayed the expected high potency (EC_{50} ~ 1 nM).

The activity of AH3960 was further investigated in a human mesenchymal stem cell culture system designed to assess effects on cell differentiation. When applied intermittently (1h/day) at a concentration of 30 μM, AH3960 inhibited the progression of the cells along the adipogenic pathway, to thus favor an osteogenic response. This effect was qualitatively similar to that induced by intermittent PTH(1-34), although, again, a much lower concentration of PTH(1-34) (50 nM) was required.

The combined data reported for AH3960 thus suggest that the compound has some capacity to function as a weak agonist at the PTHR1, and thereby mimic at least some of the proximal and downstream signaling responses induced by PTH(1-34) in osteoblasts. No cAMP or iCa^{++} response was detected in un-transfected HEK293 cells, indicating that the signaling effects observed were PTHR-dependent. Direct binding assays for the compound were not reported, however. The weak signaling potencies suggest a weak binding affinity, but this needs to be assessed. It would also be useful to know whether or not the functional actions of AH3960 can be inhibited by classical, N-terminally truncated PTHR antagonists, such as PTH(7-34), or N-terminally intact antagonists having critical valine[2] replaced by Bpa or a similarly bulky residue, such as tryptophan (24). In addition, it would be useful to know whether AH3960 exhibits altered functionality on certain

mutant forms of the PTHR that exhibit altered binding and/or signaling responses to PTH(1-34). For example, PTHR-delNt, which binds and responds only poorly to PTH(1-34) (due to the absence of the N domain docking interactions), could be used to help resolve whether AH3960 interacts with the receptor's N domain, or, as expected for such an agonist, solely with the receptor's J domain. The mode of action of AH3960, in terms of its binding site on the receptor – orthosteric versus allosteric – therefore needs to be investigated more deeply to gain a better mechanistic understanding of this interesting compound. AH3960 is of interest because it is the only non-peptide agonist reported so far for the PTHR1, but additional information is needed to help evaluate whether or not it is a worthy scaffold for more extensive chemical development aimed at obtaining a high potency PTHR agonist.

SW106

In 2007 an industry research group reported the discovery of a non-peptide compound that functions as an antagonist for the PTHR (25). This compound, SW106, was identified by screening for the capacity of compounds to inhibit the binding of a modified PTH(1-14) peptide analog. The peptide analog used was developed, in part, based on the modified PTH(1-14) analogs reported by the MGH group, and thus contains similar modifications. These modifications together increase binding affinity and signaling potency by as much as 100,000 fold, relative to the very weak binding PTH(1-14) native peptide. The modifications, generally called M by the MGH group, typically consist of the conformationally constrained α-amino-isobutyric acid (Aib) in place of Ser^{1} and/or Ala^{3}; glutamine in place of $asparagine^{10}$; arginine or homoarginine in place of $leucine^{11}$; alanine in place of $glycine^{12}$; and tryptophan in place of $histidine^{14}$. In cell-based assays, M-PTH(1-14) stimulates both cAMP and IP_3 formation with potencies comparable to those observed for PTH(1-34) (26-29). The analog developed by the industry group was termed AJ1951. Before using AJ1951 for screening purposes, the industry group first needed to establish that it functions as a true PTHR1 agonist. This was accomplished by showing that it induces the same alterations in gene expression patterns in UMR106 rat osteoblast cells that PTH(1-34) does, and that it normalizes serum calcium in thyroidparathyroidectomized rats when infused via an implanted (s.c.) Alzet mini-pump. The rescue effect on blood calcium required a peptide dose-rate (≥ 3 μg/hr) that was 40-fold higher than that needed for PTH(1-34), which most likely reflects a more rapid disappearance of the 14-mer from the circulation, due to renal filtration and/or serum protease digestion, as compared to PTH(1-34). A faster PK profile for the M-PTH(1-14) scaffold was demonstrated by a second industry group in studies that use mass-spectroscopy to measure the concentration of the peptide in blood plasma at times after i.v. injection of the peptide into rats (30).

It is worth considering that the usefulness, from a drug screening standpoint, of the “M” modified N-terminal PTH analogs is based on their unique, single-site mode of action at the PTHR1 – *i.e*., they interact only with the receptor's J domain. This J domain-specific mode of action provided the rationale for the screening approach taken by the first industry group, as any “hit” compound that competitively inhibits the binding of AJ1951 to the PTHR1 would likely interact with the receptor's J domain, and be situated in the region of the binding pocket involved in receptor activation. In contrast to this, a screening approach based on inhibition of PTH(1-34) binding would be more likely to yield compounds that bind to the receptor's N domain, as this provides most of the binding energy used for PTH(1-34). An N domain-specific non-peptide ligand would be of pharmacological interest, but would not likely have agonist potential, given that the N domain is not predicted to participate importantly in the signal transduction process (despite the proviso discussed above concerning the secretin receptor). Based on this rationale, compound SW106, which inhibits the binding of ^{125}I-AJ1951 to the PTHR1 with an IC_{50} of 0.9 μM, is highly likely to interact with the PTHR1 J domain. Schild-type analyses performed on the

compound showed that it causes a rightward and parallel shift in the cAMP dose-response curve generated by AJ1951; this pharmacological behavior is consistent with a true competitive mechanism of binding. The receptor contact site used by SW106 is thus likely to at least partly overlap with that used by the PTH(1-14) agonist analog.

Additional medicinal chemistry work aimed at transforming SW106 into a potent agonist was not directly performed in the study; however, the compound had been identified previously in a screen for HIV reverse transcriptase inhibitors. Thus, a number of related structures was available for analysis. Unfortunately, none of the structures was shown to behave as a PTHR1 agonist. Nevertheless, the analysis yielded information on how the various chemical constituents of the compound might contribute to PTHR1 binding affinity.

The chemical structure of SW106 consists of an unusual 4,1-benzoxazepinone ring scaffold that contains a constrained, di-peptide motif, two ring-attached fluorine atoms, a trifluoromethane group, and two alkyl extensions: an olefin with a terminal cyclopropyl group and an ethyl side chain (Fig. 3). The halogenation pattern on the aromatic ring, and the terminal group on the olefin, were found to be generally tolerant constituents, whereas the ethyl appendage was critical for receptor binding, however, no further gains in affinity were reported in the study.

A docking scenario for SW106 was developed *in silico*, using the rhodopsin template to model the PTHR1 J domain. The procedure suggested that the compound could fit comfortably into a hydrophobic/aromatic pocket formed by residues on the inner faces of the extracellular portions of TMs 3, 4, 5 and 6 (Fig. 3). The compound was positioned manually to this region of the receptor based on prior mutational and cross-linking data that indicated potentially important roles for several residues in the vicinity, most notably, Ser^{370} (TM5) and Met^{425} (TM6), in mediating ligand-induced signal transduction, specifically as mediated by the ligand's most critical signaling residue, Val^{2} (31-33). That this receptor region is indeed a functional "hot spot" is further suggested by recent disulfide-based mapping studies of Thomas and colleagues, which show that interactions between Ile^{367} and Leu^{368} in TM5 and Met^{425} in TM6 mediate ligand-induced transmembrane-domain movements as part of the receptor activation mechanism (34). The model presented for SW106 predicts certain pharmacological behaviors for the compound that can be tested easily. For example, the proposed binding site suggests that SW106 could function as an allele-specific inverse agonist for certain constitutively active PTHR1 mutants, and thus suppress the ligand-independent cAMP signaling activity of PTHR1-H223R, but not that of PTHR1-T410P. This pharmacological profile would mimic that produced by Bpa^{2}-containing PTH peptide analogs and which is mediated by Met^{425} (32;35). Additional work is needed to characterize the mode of action used by SW106, but the compound, at this point, appears to be a mimetic ligand that binds to a site in the PTHR1 that is critically involved in the agonist-induced activation process. It could thus conceivably be transformed, via medicinal chemistry approaches, into a potent PTHR1 mimetic agonist.

High Affinity Antagonists

A third set of small molecule compounds was reported in 2007 (17). These compounds are based on a 1,3,4-benzotriazepine scaffold structure, the parent of which (compound 1) binds to the PTHR1 with an affinity of ~1 μM. Compound 1 was identified by screening compounds for the capacity to inhibit binding of ^{125}I-[$Nle^{8,18}$,Tyr^{34}]hPTH(1-34) to the hPTHR1 expressed in HEK293 cells. Subsequent chemical optimization efforts yielded a family of derivative compounds, of which compound 19 (Fig. 4) binds to the PTHR1 with an affinity in the low-nanomolar range. Compound 19 lacks cAMP agonist activity, as tested at a concentration of 10 μM in SaOS-2 cells, and functions as a competitive PTHR1 antagonist, based on its capacity to cause a rightward shift in the dose-response curve obtained with

IBMS BoneKEy. 2009 February;6(2):71-85
http://www.bonekey-ibms.org/cgi/content/full/ibmske;6/2/71
doi: 10.1138/20090364

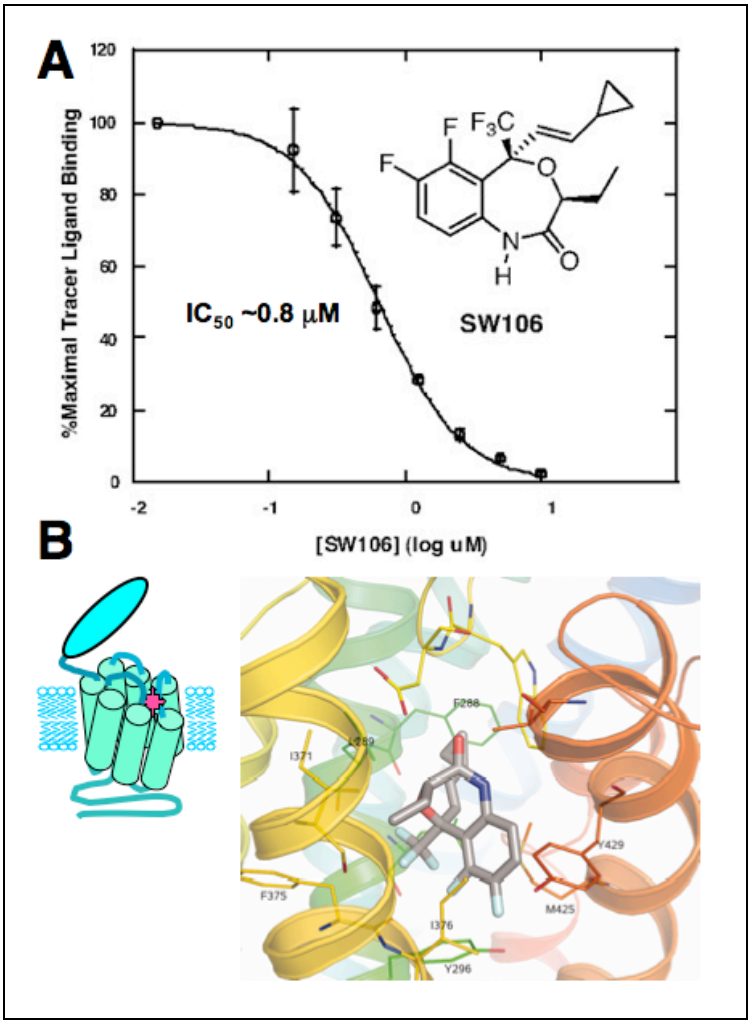

Fig. 3. A small-molecule antagonist targetred to the PTHR1 J domain. In 2006 a new antagonist compound (SW106) that binds to the PTHR1 J domain was reported (25). The J domain binding site is established by the capacity of the compound to inhibit binding of a ^{125}I-PTH(1-14) analog to the PTHR1 (A). Modeling (B) shows that the compound could fit into a hydrophobic/aromatic pocket formed by residues at the extracellular ends of TMs 3 (green), 5 (yellow) and 6 (orange). Adapted from Carter *et al*. *Proc Natl Acad Sci U S A*. 2007 Apr 17;104(16):6846-51. Copyright (2007) by the National Academy of Sciences.

PTHrP(1-34). Note that the PTHrP(1-34) peptide was used in these inhibition experiments, because the overall strategic aim of the study was to develop new therapies for malignancy-associated hypercalcemia. Given the remarkably high affinity with which compound 19 and several related compounds bind to the receptor, it is of considerable strategic interest to know the receptor-binding sites used by the analogs and whether or not the compounds function as inverse agonists at constitutively active PTHR1 mutants. If the compounds turn out to bind to the PTHR1 J domain, then they would be extremely interesting to consider for further chemical transformation aimed at generating potent PTHR1 agonists.

Rational Design Approaches

A different approach to consider for generating mimetic non-peptide agonists for the PTHR1 is conceptually based on transforming the pharmacophoric domain structure of the native PTH peptide agonist into a non-peptide compound via rational design. This approach requires that the key functional groups on the peptide ligand have

IBMS BoneKEy. 2009 February;6(2):71-85
http://www.bonekey-ibms.org/cgi/content/full/ibmske;6/2/71
doi: 10.1138/20090364

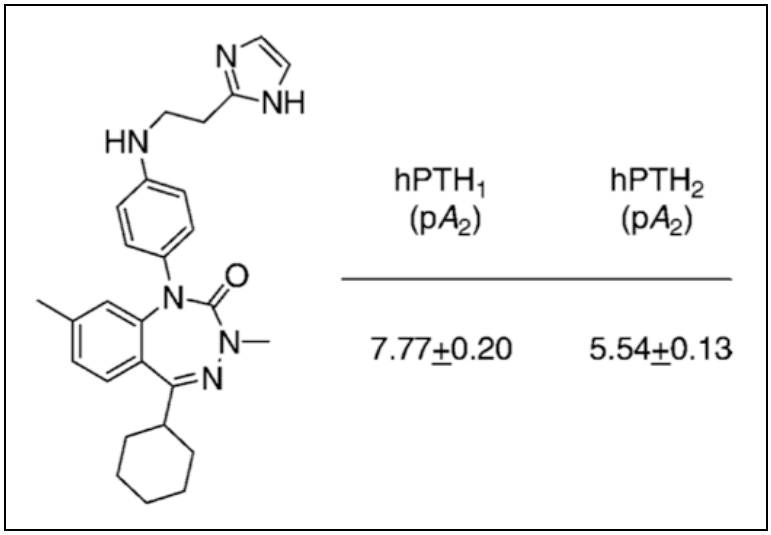

Fig. 4. High affinity PTHR1 antagonists. A family of antagonist compounds that bind to the PTHR1 with surprisingly high affinity was reported in 2007 (17). The most effective compound, compound 19, inhibits the binding of ^{125}I-PTH(1-34) to the PTHR1 with an apparent affinity of ~13 nM (affinity is ~100-fold weaker for the PTHR2). Compound 19 is the highest affinity non-peptide ligand so far identitied for the PTHR1. Adapted with permission from McDonald *et al*. *J Med Chem*. 2007 Oct 4;50(20):4789-92. Copyright (2007) ACS.

been identified, and that their relative spatial geometries, as found in the bioactive conformation, have been mapped reliably. Moreover, the bioactive pharmacophoric peptide domain structure must be amenable to chemical mimicry. For PTH, these requirements are problematic for several reasons. First, the N-terminal pharmacophore domain structure of PTH appears to be an α-helix (36-38), which is a notoriously difficult peptide fold to mimic via non-peptide chemistry (39;40). Second, the identities and spatial locations of the key functional groups within the N-terminal domain of PTH are not precisely known. A series of minimization and optimization studies performed on PTH peptides suggest that the key functional groups are contained within the first 9 to 11 amino acids, as M-PTH(1-11) ([Aib^{1,3},Gln^{10},Har^{11}]-PTH(1-11)NH_2), is the shortest length N-terminal peptide that can stimulate a measurable cAMP response in PTHR1-expressing cells, and residues 1-9 in such N-terminal PTH peptides are particularly sensitive to substitution (26;27;29). The native PTH(1-11) and PTH(1-9) sequences are inactive as free peptides, but when tethered directly to the J domain via a Gly linker between the C-terminal PTH residue and the extracellular end of TM1 of the PTHR1-delNt, they stimulate a robust cAMP production in transfected COS-7 cells, and mutagenesis studies of these tethered ligands reveal critical roles for Met^8, Ile^5 and especially Val^2 in the receptor activation process (41). Thus, the bioactive pharmacophore of PTH appears to be formed largely by the side chains of Met^8, Ile^5 and Val^2 displayed as a broad, hydrophobic ridge along approximately one face of the N-terminal α-helix (Fig. 5). Such a topological surface does not appear to lend itself to simple chemical mimicry.

A first step in peptide mimetic design strategies is often to conformationally constrain the target peptide in its bioactive structure so as to limit the rotational degrees of freedom available to the key pharmacophoric groups (42). To some extent, this has been achieved for the N-terminal PTH peptides, as they typically contain a conformationally constraining, α-α-dialkyl amino acid, such as Aib, or the slightly bulkier 1-aminocyclopentane-1-carboxylic acid (AC_5C), at positions 1 and/or 3; these substitutions alone account for increases in potency of as much as ~100-fold and marked stabilization of α-helical structure (28;29). Thus the substitutions stabilize a true bioactive confirmation. Other

IBMS BoneKEy. 2009 February;6(2):71-85
http://www.bonekey-ibms.org/cgi/content/full/ibmske;6/2/71
doi: 10.1138/20090364

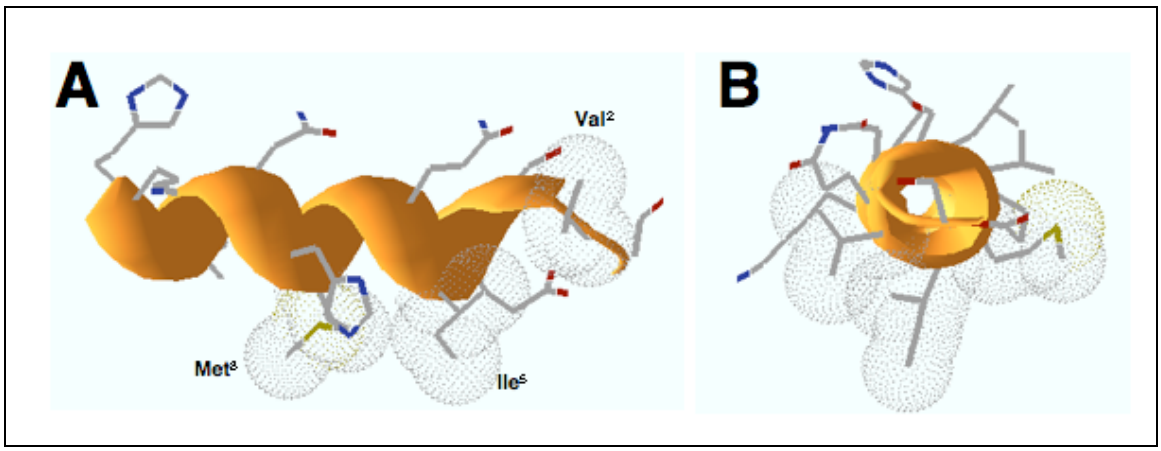

Fig. 5. Dispersed topology of the N-terminal PTH pharmacophore. Residues 1-14 of native hPTH are shown in helical configuration (views are from the side with His^{14} at the left, A; and end-on with Ser^{1} directed out from the plane, B. The backbone is shown in ribbon format (orange) and sidechains are shown as sticks (CPK coloring) with van der Waals spheres shown for Val^{2}, Met^{5} and Met^{8}. These three residues are proposed to comprise the critical agonist pharmacophore of the ligand.

attempts at conformationally constraining the N-terminal PTH domain have so far yielded only limited success in terms of maintaining activity at the receptor. Thus, the installation of a rigidified dipeptide mimetic unit – 3R-carboxy-6S-amino-7,5-bicyclic thiazolidinlactam (7,5-bTL) – at various positions in [Ala^{1},Nle^{8},Arg^{11}]hPTH(1-11)NH_2 (43), or the introduction of helix-stabilizing lactam modifications between Glu (or Asp) -Lys side-chain pairs substituted at various *i*, *i*+4 positions in the PTH(1-14) scaffold, did not result in significant gains in potency (37;44). The bioactive conformation of N-terminal PTH is thus relatively intolerant of structural modifications, presumably reflecting a precise geometric complementarity with the cognate surface of the receptor.

In parallel to the goal of optimizing and minimizing the amino-terminal bioactive domain of the PTH peptide ligand, obtaining a precise map of the cognate molecular interaction surface in the receptor would also likely facilitate the process of designing mimetic ligands for the PTHR1. Such mapping data for the PTHR1 are still largely in preliminary form. The Met^{8}-Ile^{5}-Val^{2} motif of the ligand can roughly be predicted to fit into a hydrophobic pocket formed within the extracellular portion of the juxtamembrane region of the receptor, with critical Val^{2} potentially involved in contacts with Ser^{370} and/or Met^{425} at the extracellular ends of TM5 and TM6, respectively. Less is known regarding the candidate receptor contact residues for Ile^{5} and Met^{8}. Modeling studies on the PTH(1-11)-PTHR1 tethered construct predicts that these residues penetrate deeply into the hydrophobic core of the receptor (45). Studies on the PTHR2 subtype and its capacity to discriminate between PTH and PTHrP based on the divergent residue at position 5 in the ligands – His^{5} of PTHrP produces antagonist responses, whereas Ile^{5} of PTH produces agonist responses (46;47) – functionally identify Leu^{289} and Ile^{363} (PTHR1 numbering) at or near the boundary of extracellular loop 2 and TM3 as potential interaction sites for residue 5 in the ligands (48;49). There are no biophysical data, however, that directly assess residue-5 interactions, nor are there any for Met^{8}. The information available so far is thus too limited to construct more than a preliminary view of the molecular surfaces in the receptor that mediate the critical interactions with the pharmacophoric determinants in N-terminal PTH. It is also important to consider that both mutational and cross-linking data suggest that the portion of PTH that is likely to contact the juxtamembrane region extends from residue 1 in the ligand to at least position 19 (21;45;50-52). There may thus be a myriad of ancillary interactions that help stabilize or participate in the interactions that define the

active-state complex formed between the N-terminal domain of PTH and the juxtamembrane region of the receptor. In any case, it seems clear that more experimental information on the bimolecular complex will be needed before any true rational design approach can be used to generate *de novo* mimetic ligands that potently activate the PTHR1 and faithfully stimulate the signal transduction responses induced by native PTH and PTHrP peptides.

Conclusions and Future Directions

The PTHR1 is an important and challenging target for drug discovery efforts. Several small molecule compounds have now been described for the PTHR1, and these represent potential lead compounds for future development as potent PTHR1 agonists. Identifying the binding sites in the receptor and the pharmacological modes of action used by these compounds represent important next steps to take in this direction. Further optimization and minimization of the N-terminal pharmacophoric domain of the peptide ligand, together with more precise mapping of the cognate binding pocket in the receptor, are alternative, perhaps parallel, strategies to pursue. Finally, developing new screening approaches that employ new compound libraries and some of the new methodologies and reagents that are now available for the PTHR1 and GPCRs in general (*e.g.*, FRET and fluorescent ligands), could also be fruitful paths to follow. Such approaches, while technically challenging and perhaps costly to implement, are justified by the clear need to develop the next generation of PTHR1 ligands that can be used to treat diseases of bone and mineral metabolism with greater efficacy and safety.

Conflict of Interest: The author reports receiving research funding from Chugai Pharmaceutical Co.

Peer Review: This article has been reviewed by Dale Mierke.

References

1. Canalis E, Giustina A, Bilezikian JP. Mechanisms of anabolic therapies for osteoporosis. *N Engl J Med*. 2007 Aug 30;357(9):905-16.

2. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med*. 2001 May 10;344(19):1434-41.

3. Kostenuik PJ, Ferrari S, Pierroz D, Bouxsein M, Morony S, Warmington KS, Adamu S, Geng Z, Grisanti M, Shalhoub V, Martin S, Biddlecome G, Shimamoto G, Boone T, Shen V, Lacey D. Infrequent delivery of a long-acting PTH-Fc fusion protein has potent anabolic effects on cortical and cancellous bone. *J Bone Miner Res*. 2007 Oct;22(10):1534-47.

4. Tashjian AH Jr, Gagel RF. Teriparatide [human PTH(1-34)]: 2.5 years of experience on the use and safety of the drug for the treatment of osteoporosis. *J Bone Miner Res*. 2006 Mar;21(3):354-65.

5. Greenspan SL, Bone HG, Ettinger MP, Hanley DA. Lindsay R, Zanchetta JR, Blosch CM, Mathisen AL, Morris SA, Marriott TB; Treatment of Osteoporosis with Parathyroid Hormone Study Group. Effect of recombinant human parathyroid hormone (1-84) on vertebral fracture and bone mineral density in postmenopausal women with osteoporosis: a randomized trial. *Ann Intern Med*. 2007 Mar 6;146(5):326-39.

6. Black DM, Bouxsein ML, Palermo L, McGowan JA, Newitt DC, Rosen E, Majumdar S, Rosen CJ; PTH Once-Weekly Research (POWR) Group. Randomized trial of once-weekly parathyroid hormone (1-84) on bone mineral density and remodeling. *J Clin Endocrinol Metab*. 2008 Jun;93(6):2166-72.

7. Fogelman I, Fordham JN, Fraser WD, Spector TD, Christiansen C, Morris SA, Fox J. Parathyroid hormone(1-84) treatment of postmenopausal women with low bone mass receiving hormone replacement therapy. *Calcif Tissue Int*. 2008 Aug;83(2):85-92.

8. Stewart AF, Cain RL, Burr DB, Jacob D, Turner CH, Hock JM. Six-month daily administration of parathyroid hormone and parathyroid hormone-related protein peptides to adult ovariectomized rats markedly enhances bone mass and biomechanical properties: a comparison of human parathyroid hormone 1-34, parathyroid hormone-related protein 1-36, and SDZ-parathyroid hormone 893. *J Bone Miner Res*. 2000 Aug;15(8):1517-25.

9. Horwitz MJ, Tedesco MB, Gundberg C, Garcia-Ocana A, Stewart AF. Short-term, high-dose parathyroid hormone-related protein as a skeletal anabolic agent for the treatment of postmenopausal osteoporosis. *J Clin Endocrinol Metab*. 2003 Feb;88(2):569-75.

10. Hodsman A, Bone H, Gallagher C, Kendler D, Brown JP, Greenspan S, Barclay CL, Morley P, Anderson R, MacDonald BR. Ostabolin-C™ increases lumbar spine and hip BMD after 1 year of therapy: results of a phase II clinical trial. *J Bone Miner Res*. 2007 Sep;22(Suppl 1):S38.

11. Culler MD, Dong J, Shen Y, Taylor JE, Carlile L, Sullivan T, Batista I, Bonin P, Carlson M, Lauer J, Savola A, Kasprzyk P, Morgan BA, Fisch C, Bécret A, Legrand JJ, Woon CW. BIM-44058, a novel analog of PTHrP with enhanced bone building activity, but decreased calcium-mobilization potential. *J Bone Miner Res*. 2001 Oct;16(Suppl 1):S540.

12. Legrand J, Fisch C, Guillaumat P, de Jouffrey S, Dong JZ, Woon CW, Claude J, Culler MD. BIM-44058, a novel PTHrP analog, restores in vivo spinal bone mineral density in old ovariectomized osteopenic cynomolgus monkeys. *J Bone Miner Res*. 2001 Sep;16(Suppl 1):S539.

13. Dean T, Linglart A, Mahon MJ, Bastepe, M, Jüppner H, Potts JT Jr, Gardella TJ. Mechanisms of ligand binding to the parathyroid hormone (PTH)/PTH-related protein receptor: selectivity of a modified PTH(1-15) radioligand for GalphaS-coupled receptor conformations. *Mol Endocrinol*. 2006 Apr;20(4):931-43.

14. Dean T, Vilardaga JP, Potts JT Jr, Gardella TJ. Altered selectivity of parathyroid hormone (PTH) and PTH-related protein (PTHrP) for distinct conformations of the PTH/PTHrP receptor. *Mol Endocrinol*. 2008 Jan;22(1):156-66.

15. Okazaki M, Ferrandon S, Vilardaga JP, Bouxsein ML, Potts JT Jr, Gardella TJ. Prolonged signaling at the parathyroid hormone receptor by peptide ligands targeted to a specific receptor conformation. *Proc Natl Acad Sci U S A*. 2008 Oct 28;105(43):16525-30.

16. Winer KK, Sinaii N, Peterson D, Sainz B Jr, Cutler GB Jr. Effects of once versus twice-daily parathyroid hormone 1-34 therapy in children with hypoparathyroidism. *J Clin Endocrinol Metab*. 2008 Sep;93(9):3389-95.

17. McDonald IM, Austin C, Buck IM, Dunstone DJ, Gaffen J, Griffin E, Harper EA, Hull RA, Kalindjian SB, Linney ID, Low CM, Patel D, Pether MJ, Raynor M, Roberts SP, Shaxted ME, Spencer J, Steel KI, Sykes DA, Wright PT, Xun W. Discovery and characterization of novel, potent, non-peptide parathyroid hormone-1 receptor antagonists. *J Med Chem*. 2007 Oct 4;50(20):4789-92.

18. Pioszak AA, Xu HE. Molecular recognition of parathyroid hormone by its G protein-coupled receptor. *Proc Natl Acad Sci U S A*. 2008 Apr 1;105(13):5034-9.

19. Kobilka B, Schertler GF. New G-protein-coupled receptor crystal structures: insights and limitations. *Trends Pharmacol Sci*. 2008 Feb;29(2):79-83.

20. Barbier JR, Gardella TJ, Dean T, MacLean S, Potetinova Z, Whitfield JF, Willick GE. Backbone-methylated analogues of the principle receptor binding region of human parathyroid hormone. Evidence for binding to both the N-terminal extracellular domain and extracellular loop region. *J Biol Chem*. 2005 Jun 24;280(25):23771-7.

21. Wittelsberger A, Corich M, Thomas BE, Lee BK, Barazza A, Czodrowski P, Mierke DF, Chorev M, Rosenblatt M. The mid-region of parathyroid hormone (1-34) serves as a functional docking domain in receptor activation. *Biochemistry*. 2006 Feb 21;45(7):2027-34.

22. Dong M, Pinon DI, Asmann YW, Miller LJ. Possible endogenous agonist mechanism for the activation of secretin family G protein-coupled receptors. *Mol Pharmacol*. 2006 Jul;70(1):206-13.

23. Rickard DJ, Wang FL, Rodriguez-Rojas AM, Wu Z, Trice WJ, Hoffman SJ, Votta B, Stroup GB, Kumar S, Nuttall ME. Intermittent treatment with parathyroid hormone (PTH) as well as a non-peptide small molecule agonist of the PTH1 receptor inhibits adipocyte differentiation in human bone marrow stromal cells. *Bone*. 2006 Dec;39(6):1361-72.

24. Shimizu N, Dean T, Tsang JC, Khatri A, Potts JT Jr, Gardella TJ. Novel parathyroid hormone (PTH) antagonists that bind to the juxtamembrane portion of the PTH/PTH-related protein receptor *J Biol Chem*. 2005 Jan 21;280(3):1797-807.

25. Carter PH, Liu RQ, Foster WR, Tamasi JA, Tebben AJ, Favata M, Staal A, Cvijic ME, French MH, Dell V, Apanovitch D, Lei M, Zhao Q, Cunningham M, Decicco CP, Trzaskos JM, Feyen JH. Discovery of a small molecule antagonist of the parathyroid hormone receptor by using an N-terminal parathyroid hormone peptide probe. *Proc Natl Acad Sci U S A*. 2007 Apr 17;104(16):6846-51.

26. Shimizu M, Carter PH, Khatri A, Potts JT Jr, Gardella TJ. Enhanced activity in parathyroid hormone-(1-14) and -(1-11): novel peptides for probing ligand-receptor interaction. *Endocrinology*. 2001 Jul;142(7):3068-74.

27. Shimizu M, Potts JT, Gardella TJ. Minimization of parathyroid hormone. Novel amino-terminal parathyroid hormone fragments with enhanced potency in activating the type-1 parathyroid hormone receptor. *J Biol Chem*. 2000 Jul 21;275(29):21836-43.

28. Shimizu N, Dean T, Khatri A, Gardella TJ. Amino-terminal parathyroid hormone fragment analogs containing alpha,alpha-di-alkyl amino acids at positions 1 and 3. *J Bone Miner Res*. 2004 Dec;19(12):2078-86.

29. Shimizu N, Guo J, Gardella TJ. Parathyroid hormone (PTH)-(1-14) and -(1-11) analogs conformationally constrained by alpha-aminoisobutyric acid mediate full agonist responses via the juxtamembrane region of the PTH-1 receptor. *J Biol Chem*. 2001 Dec 28;276(52):49003-12.

30. Shimizu M, Shimizu N, Okazaki M, Katsumata K, Murao K, Kato K, Potts JT Jr, Kronenberg HM, Gardella T, Makishima F, Saito H. Parathyroid hormone (1-14) fragments increase bone mass in OVX rats. Advances in Skeletal Anabolic Agents for the Treatment of Osteoporosis Meeting Abstracts;2004:M15.

31. Gardella TJ, Jüppner H, Wilson AK, Keutmann HT, Abou-Samra AB, Segre GV, Bringhurst FR, Potts JT Jr, Nussbaum SR, Kronenberg HM. Determinants of [Arg2]PTH-(1-34) binding and signaling in the transmembrane region of the

parathyroid hormone receptor. *Endocrinology*. 1994 Sep;135(3):1186-94.

32. Gensure RC, Carter PH, Petroni BD, Jüppner H, Gardella TJ. Identification of determinants of inverse agonism in a constitutively active parathyroid hormone/parathyroid hormone-related peptide receptor by photoaffinity cross-linking and mutational analysis. *J Biol Chem*. 2001 Nov 16;276(46):42692-9.

33. Behar V, Bisello A, Bitan G, Rosenblatt M, Chorev M. Photoaffinity cross-linking identifies differences in the interactions of an agonist and an antagonist with the parathyroid hormone/parathyroid hormone-related protein receptor. *J Biol Chem*. 2000 Jan 7;275(1):9-17.

34. Thomas BE, Sharma S, Mierke DF, Rosenblatt M. Parathyroid hormone (PTH) and PTH antagonist induce different conformational changes in the PTHR1 receptor. *J Bone Miner Res*. 2008 Dec 8. [Epub ahead of print]

35. Carter PH, Petroni BD, Gensure RC, Schipani E, Potts JT Jr, Gardella TJ. Selective and nonselective inverse agonists for constitutively active type-1 parathyroid hormone receptors: evidence for altered receptor conformations. *Endocrinology*. 2001 Apr;142(4):1534-45.

36. Chen Z, Xu P, Barbier JR, Willick G, Ni F. Solution structure of the osteogenic 1-31 fragment of the human parathyroid hormone. *Biochemistry*. 2000 Oct 24;39(42):12766-77.

37. Tsomaia N, Pellegrini M, Hyde K, Gardella TJ, Mierke, DF. Toward parathyroid hormone minimization: conformational studies of cyclic PTH(1-14) analogues. *Biochemistry*. 2004 Jan 27;43(3):690-9.

38. Jin L, Briggs SL, Chandrasekhar S, Chirgadze NY, Clawson DK, Schevitz RW, Smiley DL, Tashjian AH, Zhang F. Crystal structure of human parathyroid hormone 1-34 at 0.9-A resolution. *J Biol Chem*. 2000 Sep 1;275(35):27238-44.

39. Che Y, Brooks BR, Marshall GR. Protein recognition motifs: design of peptidomimetics of helix surfaces. *Biopolymers*. 2007 Jul;86(4):288-97.

40. Davis JM, Tsou LK, Hamilton AD. Synthetic non-peptide mimetics of alpha-helices. *Chem Soc Rev*. 2007 Feb;36(2):326-34.

41. Shimizu M, Carter PH, Gardella TJ. Autoactivation of type-1 parathyroid hormone receptors containing a tethered ligand. *J Biol Chem*. 2000 Jun 30;275(26):19456-60.

42. Vagner J, Qu H, Hruby VJ. Peptidomimetics, a synthetic tool of drug discovery. *Curr Opin Chem Biol*. 2008 Jun;12(3):292-6.

43. Fiori N, Caporale A, Schievano E, Mammi S, Geyer A, Tremmel P, Wittelsberger A, Woznica I, Chorev M, Peggion E. Structure-function relationship studies of PTH(1-11) analogues containing sterically hindered dipeptide mimetics. *J Pept Sci*. 2007 Aug;13(8):504-12.

44. Shimizu N, Petroni BD, Khatri A, Gardella TJ. Functional evidence for an intramolecular side chain interaction between residues 6 and 10 of receptor-bound parathyroid hormone analogues. *Biochemistry*. 2003 Mar 4;42(8):2282-90.

45. Monticelli L, Mammi S, Mierke DF. Molecular characterization of a ligand-tethered parathyroid hormone receptor. *Biophys Chem*. 2002 Feb 19;95(2):165-72.

46. Behar V, Nakamoto C, Greenberg Z, Bisello A, Suva LJ, Rosenblatt M, Chorev M. Histidine at position 5 is the specificity "switch" between two parathyroid hormone receptor subtypes. *Endocrinology*. 1996 Oct;137(10):4217-24.

47. Gardella TJ, Luck MD, Jensen GS, Usdin TB, Jüppner H. Converting parathyroid hormone-related peptide (PTHrP) into a potent PTH-2 receptor agonist. *J Biol Chem*. 1996 Aug 16;271(33):19888-93.

48. Bergwitz C, Jusseaume SA, Luck MD, Jüppner H, Gardella TJ. Residues in the membrane-spanning and extracellular loop regions of the parathyroid hormone (PTH)-2 receptor determine signaling selectivity for PTH and PTH-related peptide. *J Biol Chem*. 1997 Nov 14;272(46):28861-8.

49. Turner PR, Mefford S, Bambino T, Nissenson RA. Transmembrane residues together with the amino terminus limit the response of the parathyroid hormone (PTH) 2 receptor to PTH-related peptide. *J Biol Chem*. 1998 Feb 13;273(7):3830-7.

50. Bisello A, Adams AE, Mierke DF, Pellegrini M, Rosenblatt M, Suva LJ, Chorev M. Parathyroid hormone-receptor interactions identified directly by photocross-linking and molecular modeling studies. *J Biol Chem*. 1998 Aug 28;273(35):22498-505.

51. Gensure RC, Shimizu N, Tsang J, Gardella TJ. Identification of a contact site for residue 19 of parathyroid hormone (PTH) and PTH-related protein analogs in transmembrane domain two of the type 1 PTH receptor. *Mol Endocrinol*. 2003 Dec;17(12):2647-58.

52. Monaghan P, Thomas BE, Woznica I, Wittelsberger A, Mierke DF, Rosenblatt M. Mapping peptide hormone-receptor interactions using a disulfide-trapping approach. *Biochemistry*. 2008 Jun 3;47(22):5889-95.