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A Parathyroid Hormone Inhibitor in vivo: Design and Biological Evaluation of a Hormone Analog

Abstract. *A synthetic analog of bovine parathyroid hormone (bPTH), [tyrosine-34]bPTH-(7–34)NH_2, was found to inhibit parathyroid hormone action in vivo. When the analog and parathyroid hormone were infused simultaneously to rats at a molar ratio of 200 to 1, the analog inhibited the excretion of urinary phosphate and adenosine 3′,5′-monophosphate. When infused alone at the same dose rate, the analog was devoid of agonist activity. The compound was prepared by following design principles developed for inhibitors of parathyroid hormone, and is believed to be the first antagonist of parathyroid hormone that is effective in vivo.*

Parathyroid hormone (PTH) is a single-chain polypeptide of 84 amino acids which, through its action on kidney and bone, serves a critical role in mineral ion homeostasis. Elucidation of the complex regulatory events involved in mineral ion homeostasis and the physiological role of PTH would be facilitated by availability of an inhibitor of PTH effective in vivo. Hypersecretion of PTH by the parathyroid glands occurs commonly in humans (*1*); the complex diagnostic and therapeutic issues that this disorder poses provide considerable impetus for the development of antagonists of PTH action in vivo.

All of the structural determinants necessary for full biological activity of PTH reside within the NH_2-terminal one-third (residues 1–34) of the native hormone, as has been determined in vitro and in vivo in multiple assays of PTH-stimulated effects on concentrations of 3′,5′-adenosine monophosphate (cyclic AMP) and fluxes in calcium and phosphate (*2, 3*).

Over the last decade, extensive studies of the structure and activity of PTH have delineated within the hormone molecule separable domains responsible for receptor binding and activation (*3–6*). There is an address core, or domain, responsible for receptor binding (that is, the 3–34 sequence) and a small but critical message region (positions 1 and 2) necessary for hormone action. These studies led to the design and synthesis of bovine parathyroid hormone (bPTH) analogs that are potent antagonists in multiple assay systems in vitro (*3*). An analog of the 3–34 sequence, with norleucine (Nle) and tyrosine (Tyr) as substitutions, [Nle^8 , Nle^{18} , Tyr^{34}]bPTH - (3–34)NH_2, proved to be the most potent in vitro antagonist of PTH yet designed (*5*). In vitro, this compound is a true competitive inhibitor of PTH that possesses an avidity for PTH receptors comparable to that of PTH and displays an inhibitory constant (K_i) approximately equal to the affinity constant (K_m) for PTH (*5, 6*). In vitro, this analog is devoid of PTH-like agonist activity (*3, 5, 7*). However, when tested in vivo (*8, 9*), [Nle^8, Nle^{18}, Tyr^{34}]bPTH-(3–34)NH_2 failed to display inhibitory properties; in other investigations in vivo, the analog demonstrated weak, but definite, PTH-like agonist properties in the dog (*10*), the rat (*11*), and in a canine isolated perfused bone system (*12*). Taken together, these studies revealed this 3–34 analog to be an agonist for several parameters of PTH action, including stimulation of hypercalcemia, urinary phosphate and cyclic AMP excretion, and production of 1α,25-dihydroxyvitamin D_3 (*3*). The biological response to this analog in these several systems was less than 1 percent of the response to PTH (on a molar basis).

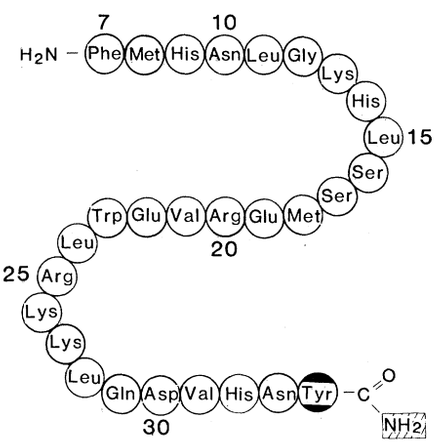

Fig. 1. The amino acid sequence of the analog [Tyr^{34}]bPTH-(7–34)NH_2. Tyrosine at position 34 replaces the phenylalanine of the native sequence and the carboxyl terminus is modified from a carboxylic acid to a carboxamide.

Our approach to designing an inhibitor for PTH in vivo was to modify the NH_2-terminus, either through substitution (*13*) or further NH_2-terminal truncation, in an attempt to eliminate the last traces of agonist activity from the 3–34 sequence without losing completely the avidity for PTH receptors. Substitutions at positions 3 and 4 failed to remove weak agonist activity (*14*).

A series of fragments of PTH containing NH_2-terminal deletions were therefore synthesized and evaluated in a renal radioactive-ligand–receptor binding assay for PTH (*15*). Stepwise deletions at the NH_2-terminus resulted in a progressive decline in receptor avidity, and identified the region 25–34, most distal from the NH_2-terminus, as the principal binding domain of the hormone (*6*). The intermediate sequence 7–34 still bound strongly to PTH receptors and inhibited completely the specific binding of a radioactive-ligand analog of PTH that was biologically active in vitro, although binding avidity was diminished from one-tenth to one-hundredth of that of the 3–34 sequence (*15*). Substitution of a tyrosine amide enhanced the activity of both agonists and antagonists in vitro (*3*). However, other studies revealed that substitution of norleucine for methionine diminished the activity in vitro and was very poorly tolerated in vivo in terms of agonist activity: a 15-fold decline in potency was observed in agonist analogs containing the 1–34 sequence and incorporating this substitution (*16*).

It thus appeared necessary to adopt the following strategy in designing an inhibitor that would be effective in vivo: (i) The amino terminus would have to be truncated beyond position 3 to remove PTH-like agonist properties. (ii) Because this structural manipulation alone would not be sufficient to remove the agonist properties, since the receptor-binding avidity of analogs truncated at the amino terminus is too weak, activity-enhancing modifications would have to be incorporated. (iii) Consideration would have to be given to the fact that alterations of the

carboxyl terminus, such as substitution of tyrosine carboxyamide at position 34, enhance analog–receptor binding. (iv) Although the substitution of norleucine for methionine (at positions 8 and 18) is well tolerated in vitro, methionine would be required for adequate receptor-binding avidity in vivo.

By following this strategy we developed the analog [Tyr^{34}]bPTH-(7–34)NH_2 (Fig. 1) as the best candidate for an antagonist devoid of intrinsic agonist properties in vivo. The peptide was synthesized by the solid-phase method of Merrifield (*17, 18*) with modifications previously described (*19*). The compound was purified by gel-filtration chromatography followed by semipreparative high-pressure liquid chromatography (*5, 19, 20*).

The properties of this analog in vivo were evaluated in an assay system (*21*) modified to assess the peptide's effects on the rates of excretion of urinary phosphate and adenosine 3′,5′-monophosphate (cyclic AMP) that normally increase rapidly in response to PTH. Male weanling Holtzman rats were maintained on a vitamin D–deficient diet (to exclude the effects of PTH-stimulated synthesis of 1α,25-dihydroxyvitamin D_3) for 6 to 7 weeks before being used in the assay. Thyroparathyroidectomy was performed and the animals were sustained for 24 hours by intravenous infusion (3 ml/hour) of a solution of 7.5 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, 20 m*M* NaCl, 2.5 m*M* KCl, and 4 percent glucose. Highly purified native bPTH-(1–84) or the antagonist or a combination of both peptides was infused intravenously. When both peptides were used, they were infused through separate femoral-vein cannulas. Urine volume and phosphate (*22*) and cyclic AMP concentrations (*23*) were determined every 30 minutes for 2.5 to 3.5 hours. When the antagonist was administered, infusion began 1 hour before the beginning of the PTH infusion.

The inhibitor was infused at molar dose rates that were 50, 100, and 200 times greater than a dose of native PTH (0.27 nmole/hour) that stimulated phosphaturia and cyclic AMP excretion. Four animals were tested at each dose rate. The preliminary data indicated that molar ratios of 100 or 200 to 1 for the analog and PTH, respectively, would be required to demonstrate efficacy of the antagonist.

At the highest dose rate used, the analog displayed no PTH-like agonist properties for phosphaturia in vivo. When administered in a 200-fold molar excess over native PTH, the analog inhibited greater than 70 percent of the maximum PTH-stimulated phosphaturic response (Fig. 2A). When phosphate excretion was normalized for urinary creatinine excretion (Fig. 2C), phosphaturia was completely inhibited (differences between animals receiving inhibitor plus PTH as opposed to PTH alone were not statistically significant, hence the inhibition observed is indistinguishable from 100 percent antagonism of PTH action). Comparable antagonist properties and lack of agonist activity were observed for the PTH-stimulated cyclic AMP response (Fig. 2, B and D). Urine volumes at each of the test intervals did not differ significantly between the group receiving PTH and the group receiving the combination of PTH and inhibitor. The experiment was continued for an additional hour in seven animals receiving both antagonist and PTH, and in four animals receiving PTH alone (not shown). Comparable inhibition of PTH-stimulated phosphaturia and cyclic AMP excretion by the analog was maintained during this period, suggesting that the analog can antagonize PTH action for at least 2.5 hours when coadministered with PTH. Assessment of inhibition of PTH-mediated effects on urine calcium excretion and serum calcium levels was precluded in this assay system because the model requires a constant sustaining infusion of calcium.

It may be possible to synthesize more potent antagonists by further extension of the NH_2-terminus, provided agonist properties are not restored to the molecule. Analogs of PTH with a sequence length of 6–34 or 5–34 may enhance inhibitory potency. In a few peptide hormonal systems it has been possible to generate antagonists effective in vivo (*24–26*). The PTH antagonist described here is presumed, on the basis of previous studies (*3, 6, 15*), to exert its biological effects through competing with PTH for occupancy of hormone-specific receptor sites. When infused simultaneously with PTH, its inhibitory effects were sustained for at least 1.5 hours.

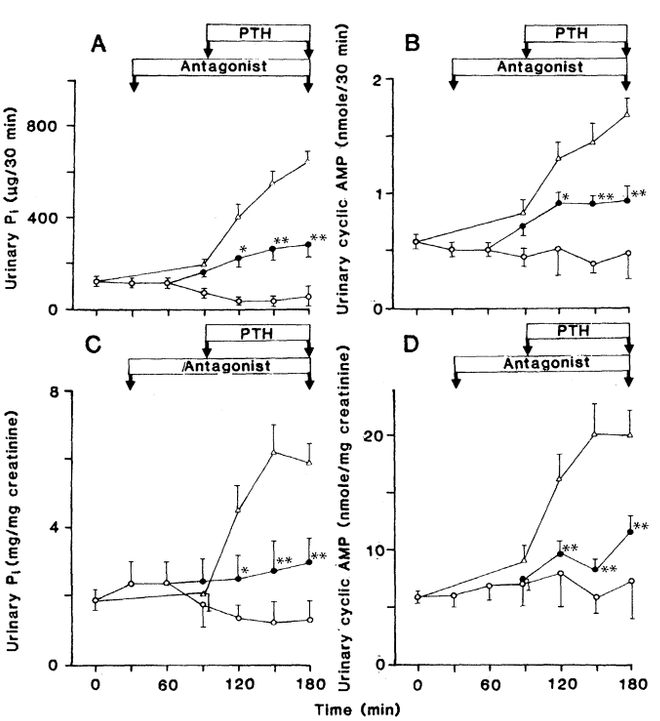

Fig. 2. Evaluation of the effects of [Tyr^{34}]bPTH-(7–34)NH_2 on (A) urinary phosphate and (B) urinary cyclic AMP excretion in rats. The data shown are compiled from a total of 40 animals. Fifteen control rats (△) received native bPTH-(1–84) alone intravenously at a rate of 0.27 nmole/hour. Twenty-one rats (●) received [Tyr^{34}]bPTH-(7–34)NH_2 and the native peptide through separate intravenous cannulas at rates of 54 nmole and 0.27 nmole per hour, respectively (a molar ratio of 200 to 1). Zeros on the abscissa represent values prior to peptide infusion. Infusion of the analog was begun 1 hour before the beginning of the infusion of native PTH. Four animals (○) received antagonist alone. (C) Urinary phosphate and (D) cyclic AMP excretion, corrected for creatinine excretion, are depicted for nine animals receiving native PTH alone (△), 11 animals receiving both the analog and native PTH (●) at a molar dose ratio of 200 to 1, and four animals (○) receiving the analog alone. The quantities of urine did not always permit determination of creatinine after quantitation of phosphate and cyclic AMP, accounting for the smaller sample size in (C) and (D). Determinations in animals receiving antagonist and PTH differ significantly from animals receiving PTH alone; $*P < .025$ and $**P < .01$ by Student's *t*-test.

These studies establish the feasibility of designing inhibitors of PTH in vivo, and may facilitate further studies of the mechanism of PTH action. Such analogs may ultimately prove useful as diagnostic and therapeutic agents in clinical disorders of the parathyroid glands.

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DNA Methylation Decreases in Aging but Not in Immortal Cells

Abstract. *When normal diploid fibroblasts from mice, hamsters, and humans were grown in culture, the 5-methylcytosine content of their DNA's markedly decreased. The greatest rate of loss of 5-methylcytosine residues was observed in mouse cells, which survived the least number of divisions. Immortal mouse cell lines had more stable rates of methylation.*

Normal diploid cells do not replicate indefinitely in vitro unless they are altered by some heritable transformation (*1*) to produce immortal cell lines that may or may not be tumorigenic in nude mice (*2*). The mechanism underlying the spontaneous conversion of cells from senescence to immortality is unknown, but alterations in gene expression are probably involved in the process.

The methylation of DNA influences vertebrate gene expression and methylation patterns are tissue-specific (*3*). The patterns are inherited with a high but not absolute fidelity (*4, 5*), and alterations in them could cause changes in gene expression that might be involved in aging in culture. We have therefore determined the levels of cytosine methylation in senescing cells from mice, hamsters, and humans and compared them to cell lines derived from the same species.

The growth kinetics of normal populations of diploid cells from these three species were followed (Fig. 1). The number of population doublings (computed from the increases in cell number without correction for plating efficiency) that the cells could undergo before senescence was lowest for mouse embryo (C3HME) cells that showed a maximum of four to eight population doublings. Spontaneous regrowth of the culture occurred after 12 passages, which is characteristic of the well-known abilities of mouse cells to form immortal lines (*2*). Syrian hamster embryo (SHE) cells were maintained for 25 doublings before decreased plating efficiency and cell death caused a decrease in the computed growth curve. Human fibroblasts can replicate for 100 population doublings (*1*). Human fetal lung fibroblasts (IMR 90) were followed for more than 40 doublings without change in growth rate (Fig. 1).

The levels of DNA methylation in these cultures were measured as a function of population doubling (Fig. 2A). The percentage of cytosine residues modified to 5-methylcytosine in freshly explanted mouse fibroblasts was 3.8 percent; the ratio decreased dramatically with increased time in culture. This decrease was in marked contrast to the situation with two immortal mouse cell lines in which the level of methylation was stable, or increased slightly, over many passages representing a large number of cell divisions (Fig. 2B).

Hamster fibroblasts, which underwent approximately 25 population doublings (Fig. 1), showed a lower rate of loss of 5-methylcytosine as a function of time in culture and human fibroblasts showed the lowest rate of decrease (Fig. 2A). Thus, the cell populations that rapidly senesced in culture showed the greatest rate of loss of methyl groups from their DNA's.

The 5-methylcytosine content of other cell types available in the laboratory was also determined to assess the generality of these findings (Table 1). The rapidly

Table 1. Methylcytosine content of cells.

Cell line	Description	Passage number	5-Methylcytosine* (%)	Number of samples
		Mouse cells		
C3H C1V	Immortal	5	2.95 ± 0.19	3
		7	3.82 ± 0.31	3
		11	3.66	2
		23	3.64	2
		Hamster cells		
A(T_1) C13	Oncogenic and immortal	Unknown	2.25 ± 0.24	5
		Human cells		
T-1	Normal diploid	8	3.00	2
		22	2.88	2
		36	2.37	2
HT1080	Oncogenic and immortal	44	2.32	2

*The percentage of 5-methylcytosine (5mC) was calculated as follows: [(5mC)/(5mC + cytosine] × 100. Values represent the average of at least two samples, and standard deviations are provided where appropriate.