Bioorganic & Medicinal Chemistry Letters 23 (2013) 2455–2459

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

New potent calcimimetics: II. Discovery of benzothiazole trisubstituted ureas

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ARTICLE INFO

Article history:
Available online 8 February 2013

Keywords:
Calcium sensing receptor
Benzothiazole urea
Positive allosteric modulators

ABSTRACT

Following the identification of trisubstituted ureas as a promising new chemical series of allosteric modulators of the calcium sensing receptor (CaSR), we further explored the SAR around the urea substitution, leading to the discovery of benzothiazole urea compound **13**. This compound is a potent calcimimetic with an EC_{50} = 20 nM (luciferase assay). Evaluated in an in vivo model of chronic renal failure (short term and long term in 5/6 nephrectomized rats), benzothiazole urea **13** significantly decreased PTH levels after oral administration while keeping calcemia within the normal range.

Secondary hyperparathyroidism (sHPT) is characterized by an excessive production of parathyroid hormone (PTH) following renal failure.[1,2] PTH is secreted by parathyroid glands and PTH level is controlled by the extracellular calcium concentration via the calcium sensing receptor (CaSR), a family C GPCR.[3,4] Treatment of sHPT by positive allosteric modulators of the CaSR also called calcimimetics-proved to be a successful approach with the discovery of the exploratory compound R-568,[5,6] followed by cinacalcet (AMG-073, Sensipar®).[7,8] Other calcimimetics have also been indentified, most of them containing the α-methylbenzylamine group.[9–11]

Earlier, we described the identification of a new chemical series of trisubstituted ureas represented by **1**. Oxazole **1** is a potent in vitro agonist of the CaSR, leading to the in vivo reduction of serum PTH level in normal rats.[12] The *gem* diphenyl-propyl group and the *N*-ethylmorpholinyl substituent of the urea **1** appeared to be important for in vitro potency, while some flexibility on the aromatic substitution on the phenyl ring was tolerated.

In our search towards new compounds with improved biological profile on the CaSR, we describe here the identification of the benzothiazole compound **13** (Fig. 1) including a SAR analysis and pharmacological profile, indicating that benzothiazole **13** is a potent calcimimetic in vitro and in vivo.

Urea compounds were prepared as described in previous publications.[12–14] We present here the optimized synthesis[15] of benzothiazole **13** from two commercially available building blocks: amino-ethyl morpholine and the *gem*-diphenyl ethyl carboxylic acid (Scheme 1). The intermediate acyl chloride derived from *gem*-diphenyl ethyl carboxylic acid was generated with thionyl chloride and was coupled to amino-ethylmorpholine to give amide **2** which was collected by filtration and used in the next step without additional purification. The reduction of the amide function of compound **2** was achieved using $LiAlH_4$ in diethyl ether. However, for kg scale synthesis, replacement of diethyl ether by THF led to low yield and eventually, the use of the combination of $LiAlH_4/AlCl_3$ in THF[16] proved to be critical for improving the yield to 72% in 3 h.

The secondary amine **3** was then purified by crystallization in ethanol after acidic treatment (see Scheme 1).

Conversion of **3**–**13** was accomplished by stirring a mixture of 2-amino benzothiazole and carbonyl di-imidazole in dichloromethane overnight, followed by addition of **3**. After 5 h of stirring, the desired urea benzothiazole **13** was isolated in 84% yield. The

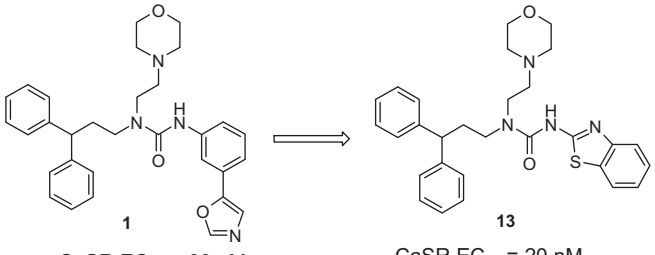

Figure 1. Identification of lead compound benzothiazole urea **13**.

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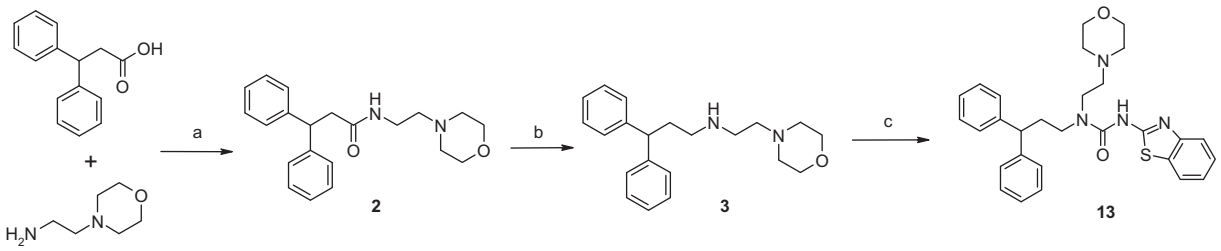
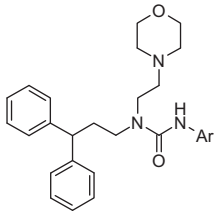
http://dx.doi.org/10.1016/j.bmcl.2013.01.077



Scheme 1. Reagents and conditions: (a) $SOCl_2$, CH_2Cl_2, rt, 88%; (b) 1 equiv $LiAlH_4$, 0.1 equiv $AlCl_3$, THF, 0 °C to rt, 3 h, then EtOH/ HCl 72%; (c) 2-aminobenzothiazole, CDI, CH_2Cl_2, rt, overnight, then addition of compound **3**, CH_2Cl_2, rt, 84%.

Table 1
Calcimimetic activity of ureas **4**–**17**[a]: replacement of phenyl by various heterocyclic rings



Compound	Ar	EC_{50} (nM)
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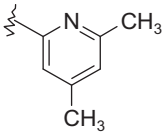
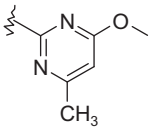
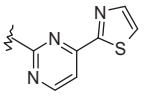
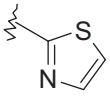
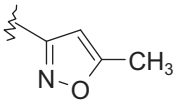
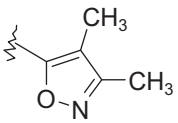
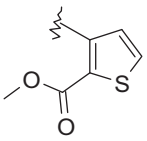
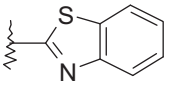
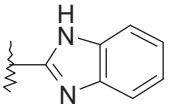
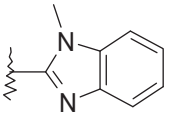
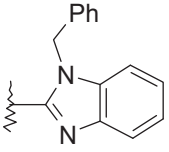
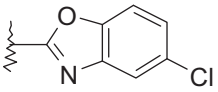
5		80
6		450
7		400
8		200
9		300
10		200
11		1700
12		400

Table 1 (*continued*)

Compound	Ar	EC_{50} (nM)
13		20
14		60
15		150
16		300
17		80

[a] EC_{50} values in μM ($n \geqslant 1$) have been determined by luciferase assay.

HCl salt was formed in EtOH and the compound was crystallized with more than 98% purity. The overall yield was 61% without any chromatography and this optimized process has been used on a kilogram scale.

Urea compounds were tested in a dose response luciferase assay, with increasing calcium concentration.[17] We observed a leftward shift of the hCaSR calcium responses by increasing concentration of calcimimetics. The values indicated in this paper correspond to an EC_{50} at 2 mM of calcium. In our assay conditions, reference compound R-568 and urea compound **1** exhibited potent activity with EC_{50} values of 80 nM and 60 nM, respectively.

Previously, we described the optimization of the trisubstituted urea series by varying the *meta* substitution of the aniline ring with a large number of functional groups.[12]

In this paper, we describe the replacement of the phenyl ring by five- and six-membered ring heterocycles and fused aromatics.

The potent activity (EC_{50} <200 nM) of a few compounds in this set confirmed the importance of the substitution of the urea for modulating potency, and also the flexibility in this region. Pyridine (**5**), thiazole (**9**) and isoxazole (**10**) substitutions of the urea proved to be active, and a heterocycle bearing a nitrogen atom adjacent to the –NH– of the urea was most efficient, as indicated by the comparison of pyridine **5** and **4** (80 nM vs 1000 nM), or isoxazole **10** and **11** (200 nM vs 1700 nM). Based on this observation, bicyclic aromatic rings with a nitrogen atom suitably positioned

were further explored (Table 1), leading to potent benzimidazole, benzoxazole and benzothiazole compounds. Benzothiazole **13** proved to be the most potent compound identified (EC_{50} = 20 nM), fourfold more potent than R-568.

Exploration of various substitutions around the benzothiazole moiety was then undertaken (Table 2).

No further improvement was observed upon substitution of the 4-, 5- and 6-positions of benzothiazole. Most of the substitutions at R^1 and R^3 resulted in analogs that were potent (EC_{50} below 100 nM).

Based on in vitro SAR studies, benzothiazole urea **13** was selected for further characterization of its pharmacological and safety profile.

Compound **13** was a potent candidate (EC_{50} = 20 nM) with fourfold improvement over R-568. It was evaluated in in vivo models as a dihydrochloride salt. This compound was stable over time in serum, intestinal fluid, and over a pH range of 2–12. Its aqueous solubility was 0.3 mg/mL at pH 2.

A PK experiment in CRF rats (Chronic Renal Failure) with benzothiazole **13** (iv dose, 0.3 mg/kg) indicated an AUC of 162 ng h/mL associated with a C_{max} of 546 ng/mL. The clearance was moderate (1.85 L/h/kg), and due to a high volume of distribution (V_d = 5.17 L/kg), the $T_{1/2}$ was 6.73 h.

Further profiling of this compound was carried out to evaluate the in vivo effect on serum PTH levels.

Benzothiazole urea **13** was evaluated[18] in CRF rats (Fig. 2), a relevant model for secondary hyperparathyroidism linked to renal insufficiency. One week following a 5/6 nephrectomy (left kidney and 2/3 of right kidney surgically removed), CRF rats were characterized by high PTH level (177 pg/mL vs 38 pg/mL for intact rats) as well as increased serum creatinin (63 vs 28 μM) and urea (10 vs 4 mM).

Benzothiazole **13** was administered orally at 10 mg/kg in CRF rats and PTH was measured over 24 h along with calcium and phosphorus. As indicated in Figure 2, compound **13** exhibited a rapid and significant decrease in PTH (95% decrease at 2 h, −75% at 6 h) and this effect was still significant after 24 h (−49%).

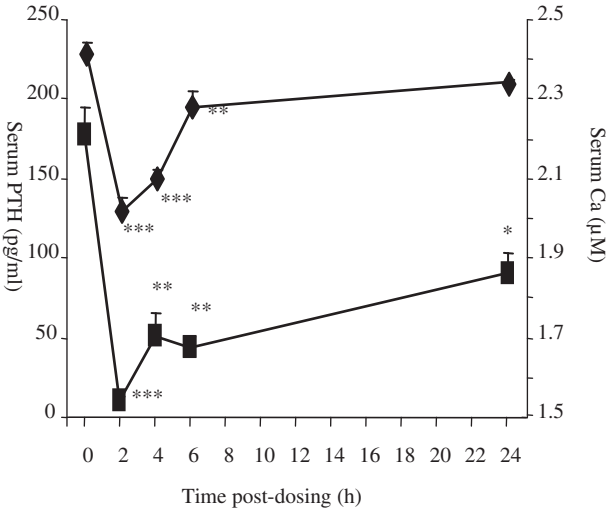
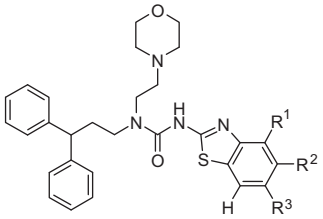


Figure 2. In vivo serum PTH and Ca after a single oral dose of compound **13** (10 mg/kg) in 5/6 nephrectomized male Sprague–Dawley rats (PTH (■), Ca (♦)). n = 9–12, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.0001 significantly different from 'CRF vehicle' group, ANOVA, t-test.

As described with other calcimimetics,[19] this strong decrease in PTH is usually associated with a significant decrease in serum calcium. However, interestingly, only a slight decrease in serum calcium was observed, with serum calcium being maintained above 2 mM. Therefore, no hypocalcemia was observed at the dose of 10 mg/kg despite the strong effect on PTH. In addition to its potency, this was a striking feature of benzothiazole urea **13**, indicating that a window between strong PTH decrease and hypocalcemia was possible with this compound. Serum phosphorus levels increased as expected (Fig. 3).

The in vivo potency was confirmed in a long term (2-month treatment) CRF model (Figs. 4 and 5). 20 days after surgery, 5/6 nephrectomized rats were provided with a high level of phosphorous in their drinking water (6 g/L $NaHPO_4$) for a total of 114 days.[20]

Table 2
Calcimimetic activity of urea **18**–**34**[a]: variation of substitution on the benzothiazole ring



Compound	R^1	R^2	R^3	EC_{50} (nM)
13	H	H	H	20
18	Cl	H	H	20
19	OCH_3	H	H	30
20	CH_3	H	H	600
21	H	$NHSO_2CH_3$	H	30
22	H	H	OCH_3	40
23	H	H	OCF_3	90
24	H	H	Cl	60
25	H	H	CH_2OH	80
26	H	H	$NHSO_2CH_3$	30
27	H	H	CH_3	30
28	H	H	COOH	100
29	H	H	$COOCH_2CH_3$	200
30	H	H	$CONH(CH_2)_2$-$N(CH_3)_2$	200
31	H	H	SO_2CH_3	80
32	H	H	OCH_2CH_3	1000
33	H	H	F	200
34	H	H	Cl	60

[a] EC_{50} values in μM ($n \geqslant 1$) have been determined by luciferase assay.

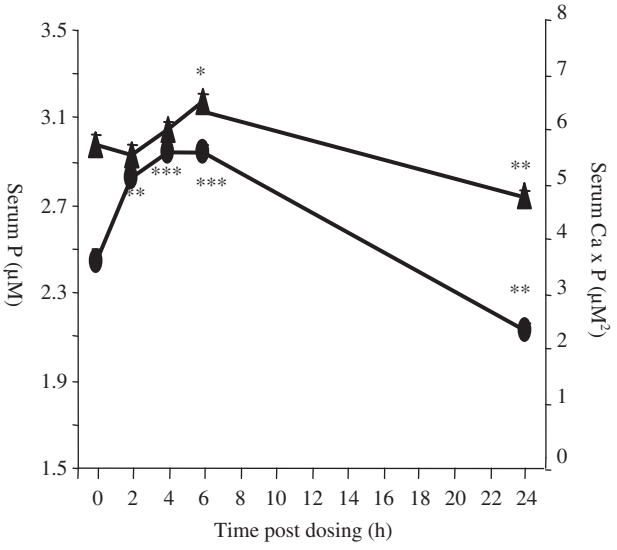


Figure 3. In vivo serum phosphorus and CaxP after a single oral dose of compound **13** (10 mg/kg) in 5/6 nephrectomized male Sprague–Dawley rats (P (●), CaxP (▲)). n = 9–12, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.0001 significantly different from 'CRF vehicle' group, ANOVA, t-test.

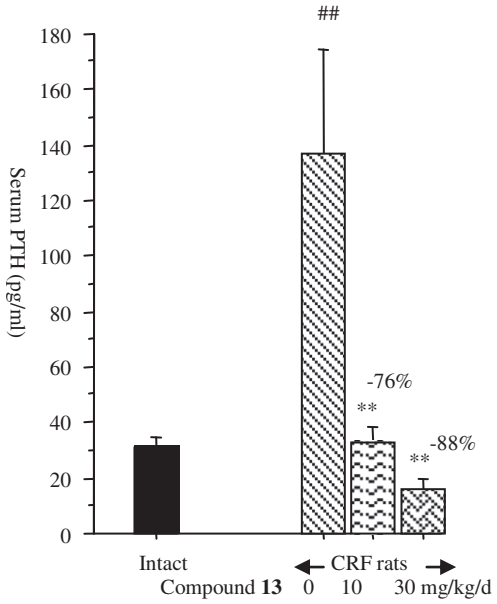


Figure 4. In vivo serum PTH after a two month-oral administration of compound **13** (10 and 30 mg/kg/day) in male 5/6 nephrectomized Sprague–Dawley rats, 24 h after last administration. $n = 6–10$, $^{\#\#}p$ <0.01 significantly different intact rats, $^{*}p$ <0.05, $^{**}p$ <0.01, significantly different from 'CRF vehicle' group, ANOVA, *t*-test.

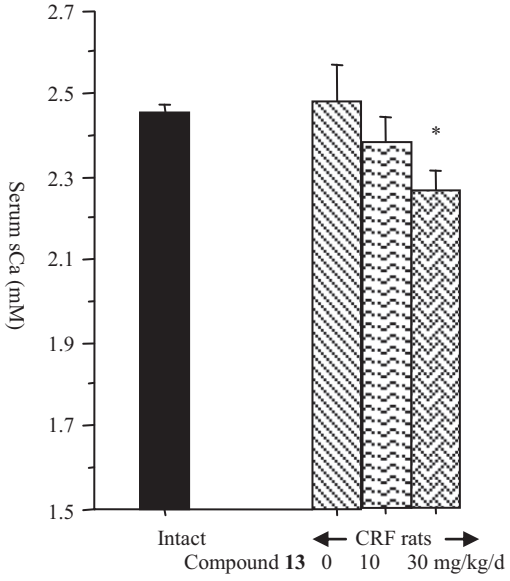


Figure 5. In vivo serum Ca after a two month-oral administration of compound **13** (10 and 30 mg/kg/day) in male 5/6 nephrectomized Sprague–Dawley rats, 24 h after last administration. $n = 6–10$, $^{*}p$ <0.05 significantly different from 'CRF Vehicle' group, ANOVA, *t*-test.

After surgery, the rats were left without treatment for 65 days. Then, compound **13** was administered orally at 10 and 30 mg/kg once a day for 2-months. No mortality or side effects were observed during the period of the treatment. Rats were then sacrificed and PTH levels measured 24 h after the last administration. We observed a decrease in PTH level of −76% (at 10 mg/kg/day) and of −88% (at 30 mg/kg/day), confirming the potency and safety of the compound in the two month-treatment (Fig. 4). As observed previously, calcium levels stayed within the normal range, with no statistical difference at 10 mg/kg/day compared with the untreated CRF rats (−4% ns), and decreased by 9% (p <0.05) at 30 mg/kg/day, (Fig. 5). Histological analysis of aortic calcification was also carried out and none of the rats at either dose were affected by aortic calcification.

In conclusion, benzothiazole compound **13** was identified as a promising compound for preclinical studies. In addition to its pharmacological properties, this compound proved to be selective over a large panel of receptors and enzymes at 10 μM (Cerep screen), without any genotoxicity (Ames) and hERG inhibition at 10 μM. A 14-day toxicity study carried out did not show serious side effect up to 1000 mg/kg.

However, despite the efficacy of benzothiazole **13** in CRF rats, we observed a difference in the PK profile between CRF rats and normal rats, with lower bioavailability in normal rats and higher clearance (4.96 L h/kg vs 1.85 L h/kg), which made it difficult to predict PK parameters in humans, thus preventing further development of this compound in clinical trials.

Acknowledgments

The authors thank Séverine Hebbe from Galapagos and Paul Harrington from Amgen for careful review of the manuscript.

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13. Deprez, P.; Patek, M. W.O. Patent 059,102, 2002.
14. Deprez, P.; Jary, H.; Temal, T. W.O. Patent 117,211, 2006.
15. *Chemical procedure for synthesis of benzothiazole urea compound **13**:* 60.1 g (0.37 mol, 1.5 equiv) of 1,1′-carbonyl-diimidazole were dissolved in 660 mL of CH_2Cl_2 in a 5-L reaction vessel. 37.1 g (0.25 mol) of 2-aminobenzothiazole solution in 660 mL of CH_2Cl_2 was then added drop wise at a temperature of between 20 and 24 °C. The mixture was left to stand with stirring at room temperature for 15 h. A solution of 117.4 g (0.30 mol, 1.2 equiv) of (3,3-diphenyl-propyl)-(2-morpholin-4-yl-ethyl)-amine dihydrochloride **3** and 59.8 g (0.60 mol, 2.4 equiv) of triethylamine in 660 mL of CH_2Cl_2 was added over 15 min with continuous stirring of the reaction medium at room temperature for 2 h. The reaction was monitored by TLC (SiO_2, mobile phase CH_2Cl_2/MeOH, 95/5, visualisation: UV).
600 mL of water and then 600 mL of saturated aqueous $NaHCO_3$ solution were added to the reaction medium. After separation of the organic phase, the aqueous phase was extracted with 1 L of CH_2Cl_2. The organic fractions were combined, washed with 1.5 L of a saturated aqueous NaCl solution, dried over Na_2SO_4, filtered and concentrated to dryness under reduced pressure to give 143 g of an orange solid. This solid was dissolved in 1.4 L of acetonitrile at 70 °C during 30 min. The resulting suspension was cooled to 0 °C and filtered. The white solid isolated was washed with 500 mL of acetonitrile then dried under a high vacuum obtained using a vane-type pump to give 103 g (84%) of 3-benzothiazol-2-yl-1-(3,3-diphenyl-propyl)-1-(2-morpholin-4-yl-ethyl)-urea **13** in the form of a white crystalline solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 1H, aromatic H), 7.70 (d, 1H, aromatic H), 7.40 (t, 1H, aromatic H), 7.35–7.10 (m, 11H, aromatic H), 4.05 (m; 4H, 2×CH_2), 4.00 (t, 1H, CH), 3.37 (t, 4H, CH_2), 2.65 (m, 6H, CH_2), 2.40 (q, 2H, CH_2).
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17. *Luciferase in vitro assay:* The human parathyroid cell Ca^{2+} receptor cDNA was subcloned into the mammalian expression vector PECE (Ref. 1). The luciferase reporter was subcloned into the mammalian expression vector pGL3basic (Promega). Resistance to neomycin (pSV2-neo) and resistance to puromycin (pSG5-puro) were used as selection markers. All these plasmids were simultaneously transfected into CHO cells by calcium phosphate precipitation. Transfected cells were grown in F12 medium containing 7.5%

foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (as 1% Pen-Strep, BioWithaker), neomycin (750 μg/mL) and puromycin (5 μg/mL). Neomycin and puromycin resistant colonies were subcloned and assayed for activation against a range of calcium concentration. Clone 8-5-5 was used to assess the effects of compounds on $[Ca^{2+}]i$. This stably transfected cell line is termed ET8-5-5.

For measurements of $[Ca^{2+}]i$, the cells were recovered from tissue culture flasks by brief treatment with Trypsin–EDTA (Invitrogen; containing 0.53 mM EDTA·4Na in HBSS) and then seeded in Culture treated 96-well plates (Greiner) at 50 K cells per well in the growth media (same as above, except neomycin 400 μg/mL). Cells were grown in 37 °C TC incubator for 24 h. The culture medium was then removed and replaced with F12 medium, 1% Pen-Strep for an overnight foetal bovine serum starvation in 37 °C TC incubator. Then the starvation medium was removed and replaced with a test buffer (20 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.5 mM, glucose, 2 g/L lysosyme and 0.3 mM $CaCl_2$) supplemented with a range of test compound concentrations crossed against a superadded range of $CaCl_2$ concentrations. The cells were incubated with the test compounds for 5 h in 37 °C TC incubator. Then the test buffer was discarded, and cells were added with the substrate for Luciferase from SteadyLite Kit (Perkin–Elmer). The luminescence was recorded.

18. *In vivo measurements:*
Single oral administration: Male Sprague–Dawley rats (250–300 g BW, CERJ, France) underwent surgical 5/6 nephrectomy or sham-operation according to a protocol approved by Internal Ethical Committee for the care and use of animals. One week post-nephrectomy, rats received a single oral gavage (5 mL/kg) of compound or vehicle. Compound **13** (dihydrochloride salt) was formulated in methylcellulose 0.5% and administered at 10 or 30 mg/kg. Vehicle-treated control rats were given the same volume of vehicle.
The rats were fasted 24 h before necropsy and were sacrificed by decapitation at various times (2, 6, 24 h) after the gavage with vehicle or calcimimetic. Blood samples were collected in Sarstedt Z tubes, allowed to clot for 20 min and centrifuged (3000 rpm, Jouan CR422) at 4 °C. Serums were removed and stored at −20 °C until assayed. Serum PTH was quantified according to the provider's instructions using a rat IRMA kit (Immutopics, 50-2000). Serum Ca, P, creatinin and urea were measured using a blood chemistry analyzer (Cobas Mira) according to the manufacturer's protocols. Increased levels of urea and creatinin confirmed the renal failure. Comparisons among the groups were made using one-way analysis of variance (ANOVA) and unpaired *t*-test.
19. Fox, J.; Lowe, S. H.; Petty, B. A.; Nemeth, E. F. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 473.
20. *In vivo measurements:*
Two-month treatment in long-term chronic renal failure:
Male Sprague–Dawley rats (250–300 g BW, CERJ, France) underwent surgical 5/6 nephrectomy or sham-operation according to a protocol approved by Internal Ethical Committee for the care and use of animals.
The dosings started 12 weeks post-nephrectomy and lasted 7 weeks. The rats were orally dosed at 10 and 30 mg/kg/day with compound **13**, (dihydrochloride salt) formulated in methylcellulose 0.5%. Vehicle-treated control rats received the same volume of vehicle (5 mL/kg).
The rats were fasted 24 h before necropsy and were sacrificed by decapitation. Blood samples were collected in Sarstedt Z tubes, allowed to clot for 20 min and centrifuged (3000 rpm, Jouan CR422) at 4 °C. Serums were removed and stored at −20 °C until assayed. Serum PTH was quantified according to the provider's instructions using a rat IRMA kit (Immutopics, 50-2000). Serum Ca, P, creatinin and urea were measured using a blood chemistry analyzer (Cobas Mira) according to the manufacturer's protocols. Increased levels of urea and creatinin confirmed the renal failure. Comparisons among the groups were made using one-way analysis of variance (ANOVA) and unpaired *t*-test.