Identification of an orally active small-molecule PTHR1 agonist for the treatment of hypoparathyroidism

Tatsuya Tamura1, Hiroshi Noda1, Eri Joyashiki1, Maiko Hoshino1, Tomoyuki Watanabe1, Masahiko Kinosaki1, Yoshikazu Nishimura1, Tohru Esaki1, Kotaro Ogawa1, Taiji Miyake1, Shinichi Arai1, Masaru Shimizu1, Hidetomo Kitamura1, Haruhiko Sato1 & Yoshiki Kawabe1

Parathyroid hormone (PTH) is essential for calcium homeostasis and its action is mediated by the PTH type 1 receptor (PTHR1), a class B G-protein-coupled receptor. Hypoparathyroidism and osteoporosis can be treated with PTH injections; however, no orally effective PTH analogue is available. Here we show that PCO371 is a novel, orally active small molecule that acts as a full agonist of PTHR1. PCO371 does not affect the PTH type 2 receptor (PTHR2), and analysis using PTHR1–PTHR2 chimeric receptors indicated that Proline 415 of PTHR1 is critical for PCO371-mediated PTHR1 activation. Oral administration of PCO371 to osteopenic rats provokes a significant increase in bone turnover with limited increase in bone mass. In hypocalcemic rats, PCO371 restores serum calcium levels without increasing urinary calcium, and with stronger and longer-lasting effects than PTH injections. These results strongly suggest that PCO371 can provide a new treatment option for PTH-related disorders, including hypoparathyroidism.
Parathyroid hormone (PTH) is an 84 amino acid peptide that is secreted from the parathyroid glands in response to low blood calcium (Ca) levels. PTH is the principal regulator of blood Ca concentration, acting primarily on bones and kidneys. Biological actions of PTH are mediated via stimulation of the PTH type 1 receptor (PTHR1), a member of the class B family of G-protein-coupled receptors (GPCRs). PTH/1 couples strongly to the adenylate cyclase-cyclic AMP (cAMP)-protein kinase A signalling pathway, and generally less robustly to the phospholipase C–protein kinase C–intracellular Ca\(^{2+}\) signalling pathway. Activation of PTH/1 can also promote recruitment of \(\beta\)-arrestins, leading to internalization of PTHR1 as well as to stimulation of intracellular signalling pathways. Although PTH injections are clinically available, orally available agonists of PTHR1 would offer several advantages over existing therapies. Whereas small molecules that activate class A GPCRs are used in clinical practice, a small-molecule agonist for class B GPCRs, which include PTHR1, is not yet available.

A lack of functional PTH results in hypoparathyroidism, which is a rare disease characterized by hypocalcemia. The clinical manifestations of hypoparathyroidism include muscle cramping and potentially life-threatening complications, such as seizure or laryngeal spasm. Conventional therapy for hypoparathyroidism involves pharmacological doses of oral Ca and active vitamin D. However, although this treatment regimen can increase intestinal absorption of Ca, it cannot restore renal Ca reabsorption, and can thereby lead to hypercalciuria. Chronic hypercalciuria can cause irreversible renal damage and eventual renal failure.

To avoid such risk, studies have focused on PTH replacement therapy using the full-length, native PTH molecule called human PTH(1–84) (hPTH(1–84)) and a synthetic fragment called human PTH(1–34) (hPTH(1–34)). PTH replacement therapy has been demonstrated to ameliorate the blood, renal, skeletal and neuropsychological features of hypoparathyroidism to a greater extent than conventional treatment. hPTH(1–84) and hPTH(1–34) can elevate serum Ca without raising urinary Ca in patients with hypoparathyroidism; however, due to the short half-lives of these compounds, frequent injections or continuous pump delivery is necessary to maintain serum Ca levels at a steady level. The peptidic nature (or poor oral bioavailability) of PTH(1–34) has limited its use in the treatment of osteoporosis, since daily subcutaneous injection of hPTH(1–34) is also used clinically for treating severe osteoporosis. Given the chronic nature of hypoparathyroidism and osteoporosis, orally active PTH mimetics are desirable and will benefit patients in their long-term treatment. However, no orally active compounds for PTH/1 have been identified so far.

In this study, we identified a nonpeptidyl small-molecule PTH/1 agonist, PCO371, through a cell-based functional screening assay. The compound activates human PTH/1 (hPTH/1) but not the human PTH type 2 receptor (hPTH/2). Analysis using hPTH/1–hPTH/2 chimeric receptors demonstrated that Proline 415 in transmembrane segment 6 (TM6) of hPTH/1 is a key residue for the receptor selectivity and activation. Orally administered PCO371 exhibits PTH-like biological activity in osteopnenic ovariectomized (OVX) rats and in hypocalcemic thyroparathyroidectomized (TPTX) rats. In the former model, intravenously administered PCO371 increases bone mineral density (BMD) and bone strength as effectively as PTH; however, when administered orally, only a limited increase in BMD and bone strength is observed. In the latter model, 4-week oral administration shows ability to restore the normal serum Ca level without increasing urinary Ca. To our knowledge, PCO371 is the first example of a small-molecule PTH/1 agonist with good oral bioavailability and efficacy in animal models. The safety and pharmacokinetics of PCO371 are currently being evaluated in humans.

Results

In vitro characterization of PCO371. PCO371 (Fig. 1a) was identified through a cell-based functional screening assay with transfected cells expressing hPTH/1. In COS-7 cells expressing hPTH/1, PCO371 stimulated cAMP production in a dose-dependent manner (EC\(_{50}\) = 2.4 \(\mu\)mol\(\cdot\)l\(^{-1}\), Fig. 1b). Similar results were obtained in COS-7 cells expressing rat PTH/1 (Supplementary Fig. 1a). PCO371 also enhanced the phospholipase C activity (EC\(_{50}\) = 17 \(\mu\)mol\(\cdot\)l\(^{-1}\)) as did hPTH(1–34) (Fig. 1c). PCO371 stimulated cAMP production in COS-7 cells transfected with hPTH/1-delNT, which lacks the N-terminal extracellular domain of hPTH/1 (ref. 19), with an EC\(_{50}\) value (EC\(_{50}\) = 2.5 \(\mu\)mol\(\cdot\)l\(^{-1}\)) similar to that of cells expressing full-length hPTH/1. On the other hand, the ability of hPTH(1–34) to induce cAMP production was significantly lower in cells transfected with hPTH/1-delNT than in those with the full-length hPTH/1 (Fig. 1b,d). We therefore examined whether PCO371 binds to the transmembrane domain of PTHR1 using cell membranes prepared from COS-7 cells expressing hPTH/1 and using \(^{125}\text{I}-[\text{Aib}^1,3,\text{Nle}^8,\text{Gln}^{10},\text{Har}^{11},\text{Ala}^{12},\text{Trp}^{14},\text{Ty}^{15}]-\text{PTH}(1–15)\) (PCO371), which interacts mainly with the receptor’s transmembrane domain, as a radiolabelled tracer. PCO371 at a concentration of 100 \(\mu\)mol\(\cdot\)l\(^{-1}\) inhibited the binding of \(^{125}\text{I}-[\text{Aib}^{1,3,\text{M}}]–\text{PTH}(1–15)\) almost completely (Fig. 1e, suggesting that PCO371 exerts its agonist activity by interacting with PTHR1’s transmembrane domain.

To assess the selectivity of PCO371 for PTHR1, we first examined the reactivity of PCO371 with PTHR2, which also belongs to the class B GPCRs and is most abundantly expressed in the central nervous system. hPTH/2 shares ~50% amino acid sequence identity with hPTH/1, and ~82% amino acid sequence identity with rat PTHR2 (ref. 21). PCO371 did not stimulate cAMP production in transfected COS-7 cells expressing human or rat PTHR2, while tuberoinfundibular peptide of 39 residues (TIP39), the cognate ligand for PTHR2, showed an increase in cAMP production (Fig. 1f and Supplementary Fig. 1b). The specificity of PCO371 to hPTH/1 was further examined in functional GPCR cell-based assays to determine agonistic and antagonistic activities against 12 class B GPCRs. PCO371 did not exhibit activity at 1 or 10 \(\mu\)mol\(\cdot\)l\(^{-1}\), whereas the cognate ligands and relevant reference compounds showed significant activity in these assays (Supplementary Table 1).

We also examined whether PCO371 induces cAMP production via endogenously expressed PTHR1 by using UMR-106 cells (a rat osteosarcoma cell line) that express endogenous rat PTHR1. Although the potency was much weaker than that of hPTH(1–34), PCO371 induced cAMP production as did hPTH(1–34), suggesting that PCO371 acts as a full agonist of PTHR1 (Fig. 2a).

PTHR1 has the capacity to form two distinct high-affinity conformational states: one is G-protein-uncoupled conformation (R\(_0\)), and the other is G-protein-coupled conformation (RG)\(_2\). Whereas a PTH-related peptide binds weakly to R\(_0\) and exhibits only a transient response, PTH-related peptide analogues such as M-PTH(1–28), M-PTH(1–34) and LA-PTH, bind with greater affinity to R\(_0\) and produce prolonged calcemic responses in vivo. The peptides that bind R\(_0\) with high affinity are able to produce CAMP signalling responses in PTHR1-expressing cells for a certain amount of time after initial binding to PTHR1. A previous study also suggested that there is a good correlation between R\(_0\) binding affinity and the duration of the CAMP response induced by a given PTH ligand after initial binding to PTHR1.
We therefore examined the sustained cAMP signalling responses by PCO371 or LA-PTH in comparison with hPTH(1–34) in a cAMP washout assay in UMR-106 cells. The duration of cAMP-signalling response induced by PCO371 was much shorter than that of hPTH(1–34), whereas LA-PTH, a long-acting PTH analogue, showed more prolonged cAMP signalling than hPTH(1–34) (Fig. 2b).

In UMR-106 cells, PCO371 also dose-dependently increased expression of bone-related messenger RNAs (mRNAs) for Fos (encoded by Fos), osteocalcin (encoded by Bglap) and receptor activator of nuclear factor-kB ligand (RANKL, encoded by Tnfsf11), and dose-dependently decreased expression of mRNAs for osteoprotegerin (encoded by Tnfrsf11b) and sclerostin (encoded by Sost), as did hPTH(1–34) (Fig. 2c–g). In these assays, 1 μmol l⁻¹ of PCO371 was able to cause changes in mRNA expressions in UMR cells even when PCO371 did not induce cAMP production in the cells (Fig. 2a). This anomaly may be due to the difference in incubation time (6 h for gene expressions versus 20 min for cAMP production) or due to a level of cAMP elevation that despite being undetectable has the ability to transmit signals in cells²⁵–²⁷.

We next examined whether PCO371 induces PTH-like bone-resorbing activity in organ cultures with fetal rat long bones. PCO371 at 1 μmol l⁻¹ or more had the ability to stimulate forty5Ca release from prelabeled bones as did hPTH(1–34) (Fig. 3).

Mechanism of selectivity for PTHR1 over PTHR2. To investigate the mechanisms underlying the selectivity of PCO371 for hPTHR1 over hPTHR2, we performed cAMP production assays using transfected COS-7 cells expressing hPTHR1 mutants in which amino acid residue(s) were replaced by the corresponding hPTHR2 amino acid residue(s) (Fig. 4a,b). hPTH(1–34) increased cAMP in cells expressing hPTHR1–hPTHR2 chimeric receptors (C1, C2 or C3), whereas PCO371 did not do so in cells expressing C1 or C2 (Fig. 4c). Since the common replaced segment in C1 and C2 mutants is TM regions 6 and 7, the critical role of these
regions is indicated. Furthermore, because cells expressing C3 responded well to PCO371, we examined the effect of replacing the amino acid residues in the TM6 and TM7 intracellular region with those of hPTHR2 (mutant receptors M24, M25 and M26; Fig. 4b). A lack of cAMP increase by PCO371 was shown only in mutant receptor M25, which contains three mutated amino acids (Methionine 414, Proline 415 and Lysine 416; Fig. 4c). In subsequent experiments with single amino acid replacement, Methionine 414 to Valine (M414V) and Leucine 416 to Valine (L416V) had only modest effects on cAMP response, whereas Proline 415 to Leucine (P415L) resulted in a dramatic reduction of cAMP production by PCO371 (Fig. 4c,d). These results indicate that Proline 415 is essential for the selective activation of PTHR1 by PCO371.

Figure 2 | Effects of PCO371 on PTHR1 signalling in UMR-106 cells. (a) cAMP production by hPTH(1–34) and PCO371 in UMR-106 cells, which natively express rat PTHR1. (b) Duration of cAMP-signalling responses induced by PCO371, hPTH(1–34) and LA-PTH in UMR-106 cells. The time courses of cAMP responses produced by PCO371 (0.1 mmol l$^{-1}$), hPTH(1–34) (1 mmol l$^{-1}$) or LA-PTH (0.1 mmol l$^{-1}$) in UMR-106 cells were examined by cAMP washout assay. The cAMP response is expressed as a percentage of the maximal cAMP produced in the cells treated with each ligand (determined by incubating cells concomitantly with the ligand for 10 min, and further incubating with IBMX for 5 min without a washing-out phase). The range of maximum cAMP values was PCO371: 17.4 ± 0.2 pmol per well, hPTH(1–34): 21.3 ± 0.9, LA-PTH: 42.2 ± 0.9, and basal cAMP: 1.8 ± 0.2. (c–g) Effects of PCO371 and hPTH(1–34) on the expression of mRNAs for c-fos (Fos) (c), osteocalcin (Bglap) (d), RANKL (Tnfsf11) (e), osteoprotegerin (Tnfrsf11b) (f) and sclerostin (Sost) (g). mRNA levels are shown as fold-change over control. Gene expression was analysed at 1 h (c) or 6 h (d–g) after treatment of UMR-106 cells with hPTH(1–34) or PCO371. All genes were normalized to 18 S ribosomal RNA. Data are represented as the mean ± s.d. of one experiment ($n=3$ for a–g).
Pharmacokinetics profile in normal rats. Pharmacokinetics profiles of PCO371 and hPTH(1–34) after single administration to normal rats are summarized in Table 1. Exposures following oral dosing of PCO371 dose-dependently increased within the dose range tested. The maximum plasma concentrations ($C_{\text{max}}$) of PCO371 were attained at 1–1.5 h, and the terminal half-lives ($T_{1/2}$) were 1.5–1.7 h. Oral bioavailability of PCO371 was 34% at a dose of 2 mg kg$^{-1}$. After a single subcutaneous injection of hPTH(1–34), the concentration in serum was elevated within 15 min in a dose-dependent manner, and the $T_{1/2}$ was 15 min.

**Figure 3 | Effects of PCO371 on 45Ca release from pre-labelled fetal rat long bones.** Control medium contained only vehicle for PCO371 (0.1% DMSO) or hPTH(1–34) (10 µmol l$^{-1}$ acetic acid). Data are represented as the mean ± s.d. of one experiment ($n = 4$). Williams’s test was used to compare PCO371- or hPTH(1–34)-treated groups with vehicle-treated group; **$P < 0.01$, ***$P < 0.001$.

**Figure 4 | Mutation studies using chimeric receptors of hPTHR1 and hPTHR2.** (a) Schematic structures of hPTHR1, hPTHR2 and chimeric receptors in which blue indicates amino acids comprising hPTHR1 and pink indicates amino acids comprising hPTHR2. C1 is a chimeric hPTHR1 in which the C-terminal region (from 398 to 593) is replaced by the corresponding region of hPTHR2 (from 353 to 550). C2 is a chimeric hPTHR1 in which two regions (from 325 to 390 and from 407 to 461) are replaced by the corresponding regions of hPTHR2 (from 280 to 345 and from 362 to 415). Similarly, in C3 two regions (from 334 to 378 and from 425 to 446) are replaced by the corresponding regions of hPTHR2 (from 289 to 333 and from 425 to 446). (b) Snake diagram of TM regions 6 and 7 of hPTHR1 based on the topological arrangement of class B GPCRs (ref. 4). The amino acid residues of hPTHR1 that were replaced with the corresponding residues of hPTHR2 in C3 (filled black circles) and in mutant receptors M24 (L407), M25 (M414, P415 and L416), M26 (A456 and F461), M414V, P415L and L416V (filled blue circles) are indicated. (c) cAMP production stimulated by PCO371 (0.1 µmol l$^{-1}$) in COS-7 cells expressing hPTHRs or mutant hPTHRs, indicated as a percentage of cAMP production stimulated by 0.1 µmol l$^{-1}$ hPTH(1–34). Data are represented as the mean ± s.d. of one experiment ($n = 3$). (d) Stimulation of cAMP production by hPTH(1–34) and PCO371 in cells expressing hPTHR1, hPTHR2 and mutant hPTHRs. Data are represented as the mean ± s.d. of one experiment ($n = 3$).

In vivo experiments in OVX rats. To evaluate the effect of PCO371 on bone metabolism, we examined its effects on BMD, bone strength and serum biochemistry in mature OVX rats. Treatment began 3 months after ovariectomy when the BMD of lumbar vertebrae in OVX rats was significantly reduced compared with sham-operated rats. The anabolic and catabolic actions of PTH depend on the dosage regimen, and intermittent, not continuous, administration of PTH increases bone mass$^{28}$. We therefore examined the effect of PCO371 on OVX rats after oral or intravenous administration, which have different pharmacokinetics profiles (Supplementary Fig. 2 and Supplementary Table 2). Twelve-week treatments of once-daily intravenous PCO371 (10 mg kg$^{-1}$) or subcutaneous hPTH(1–34) injections caused significant increase in BMD and bone strength in the lumbar vertebrae of OVX rats compared with OVX rats dosed with vehicle (Fig. 5a,c). Whereas daily oral PCO371 (30 mg kg$^{-1}$) and intravenous PCO371 (3 mg kg$^{-1}$) did not affect BMD or bone strength in the lumbar spine (Fig. 5a,c), daily oral PCO371 (30 mg kg$^{-1}$) partially increased BMD in the proximal femur (Fig. 5b), but not in total femur (Supplementary Fig. 3). Both oral and intravenous (10 mg kg$^{-1}$) PCO371 induced a significant increase in serum osteocalcin and urinary collagen type 1 cross-linked C-telopeptide (CTX)/creatinine (Fig. 5d,e), which suggests accelerated bone turnover, and this was supported histologically by an increase in bone formation (Fig. 5f and Supplementary Table 3) and bone resorption parameters in the lumbar vertebrae (Supplementary Table 3). Serum Ca was within the normal range in all rats treated with PCO371 or hPTH(1–34) (Fig. 5g).

NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13384

NATURE COMMUNICATIONS | ARTICLE

NATURE COMMUNICATIONS | 7:13384 | DOI: 10.1038/ncomms13384 | www.nature.com/naturecommunications
Table 1 | Pharmacokinetics parameters of hPTH(1–34) and PCO371 in normal rats.

| Route | Dose | T_{1/2} (h) | T_{max} (h) | C_{max} (ng ml^{-1}) | AUC_{inf} (ng ml h^{-1}) | BA % |
|-------|------|-------------|-------------|----------------------|------------------------|------|
| s.c.  | 3    | 0.3 ± 0.1   | 0.1 ± 0.1   | 2.42 ± 0.53          | 0.99 ± 0.17            | 22   |
| p.o.  | 2    | 1.5 ± 0.1   | 1.5 ± 0.9   | 92.5 ± 37.1          | 374 ± 187              | 34   |
|       | 6    | 1.4 ± 0.2   | 1.0 ± 0.0   | 727 ± 103            | 2,360 ± 280            | NE   |
|       | 18   | 1.7 ± 0.3   | 1.3 ± 0.6   | 4,560 ± 710          | 18,600 ± 1,900         | NE   |

In *vivo* experiments in TPTX rats. We next examined the effect of PCO371 on serum Ca and inorganic phosphate (P_i) in TPTX rats. After a single oral administration, PCO371 dose-dependently increased serum Ca and decreased P_i, with greater efficacy and longer-lasting effects than those of hPTH(1–84) (Fig. 6a,b) or hPTH(1–34) (Supplementary Fig. 4a,b). Pharmacokinetics profiles of PCO371 and hPTH(1–84) were determined in TPTX rats. PCO371 had longer serum T_{1/2} and T_{max} than hPTH(1–84) (Supplementary Table 4). We then examined the efficacy of PCO371 in 4-week multiple oral dosing studies in TPTX rats. In this model, subcutaneous treatment with hPTH(1–84) or hPTH(1–34) transiently increased serum Ca to within the target therapeutic range (7.6–11.2 mg dl^{-1})^{10,11}, but the serum Ca level dropped quickly to the basal level following each injection (Supplementary Fig. 4c,d). No increase in urinary Ca excretion was observed after 4-week treatment of hPTH(1–84) (Supplementary Fig. 4e). Twice-daily oral PCO371 at 3 mg kg^{-1} or more dose-dependently increased serum Ca to within the target therapeutic range, and maintained the Ca level within this range for at least 6 h after each administration from the first administration until the end of the study (Fig. 6c). In this experiment, no hypercalcemia (>11.2 mg dl^{-1}) was observed at 0, 6 and 10 h after administrations of PCO371 on days 1, 7, 14, 22 and 29. Urinary Ca excretion after 4-week treatment was not increased by treatment with PCO371 up to 9 mg kg^{-1}, even when the serum Ca had reached the target therapeutic range (Fig. 6d), possibly because renal Ca reabsorption was stimulated. Serum 1x,25-dihydroxyvitamin D_3 [1,25(OH)2D_3] levels increased significantly in rats treated with 9 mg kg^{-1} PCO371 (Fig. 6e), similarly to animals treated with hPTH(1–34) (Supplementary Fig. 4f). There was no significant difference in body weight or general condition between vehicle-treated rats and PCO371-treated rats (Supplementary Fig. 4g).

An important benefit of PTH replacement therapy when treating hypoparathyroidism is that it can reduce the doses of Ca and vitamin D_3 while maintaining serum Ca levels, and this has been clinically demonstrated by combining hPTH(1–84) with conventional vitamin D and Ca therapy^{12}. We therefore examined the effect of PCO371 in combination with alfalcidol (1x-hydroxycholecalciferol) in TPTX rats. Alfalcidol alone at 75 ng kg^{-1} normalized the serum Ca to within or above the target therapeutic range, but was accompanied by considerable urinary Ca excretion (Fig. 6f.g). Once-daily oral add-on treatment with 6 mg kg^{-1} of PCO371 plus 38 ng kg^{-1} of alfalcidol increased serum Ca to levels comparable with that by 75 ng kg^{-1} of alfalcidol alone (Fig. 6f). The combination of PCO371 plus alfalcidol did not elevate urinary Ca excretion, indicating that PCO371 can enable the dose of alfalcidol to be lowered, thereby reducing urinary Ca excretion (Fig. 6g). These results indicate that PCO371 can be useful for the treatment of hypoparathyroidism both in monotherapy and as add-on therapy to conventional therapy.

**Discussion**

As a principal regulator of Ca homeostasis, PTH increases tubular reabsorption of Ca in the kidney and promotes renal excretion of phosphate. In blood, PTH stimulates activity of 25-hydroxyvitamin D_3 1x-hydroxylase, which increases production of 1,25(OH)_2D_3, which then stimulates intestinal absorption of Ca; whereas in bone, PTH stimulates bone turnover and mobilizes Ca into the circulation^{1,2}. Accordingly, insufficient levels of PTH can cause hypocalcemia. Patients with hypoparathyroidism require very high doses of vitamin D and Ca supplements to normalize serum Ca; however, this treatment risks hypercalcemia and may lead to nephrocalcinosis and subsequent loss of renal function. To reduce (or prevent) hypercalcemia, thiazide diuretics can be used to lower urinary Ca (ref. 14). Recently, PTH replacement therapy with recombinant hPTH(1–84) has been approved to treat hypoparathyroidism; however, as a peptide it requires daily subcutaneous injections^{12,29}. Once-daily dosing of hPTH(1–84) can increase Ca to peak levels at around 7 h after administration, and the effects are observed for up to 24 h. However, hypercalcemia was observed at peak times in 71% of hPTH(1–84)-treated patients, and this suggests that a lower dose or more frequent daily dosing regimens may be more beneficial for some patients^{30}. Studies with hPTH(1–34) have also demonstrated that pump delivery or twice-daily delivery of the peptide provides higher physiological Ca levels than once-daily injections^{15}.

The present study demonstrates that PCO371 exhibits PTH-like activity with regard to Ca homeostasis, such as renal Ca reabsorption, 1,25(OH)_2D_3 production, and Ca release from the bone (Figs 3 and 6a,c–e). PCO371 was also able to mimic the effects of PTH on bone metabolism (Fig. 5a–f and Supplementary Table 3) and phosphate homeostasis (Fig. 6b) in *vivo*, which demonstrates the compound’s potential as a PTH mimetic. Although the *in vitro* activity of PCO371 was 1,000- to 10,000-fold less potent than that of hPTH(1–34), oral PCO371 exhibited more robust and longer-lasting calcemic effects than did subcutaneous hPTH(1–34) or hPTH(1–84) *in vivo*. The duration of CAMP-signalling response induced by PCO371 in UMR-106 cells was not prolonged compared with PTH(1–34) or LA-PTH (Fig. 2b). These results suggest that the superior calcemic activity in response to PCO371 *in vivo* presumably stems from its good bioavailability and longer half-life than that of the hPTHs. Differences between *in vitro* activity and *in vivo* response, which are mainly due to the difference in pharmacokinetics, were found in earlier studies with PTH peptide analogues^{18}.

In the present study, we used TPTX rats as an animal model for hypoparathyroidism, and demonstrated that complementing alfalcidol therapy with PCO371 increased serum Ca to a level comparable with that produced by twice the dose of alfalcidol without elevating urinary Ca excretion. Our results suggest that replacing conventional therapy for hypoparathyroidism with...
PCO371 is expected to provide three advantages: robust serum Ca control, low risk of hypercalciuria and a decrease in pill burden. However, we should bear in mind that rat skeleton is different from human skeleton, thus non-rodent animal models for studying human bone metabolism are needed to bridge the gap between animal studies and clinical trials. We therefore examined the effects of PCO371 on normal dogs, since dogs have been used to study the human skeleton because of their extensive basic
multicellular unit-based remodelling. In a pharmacological study with normal dogs, serum Ca was significantly increased by single oral administrations of PCO371 (3.0 – 30 mg kg⁻¹; Supplementary Fig. 5). Since no serious side effects of PCO371 were observed in preclinical toxicology studies, a phase 1 clinical study of PCO371 is currently being performed.

In the treatment of osteoporosis, in contrast to hypoparathyroidism, a sharp, transient increase in PTH levels is warranted to obtain a desirable anabolic effect on bone. Once-daily injection of hPTH(1–34) giving a spiked pharmacokinetics profile is a widely applied osteoporosis therapy in clinic. In the present study, orally administered PCO371 significantly increased bone

| Administration | Day 1 | Day 7 | Day 14 | Day 22 | Day 29 |
|----------------|-------|-------|--------|--------|--------|
| Sham           | 11    | 10    | 9      | 8      | 7      |
| TPTX           | 6     | 10    | 3      | 6      | 0      |
| PCO371 1 mg kg⁻¹ | 6     | 10    | 3      | 6      | 0      |
| PCO371 3 mg kg⁻¹ | 6     | 10    | 3      | 6      | 0      |
| PCO371 9 mg kg⁻¹ | 6     | 10    | 3      | 6      | 0      |

Figure 6 | Effects of PCO371 on serum parameters in TPTX rats. (a, b) Calcemic (a) and hypophosphatemic (b) effects of oral PCO371 or subcutaneous hPTH(1–84) in single administration. (c–e) Changes in serum Ca level (c), urinary Ca excretion (d), and serum 1,25(OH)₂D₃ level (e) from twice-daily repeated oral dosing of PCO371 (arrows in c) for 4 weeks. (f, g) Changes in serum Ca level (f), urinary Ca excretion (g) in once-daily repeated oral dosing of PCO371 (arrows) as an add-on to oral alfacalcidol treatment for 4 weeks. The shaded area shows the target therapeutic range (7.6–11.2 mg dl⁻¹) of serum Ca level. Data are represented as the mean ± s.e.m. of one experiment (n = 6 in a, b, f, g, n = 5 in c–e). Student’s t test was used to compare the sham and TPTX vehicle-treated groups (#P < 0.05 in g) and the alfacalcidol 75 ng ml⁻¹ and TPTX control groups (**P < 0.05 in g). Parametric Dunnett’s test was used to compare each treated group with the TPTX control group; *P < 0.05, **P < 0.01 in e and g.
turnover, but induced limited increase in bone mass in OVX rats. In contrast, intravenous injection of PCO371 (10 mg kg⁻¹) showed a spiked pharmacokinetics profile and significantly increased both BMD and bone strength to levels comparable with those produced by hPTH(1–34) (0.9 nmol kg⁻¹), although markers of bone formation and resorption were more increased by PCO371 than they were by hPTH(1–34).

The results obtained in the present study, however, do not support the benefits of oral PCO371 as an anti-osteoporosis drug; further studies at different doses and intervals or studies in other animals, such as dog and monkey, are needed to better evaluate the potential of the compound for the treatment of osteoporosis.

Supra-therapeutic doses of hPTH(1–34) and hPTH(1–84) have been shown to increase the incidence of osteosarcoma in rat carcinogenicity studies. Because of this potential risk, hPTH(1–34) currently carries a black-box warning for the treatment of osteoporosis, although there appears to be no evidence of an increased risk of osteosarcoma in human subjects receiving the PTH peptide. The exact mechanism is not yet elucidated, but it is postulated that profound bone formation in rats caused by PTH peptides is somewhat causative for the development of osteosarcoma. The presence of a constitutively activating mutation (T410P) within the transmembrane domain of PTHR1, or more specifically, by interacting entirely or partially with TM6. We base this suggestion on the finding that Proline 415 within TM6 plays a critical role in the selective activation of PTHR1 by PCO371. The importance of TM6 in GPCR-mediated signalling has also been demonstrated by crystal structure analyses of corticotropin-releasing factor receptor 1 (ref. 38) and the β₂ adrenergic receptor-Gs protein complex. Studies involving the mechanism of action of PCO371 on PTHR1 will provide useful insights into the mechanism of activation of class B GPCRs.

In conclusion, the small-molecule PTHR1 agonist PCO371 is a potent compound with potential to be developed and used as an orally available drug to treat hypoparathyroidism. PCO371 is also useful to more fully understand the mechanism of activation of class B GPCRs.

**Methods**

**Synthesis of PCO371.** Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification (Fig. 7). Silica gel
chromatography purification was performed using prepacked silica gel cartridges (Biotage, Shoko Scientific). Reverse-phase column chromatography purification was performed using WakoSil 2515R (Wako Pure Chemical Industries). Nuclear magnetic resonance (NMR) spectra were determined with a Varian MR-400 spectrometer (400 MHz, Agilent).

Chemical shifts are shown in parts per million (p.p.m., δ units). The following NMR abbreviations are used: s = singlet, d = doublet, q = quartet, m = multiplet, dd = doublet of doublets, brs = broad singlet. High-resolution mass spectrometry (HRMS) was performed on a Xevo G2-S ToF instrument (Waters).

Synthesis of compound 2. A mixture of tert-butyl 4- amono-4- cyanopyrrolidin-1-carboxylic acid (1) (11.5 g, 59.0 mmol, 4-trifluoromethoxybenzoic acid (10 g, 48.5 mmol), and diisopropylethylamine (DIEPA) (8.78 g, 67.9 mmol) in anhydrous DMF (150 ml) was added 2-(3H-[1,2,3]triazol-4-yl)-pyridin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate(V) (HATU, 22.1 g, 58.2 mmol). To a stirred mixture at 0 °C for 1 h under N2, EtOAc (300 ml)–EtOH/C0 aq. (15.5 ml) at 0 °C-NMR abbreviations are used: s = singlet, d = doublet, q = quartet, m = multiplet, dd = doublet of doublets, brs = broad singlet. High-resolution mass spectrometry (HRMS) was performed on a Xevo G2-S ToF instrument (Waters).

Synthesis of compound 6. A mixture of 2-[4-(trifluoromethoxy)phenyl]-8-(vinyl)-1,3,8-triazaspiro[4.5]dec-1-ene-8-carboxylate ([4,5]-dec-1-ene-4-one (5) (3.07 g, 7.60 mmol), 1-(4-bromo-3,5-dimethylphenyl)-5,5-dimethylimidazolidine-2,4-dione (9) (2.60 g, 8.36 mmol), Pd(OAc)2 (1.33 g, 2.31 mmol) in anhydrous dioxane (68 ml) was added 2 mol l−1 HCl in dioxane (68.2 ml, 273 mmol) in CH2Cl2 (68 ml) was stirred at 0 °C for 1 h under N2. The mixture was stirred at room temperature overnight, filtered and concentrated in vacuo.

Synthesis of compound 2. To a stirred mixture of tert-butyl 4- amino-4-[trifluoromethoxy]benzamido]piperidine-1-carboxylate (2) (18.9 g, 94%) as a white solid. 1H-NMR (400 MHz, CDCl3) δ: 7.84–7.81 (2H, m), 7.29 (2H, d, J = 8.1 Hz), 6.25 (1H, s), 4.14–3.97 (2H, m), 3.34–3.24 (2H, m), 2.61–2.47 (2H, m), 1.90–1.81 (2H, m), 1.47 (9H, s; Supplementary Fig. 6). 13C-NMR (100 MHz, CDCl3) δ: 165.54, 154.31, 152.15 (q, JCF ¼ 257.5 Hz), 131.39, 129.16, 120.79, 120.27 (q, JCF ¼ 129.7 Hz), 118.27, 80.57, 50.97, 40.00, 34.82, 28.37 (Supplementary Fig. 7). HRMS (electrospray ionization-time-of-flight (ESI-TOF)) m/z calcd for C21H26F3N4O4 (M + H) +, 412.1494; found 412.1493.

Synthesis of compound 3. A mixture of tert-butyl 4-cyano-4-[trifluoromethoxy]benzamido]piperidine-1-carboxylic acid (2) (12.6 g, 30.5 mmol) in EtOH (150 ml) was added 2 mol l−1 NaOH aq. (30.4 ml) followed by 30% H2O2 (15 ml; twice). The organic layer was washed with H2O and sat. aq. NaCl and concentrated in vacuo. The residue was purified by column chromatography (silica gel, C2 for 1 h under N2). The reaction mixture was diluted with EtOAc. The organic layer was washed with H2O, dried over anhydrous MgSO4 and evaporated in vacuo. The crude residue was purified by column chromatography (silica gel, C176 for 2 h under N2). The mixture was stirred at room temperature for 3 h and poured into sat. aq. NaHCO3 and concentrated in vacuo, and dissolved in EtOAc. The organic layer was washed with H2O, dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (silica gel, C176 for 1 h under N2). The mixture was stirred at room temperature for 3 h and poured into sat. aq. NaHCO3 and concentrated in vacuo, and dissolved in EtOAc. The organic layer was washed with H2O, dried over anhydrous MgSO4 and evaporated in vacuo.
and the product was extracted with EtOAc. The organic layer was dried over anhydrous MgSO₄ and evaporated in vacuo. The crude solid was washed with EtOAc-hexane and CH₂Cl₂-hexane, consecutively, and dried to afford 1-(4-bromo-3,5-dimethylphenyl)-5,5-dimethylimidazolidine-2,4-dione (9) (3.80 g, 71% from compound 7). "H-NMR (400 MHz, CDCl₃): δ 8.64 (1H, s), 6.96 (2H, s), 2.44 (6H, s, C₆H₄), 2.33 (4H, s, 8H, 1NH₂), 1.65 (6H, s, 1NH₂), 1.45 (6H, s, 8H, 1NH₂), 1.23 (1H, m, 2H, 1NH₂). "C-NMR (100 MHz, CDCl₃): δ 176.50, 154.57, 140.74, 131.92, 128.47, 128.12, 65.11, 24.03, 23.68. (Supplementary Fig. 19) HRMS (ESI-TOF) m/z calcd for C₁₃H₁₄BrN₂O₂ (M-H) − 280.97, found 280.99 (M-H)."
assessed in a time-course experiment in UMR-106 cells (cAMP washout assay). The cells were treated with assay medium (2 mg ml−1 BSA, and 2 mmol l−1 HEPES/McCoy’s 5A medium, Life Technologies) alone (basal) or with ligands (1 mmol l−1 for hPTH(1–34), 0.1 mmol l−1 for LA-PTH and 0.1 mmol l−1 for PCO371) in the assay medium for 10 min, washed (t = 0), incubated for 0, 30, 60 and 120 min (washout phase), and then further incubated for 5 min in the assay medium containing IBMX (2 mmol l−1). Incubations were terminated with 50 mmol l−1 4% HCl. The cAMP concentrations were determined using a cAMP EIA kit (GE Healthcare).

**Selectivity assay.** Selectivity assays against class B GPCRs were performed by Cerep (France). Agonistic and antagonistic effects of PCO371 against a panel of secretin, GHRH, PAC1, VPAC1 and VPAC2 were examined at PCO371 concentrations of 1–10 mmol l−1. The following cell lines were used: T47D (calcinon), BT6 (GLP-1), HT-29 (VPA1, VPA2), CHO (CGRP, CFFI, GLP-2, Glucagon, Secretin, GHRH and PAC1, recombinant). Their antagonistic activity was measured in the presence of the control agonists shown in Supplementary Table 1. In agonist and antagonist experiments, intracellular cAMP levels were determined with the HTRF detection system. EC50 and IC50 values of control agonists or antagonists were calculated by non-linear regression analysis of the concentration-response curves generated with mean replicate values using Hill equation curve-fitting using the formula: equation (2).

\[
Y = \frac{D}{(A - D)/((1 + (C_{50}/C_{60}) \times nH)}
\]

(2) Y = response, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, C50 = EC50 or IC50 = nH = slope factor. Agonistic activity of PCO371 at 1 or 10 mmol l−1 was calculated as a per cent of the control agonist response; intracellular cAMP accumulation by PCO371 × 100/intracellular cAMP accumulation by the control agonist at the IC50 concentration

(3) A value >50% was defined as positive (agonist or antagonist activity), a value between 25 and 50% as weak to moderate, and a value <25% as considered as negative.

**Animal ethics.** In accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical, all animal studies were performed under the approval of the company’s Institutional Animal Care and Use Committee. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (http://www.aaacal.org). The studies were also carried out in compliance with the ‘Act on Welfare and Management of Animals’ in Japan.

**Fetal rat long bone cultures.** Bone-resorbing activity was assessed by fetal rat long bone cultures.44, 45CaCl2 (1,480 kBq, PerkinElmer) was subcutaneously injected into a pregnant Crl:CD(SD) rat (Charles River Laboratories) on its 18th day of gestation. At 48 h after the injection, the pregnant rat was euthanized, and the ulnae and radial bones were isolated from euthanized fetal rats. The long bones were placed on nylon mesh and covered with paraffin paper, and a floating culture in BGJb medium (Life Technologies) was carried out for 3 days at 37°C in 95% air and 5% CO2 atmosphere. On the 4th day, the medium was replaced with fresh BGJb medium containing hPTH(1–34) (1 mmol l−1 to 1 mmol l−1) or PCO371 (0.1 mmol l−1 to 3 mmol l−1) or vehicle (0.1% DMSO and 0.1 mmol l−1 acetic acid at final concentration) and cultured for a further 4 days at 37°C. Bone samples were then treated with 10% TCA (500 μl/bone) overnight at 37°C. Radioactivity of the bone and the medium samples was measured44. Four to five long bones were used for each individual dose. 45Ca release (%) was calculated by the following formula (equation (4)):

\[
\text{Ca release} (\%) = \left( \frac{\text{medium radioactivity (cpm)}}{\text{medium radioactivity (cpm) + bone radioactivity (cpm)}} \right) \times 100
\]

(4) Repeated administration study in OVX rats. OVX was carried out in 32-week-old female Crl:CD (SD) rats (Charles River Laboratories). Twelve weeks after surgery, lumbar vertebral BMD (L3–L5) was measured in vivo by dual-energy X-ray absorptiometry (DXA) using a DCN-600EX–T8 bone densitometer (Hachiri Aloka Medical), and the rats were divided into five groups, each with a similar mean value of BMD and body weight. Sample sizes were determined based on previous experiments with hPTHs and a published study with ibandronate and dexamethasone in our laboratories43 (n = 7–10). Then, OVX rats were treated once daily subcutaneously (vehicle or PCO371 at 30 mg kg−1) or subcutaneously (vehicle or hPTH(1–34) at 0.9 mmol kg−1) for 12 weeks. Ten to twelve OVX rats were assigned to each dose group. Nine rats were assigned to the sham-operated control group and were treated with vehicle for PCO371. Ten per cent DMSO (Wako Pure Chemical Industries)/10% Kodhiphor EL (Sigma-Aldrich) in 10% hydroxypropyl cyclodextrin (HPCD; Nihon Shokuhin Kako)/0.752% glycine (Wako Pure Chemical Industries) buffer was used as vehicle for PCO371, and phosphate–citrate buffer (pH 6.0) was used as vehicle for hPTH(1–34). To label bone-forming surfaces, tetracycline (25 mg ml−1) was subcutaneously injected on Day 78 and calcitonin (8 mg kg−1). Dajoindo Laboratories) was subcutaneously injected on day 83. Under isoflurane anaesthesia, jugular vein blood was collected immediately before and at 1, 2, 6, 10 and 24 h after administration on day 82 to measure plasma concentration of PCO371 or on day 84 to measure serum Ca levels (o-CPC method, Wako Pure Chemical Industries). Urine was collected for 24 h from metabolic cages on days 84–85 to measure urinary collagen type 1 cross-linked C-telopeptide (CTX) by using the Rat-Laps ELISA System (Immunodiagnostic Systems). Under isoflurane anaesthesia, blood was collected on day 85 from the abdominal aorta to measure serum osteocalcin level using the Rat Osteocalcin ELISA System (GE Healthcare). The lumbar spine and bilateral femurs were excised after 12 weeks of treatment. The first lumbar vertebra (L1), 3rd to 5th lumbar vertebrae (L3–L5), the right femur, and the right tibia were preserved in 70% ethanol to measure bone histomorphometry and BMD (DCS-600EX; Hitachi Aloka Medical). Measurement of bone histomorphometry was performed by the Ito Bone Histomorphometry Institute (Niigata, Japan). The second lumbar vertebra (L2) was wrapped in saline-soaked gauze and stored at −80°C until measurement. Ultimate compressive strength (N) of the L2 vertebra was measured by using a mechanical testing machine (TK-252C; Muromachi Kikai).

**Single administration study in TPTX rats.** TPTX was carried out in 6-week-old female Crl:CD(SD) rats (Charles River Laboratories). Rats with serum Ca levels of <8.0 mg dl−1 at 5 days after surgery were used for the experiment. The rats were divided into six groups, each with a similar mean value of serum Ca levels and body weight. TPTX rats were treated once orally (vehicle or PCO371 at 1.3, 6.5 or 33 mg kg−1) or subcutaneously (vehicle or hPTH(1–84) (Chugai Pharmaceutical) at 9 mg kg−1). Six TPTX rats were assigned to each dose group. The sample size was determined based on previous experiments and a published study with hPTHs and LA-PTH in our laboratories43 (n = 5–6). Vehicle for PCO371 was the same as in the OVX rat study, and phosphate–citrate buffer (pH 6.0) was used as vehicle for hPTH(1–84). Under isoflurane anaesthesia, jugular vein blood was collected immediately before and at 1, 2, 6, 10 and 24 h after administrations to measure serum Ca (o-CPC method, Wako Pure Chemical Industries) and P, levels (xanthine oxidase method, Wako Pure Chemical Industries). In another experiment, the effects of a single treatment with PCO371 or hPTH(1–34) on serum Ca and P, in TPTX rats (6-week-old) were evaluated at the dose of 3.9 or 30 mg kg−1 (PCO371, p.o.) or 9 mg kg−1 (hPTH(1–34), s.c.) according to the same method as that described above.

**Repeated administration study in TPTX rats.** TPTX was carried out in 6-week-old female Crl:CD (SD) rats (Charles River Laboratories). Rats with serum Ca levels of <8.0 mg dl−1 at 5 days after surgery were used in the following experiments. The rats were divided into four groups both in study 1 and 2, each with a similar mean value of serum Ca levels and body weight. Sample sizes were determined as described in the above single administration study. To evaluate the calcemic effects of PCO371 and hPTHs, we defined a target therapeutic range for serum Ca of 7.6–11.2 mg dl−1, which corresponds to the low normal to normal range of serum Ca levels3,31,33.

**Study 1.** TPTX rats were treated twice daily (at around 09:00 and 19:00 hours) orally (vehicle or PCO371 at 1, 3 or 9 mg kg−1) or subcutaneously [hPTH(1–34) or vehicle at 9 mg ml−1] for 4 weeks. Four rats were assigned to sham control, and treated with vehicle for PCO371. Vehicles for PCO371 and hPTH(1–34) were the same as in the OVX rat study. Under isoflurane anaesthesia, jugular vein blood was collected immediately before and at 6 and 10h (for PCO371) or at 1 and 10h (for hPTH) after administrations to measure the serum Ca levels on days 1 (the first dosing day), 7, 14, 22 and 29. Urine was collected for 24 h by using metabolic cages on days 29–30, and Ca excretion was calculated from urine volume and Ca level. Under isoflurane anaesthesia, jugular vein blood was collected after administrations to measure serum 1,25(OH)2D3 levels on Day 29 by using a 1,25(OH)2D3 RIA kit (Fuji). Four or five TPTX rats were assigned to each dose group. Serum and urine Ca levels were measured in the same way as in the single-dosing study. In another experiment, TPTX rats were treated subcutaneously once daily with hPTH(1–84) at 10 mg kg−1 for 4 weeks. Serum Ca was measured immediately before and at 2 and 6 h after administration on day 1 (the first dosing day), 7, 14, 21 and 28 using the same method as described above. Urine was
collected for 24 h by using metabolic cages on days 28 and 29, and Ca excretion was calculated from urine volume and Ca level.

**Study 2.** TPTX rats were orally treated once daily (vehicle alfacalcidol (Chugai Pharma Manufacturing) at 38 or 75 ng kg\(^{-1}\), or alfacalcidol at 38 ng kg\(^{-1}\) plus PCO371 at 6 mg kg\(^{-1}\)) for 4 weeks. Five or six TPTX rats were assigned to each dose group. The vehicle for alfacalcidol was medium-chain triglyceride (Chugai Pharma Manufacturing). Under isoflurane anesthesia, jugular vein blood was collected immediately before and at 6 and 10 h after administration to measure serum Ca levels on day 1 (the first dosing day), 8, 14, 22 and 28. Urine was collected for 24 h by using metabolic cages on days 28 and 29. Serum and urine Ca levels were measured in the same way as in the single-dosing study.

**Single administration study in normal dogs.** Marshall Beagle dogs (20 months old, male, Marshall BioResources Japan Inc) were treated once orally (vehicle or PCO371 at 3, 10 or 30 mg kg\(^{-1}\)). Six dogs were assigned to each group. Vehicle for PCO371 was the same as in the rat studies. The sample size was determined based on previous experiments with hPHTs in our laboratories (n = 6). Blood was collected from the cephalic vein immediately before and at 1, 2, 4, 6, 8, 10 and 24 h after administration to measure serum Ca levels (o-CPC method, Wako Pure Chemical Industries).

**Pharmacokinetics studies.** PCO371 was administered at a dose of 2, 6 or 18 mg kg\(^{-1}\) orally to 7-week-old normal female rats (RccHan: WIST, JLA; three rats for each dose group), or at a dose of 2, 6 or 18 mg kg\(^{-1}\) orally to 7-week-old female TPTX rats [Cr:CD(CD), Charles River Laboratories; three TPTX rats for each dose group] or at 30 mg kg\(^{-1}\) orally or 3, 10 mg kg\(^{-1}\) intravenously to 55-week-old O VX rats as described above. Blood samples were collected at 15 and 30 min, 1, 2, 4, 8 and 24 h after administration. Blood samples were also collected 2 min after intravenous administration in mature O VX rats. The concentrations of plasma PCO371 were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (AB SCIEX), detection limit: 1 ng ml\(^{-1}\). Pharmacokinetic parameters (\(T_{1/2}, C_{\text{max}}, C_{\text{AUC}}\text{and } AUC_{\text{z}}\) were calculated by a non-compartmental model using WinNonlin 6.4. Oral bioavailability (BA) was calculated with \(AUC_{\text{inf}}\) after oral and intravenous administration at a dose of 2 mg kg\(^{-1}\) by using the following equation (5):

\[
\text{BA} (\%) = \frac{AUC_{\text{oral}}}{AUC_{\text{inf}}} \times 100
\]

hPHT(1-34) was subcutaneously injected at a dose of 3 nmol kg\(^{-1}\) to normal female 10-week-old Cr:CD(CD) rats (Charles River Laboratories). Four rats were assigned to each group. Blood samples were collected at pre-dose and at 2, 5, 10, 15, 30 and 45 min, and 1 and 2 h after administration. The concentrations of plasma hPHT(1-34) were determined by an enzyme immuno assay (EIA) system (Immunotronics International). hPHT(1-84) was subcutaneously injected at a dose of 30, 100 or 300 nmol kg\(^{-1}\) to TPTX female 8-week-old Cr:CD(CD) rats (Charles River Laboratories). Blood samples were collected from the jugular vein at pre-dose and at 5, 10, 15, 20, 30 and 45 min and at 1, 2, 4 and 24 h after administration. Five TPTX rats were assigned to each dose group. The concentrations of plasma hPHT(1-84) were determined by an EIA system (Immunotronics International).

**Statistical analysis.** Statistical significance was assumed at the two-sided 5% level (\(P<0.05\)). The data displayed normal variance. The dose-dependent effect was tested with Williams’ test. Dunnett’s test was performed to test for significant difference between two groups. SAS Preclinical Package (SAS Institute) software was used for statistical analyses for all other experiments. Quantitative results are represented as the mean ± s.d. or s.e.m., unless otherwise noted.

**Data availability.** The data that support the findings of this study are available from the corresponding author on request.

**References**

1. Potts, J. T. & Gardella, T. J. Progress, paradox, and potential: parathyroid hormone research over five decades. *Ann. N. Y. Acad. Sci.* 1117, 196–208 (2008).

2. Goltzman, D. Interactions of PTH and PTHrP with the PTH/PTHrP receptor and with downstream signaling pathways: exceptions that provide the rules. *Ann. N. Y. Acad. Sci.* 88, 16525–16530 (2008).

3. Augustine, M. & Horwitz, M. J. Parathyroid hormone and parathyroid hormone receptor by using an N-terminal parathyroid hormone peptide probe. *Proc. Natl Acad. Sci. USA* 104, 6846–6851 (2007).

4. Winer, K. K. Efficacy and safety of recombinant human parathyroid hormone (1-34) versus calcitriol and calcium. *J. Clin. Endocrinol. Metab.* 88, 4214–4220 (2003).

5. Shimizu, M., Guo, J. & Gardner, P. J. 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.* 276, 49003–49012 (2001).

6. Dean, T. et al. Mechanisms of ligand binding to the parathyroid hormone (PTH)/PTH-related protein receptor: selectivity of a modified PTH(1-15) radioligand for Galphα(5) coupled-receptor conformations. *Mol. Endocrinol.* 20, 931–943 (2006).

7. Hoare, S. R., Bonner, T. I. & Usdin, T. B. Comparison of rat and human parathyroid hormone 2 (PTH2) receptor activation: PTH is a low potency partial agonist at the rat PTH2 receptor. *Endocrinology* 140, 4419–4425 (1999).

8. Dean, T., Vilaradaga, J. P., Potts, J. T. & Gardella, T. J. Altered selectivity of parathyroid hormone (PTH) and PTH-related protein (PTHrP) for distinct conformations of the PTH/PTHrP receptor. *Mol. Endocrinol.* 22, 156–166 (2008).

9. Okazaki, M. et al. Prolonged signaling at the parathyroid hormone receptor by peptide ligands targeted to a specific receptor conformation. *Proc. Natl Acad. Sci. USA* 105, 16525–16530 (2008).

10. Shimer, M. & et al. Pharmacodynamic actions of a long-acting PTH analog (LA-PTH) in thyroparathyroidectomized (TPTX) rats and normal monkeys. *J. Bone Miner. Res.* 31, 1405–1412 (2016).

11. Dufau, M. L., Tsuruhara, T., Horner, K. A., Podesta, E. & Catt, K. J. Intermediate role of adenosine 3′,5′-cyclic monophosphate and protein kinase during gonadotropin-induced steroidogenesis in testicular interstitial cells. *Proc. Natl Acad. Sci. USA* 74, 3419–3423 (1977).

12. Partridge, N. C., Kemp, B. E., Livesey, S. A. & Martin, T. J. Activity ratio measurements reflect intracellular activation of adenosine 3′,5′-monophosphate-dependent protein kinase in osteoblasts. *Endocrinology* 111, 178–183 (1982).

13. Podesta, E. J., Dufau, M. L., Solano, A. R. & Catt, K. J. Hormonal activation of protein kinase in isolated Leydig cells. Electrophoretic analysis of cyclic AMP protein-coupled receptors. *J. Biol. Chem.* 268, 29964–29971 (1993).

14. Florek, C. A. et al. Anabolic and catabolic bone effects of human parathyroid hormone (1-34) are predicted by duration of hormone exposure. *Bone* 33, 372–379 (2003).
29. Cusano, N. E. et al. Therapy of hypoparathyroidism with PTH(1-84): a prospective four-year investigation of efficacy and safety. J. Clin. Endocrinol. Metab. 98, 137–144 (2013).
30. Sikjaer, T. et al. PTH(1-84) replacement therapy in hypoparathyroidism: a randomized controlled trial on pharmacokinetic and dynamic effects after 6 months of treatment. J. Bone Miner. Res. 28, 2232–2243 (2013).
31. Martin, T. J. et al. Mechanisms involved in skeletal anabolic therapies. Ann. N. Y. Acad. Sci. 1068, 458–470 (2006).
32. Kraenzlin, M. E. & Meier, C. Parathyroid hormone analogues in the treatment of osteoporosis. Nat. Rev. Endocrinol. 7, 647–656 (2011).
33. Hollenstein, K. et al. Insights into the structure of class B GPCRs. Trends Pharmacol. Sci. 35, 12–22 (2014).
34. Wang, M. W., Liu, Q. & Zhou, C. H. Non-peptidic glucose-like peptide-1 receptor agonists: aftermath of a serendipitous discovery. Acta Pharmacol. Sin. 31, 1026–1030 (2010).
35. Chen, D. et al. A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice. Proc. Natl Acad. Sci. USA 104, 943–948 (2007).
36. Shimizu, N. et al. Novel parathyroid hormone (PTH) antagonists that bind to the juxtamembrane portion of the PTH/PTH-related protein receptor. J. Biol. Chem. 280, 1797–1807 (2005).
37. Schipani, E. et al. Constitutively activated receptors for parathyroid hormone and parathyroid hormone-related peptide in Jansen’s metaphyseal chondrodysplasia. N. Engl. J. Med. 335, 708–714 (1996).
38. Hollenstein, K. et al. Structure of class B GPCR corticotropin-releasing factor receptor 1. Nature 499, 438–443 (2013).
39. Rasmussen, S. G. et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. Nature 477, 549–555 (2011).
40. Takasu, H., Guo, J. & Bringhurst, F. R. Dual signaling and ligand selectivity of the human PTH/PTHrP receptor. J. Bone Miner. Res. 14, 11–20 (1999).
41. Shaughnessy, S. G., Young, E., Deschamps, P. & Hirsh, J. The effects of low molecular weight and standard heparin on calcium loss from fetal rat calvaria. Blood 86, 1368–1373 (1995).
42. Sakai, S. et al. Treatment with the combination of ibandronate plus eldecalcitol has a synergistic effect on inhibition of bone resorption without suppressing bone formation in ovariectomized rats. Bone 81, 449–458 (2015).

Acknowledgements
We express our deep appreciation to the late E. Ogata, who emphasized to us the great need for improved therapy for hypoparathyroidism and wisely and helpfully encouraged us in our efforts to produce the compound reported here. We are grateful to H. Okabe for thoughtful discussion and helpful comments on the manuscript. We thank H. Saito, N. Inomata, H. Takasu, N. Shimizu and F. Makishima for their contribution to screening assays and hit identification. We also thank our colleagues at Chugai Medical Science Inc. for their support with the in vitro and in vivo experiments.

Author contributions
T.T. and H.N. led the pharmacological research. T.E. and Y.N. led the medicinal chemistry studies. E.J., M.H., T.W., M.K., M.S. and H.K. conducted the pharmacological studies. K.O., T.M. and S.A. conducted the pharmacokinetics studies. Y.K. and H.S. directed this programme based on their biological and chemical expertise, respectively. T.T., H.N., M.S., H.K., T.E. and Y.N. revised the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Tamura, T. et al. Identification of an orally active small-molecule PTHR1 agonist for the treatment of hypoparathyroidism. Nat. Commun. 7, 13384 doi: 10.1038/ncomms13384 (2016).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016