The effect of calcitriol, paricalcitol, and a calcimimetic on extraosseous calcifications in uremic rats

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Vitamin D derivatives and calcimimetics are used to treat secondary hyperparathyroidism in patients with chronic renal failure. We investigated the effect of calcitriol, paricalcitol, and the calcimimetic AMG 641 on soft-tissue calcification in uremic rats with secondary hyperparathyroidism. Control and uremic rats were treated with vehicle, calcitriol, paricalcitol, AMG 641, or a combination of AMG 641 plus calcitriol or paricalcitol. Parathyroid hormone levels were reduced by all treatments but were better controlled by the combination of paricalcitol and AMG 641. The calcimimetic alone did not induce extraosseous calcification but co-administration of AMG 641 reduced soft-tissue calcification and aortic mineralization in both calcitriol- and paricalcitol-treated rats. Survival was significantly reduced in rats treated with calcitriol and this mortality was attenuated by co-treatment with AMG 641. Our study shows that extraskeletal calcification was present in animals treated with calcitriol and paricalcitol but not with AMG 641. When used in combination with paricalcitol, AMG 641 provided excellent control of secondary hyperparathyroidism and prevented mortality associated with the use of vitamin D derivatives without causing tissue calcification.

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Secondary hyperparathyroidism (HPT) and vascular calcification (VC) are common complications of stage 5 chronic kidney disease. The elevations in calcium, phosphorus, and Ca × P product observed in patients with secondary HPT have been associated with VC and increased risk of cardiovascular morbidity and mortality.[1–4]

Vitamin D derivatives, such as calcitriol and paricalcitol, are commonly used to treat the elevated parathyroid hormone (PTH) associated with secondary HPT;[5–10] however, they have been shown to induce VC *in vivo* and *in vitro* animal models.[11–13] Vitamin D derivatives increase expression of several proteins involved in calcification and decrease expression of proteins that inhibit calcification.[14,15] In addition, they potentially lead to hypercalcemia and hyperphosphatemia,[6,7,10] although paricalcitol has been reported to have less calcemic effects than calcitriol.[16,17] Together, these data suggest the need for alternative treatment strategies.

One such strategy is the use of calcimimetics. These compounds bind to the calcium-sensing receptor and increase its sensitivity to extracellular calcium, thereby suppressing PTH synthesis[18] and secretion[19] without inducing hypercalcemia.[20,21] We have recently demonstrated that, when administered to uremic rats, the calcimimetic R-568 reduces PTH levels without inducing VC, attenuates the calcitriol-induced calcifying effects on vascular tissue, and decreases mortality associated with calcitriol.[22] AMG 641 is a research calcimimetic that has a more sustained action than R-568, allowing administration every 48 h.

In this study, we investigate the effect of calcitriol, paricalcitol, and the research calcimimetic AMG 641, alone or in combination, on the development of vascular and other soft-tissue calcifications in a rat model of uremia-associated secondary HPT.

RESULTS

Animal survival

During the first 14 days following nephrectomy and the change in diet, survival was >90% in all treatment groups, except in rats treated with calcitriol, in which survival was 80%. However, marked differences in survival were found between treatment groups from days 15 through 28. Survival

was not different in the five-sixth (5/6) Nx + vehicle (100%) and in the AMG 641 groups (86%). However, treatment with vitamin D derivatives resulted in a significant reduction in survival that was more marked with calcitriol (18%, $P<0.001$ vs 5/6 Nx + vehicle) than with paricalcitol (50%, $P=0.02$ vs 5/6 Nx + vehicle). The addition of AMG 641 to the vitamin D derivatives improved survival both in calcitriol (40%)- and in paricalcitol (70%, NS vs 5/6 Nx + vehicle)-treated rats (Figure 1).

Serum biochemical parameters

At day 14, mean plasma creatinine concentration in sham-operated rats was 0.57 ± 0.01 mg per 100 ml. As expected, all 5/6 Nx rats had significantly ($P<0.05$) higher creatinine levels, but significant differences were found between treatment groups. Plasma creatinine concentrations in rats treated with either calcitriol (1.86 ± 0.68 mg per 100 ml) or paricalcitol (1.83 ± 0.85 mg per 100 ml) were significantly higher ($P<0.05$) than in rats treated with vehicle (1.16 ± 0.05 mg per 100 ml) or AMG 641 (0.94 ± 0.14 mg per 100 ml). Addition of AMG 641 to vitamin D derivatives significantly ($P=0.001$) reduced plasma creatinine in paricalcitol-treated rats (1.15 ± 0.27 mg per 100 ml) but not in rats treated with calcitriol (1.58 ± 0.51 mg per 100 ml).

Plasma levels of ionized calcium, phosphorus, and PTH at day 14 are depicted in Figure 2. Plasma ionized calcium levels were significantly ($P<0.001$) reduced in all 5/6 Nx groups (range 0.92–1.05 mmol l^{-1}) when compared with sham-operated rats (1.18 ± 0.02 mmol l^{-1}). A higher ionized calcium concentration ($P<0.05$) was identified in rats treated with calcitriol (1.05 ± 0.03 mmol l^{-1}) than in groups treated with AMG 641 (0.94 ± 0.02 mmol l^{-1}) and paricalcitol (0.92 ± 0.03 mmol l^{-1}) (Figure 2a). Plasma phosphorus levels (Figure 2b) were consistently ($P<0.01$) elevated in 5/6 Nx rats, except in groups treated with AMG 641. Plasma phosphorus was higher in rats treated with calcitriol (19.7 ± 1.6 mg per 100 ml), and lower in rats treated with AMG 641 alone (9.1 ± 0.7 mg per 100 ml). Paricalcitol treated rats had significantly higher ($P=0.002$) plasma phosphorus (16.0 ± 2.1 mg per 100 ml) than rats treated with AMG 641 (9.1 ± 0.7 mg per 100 ml). It is also interesting to note that addition of AMG 641 achieved a reduction in plasma phosphorus both in rats treated with paricalcitol (10.2 ± 0.8 mg per 100 ml) and in rats treated with calcitriol (14.8 ± 1.8 mg per 100 ml). Plasma PTH concentration was significantly ($P<0.001$) increased in 5/6 Nx rats (450.1 ± 45.9 pg ml^{-1}) when compared with sham-operated animals (44.4 ± 16.2 pg ml^{-1}). Treatment with AMG 641, alone or in combination with vitamin D derivatives, reduced plasma PTH concentrations to levels that were not significantly different from the sham-operated rats. However, PTH concentrations were higher in rats treated with calcitriol (284.1 ± 44.0 pg ml^{-1}, $P<0.001$) and with paricalcitol

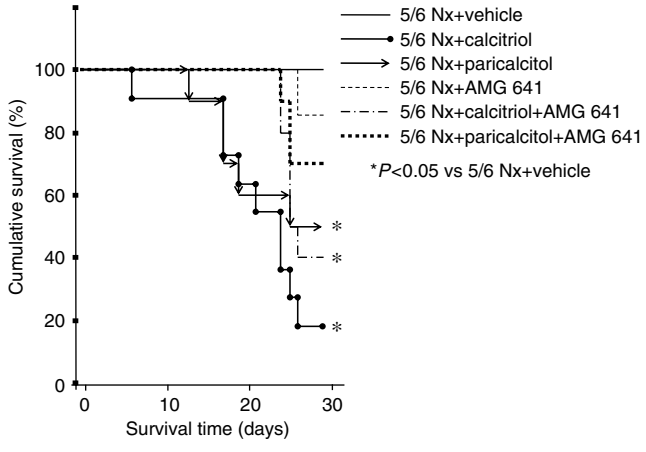

Figure 1 | Kaplan-Meier survival curve in nephrectomized rats (5/6 Nx) treated with vehicle (saline) ($n=8$), calcitriol 80 ng kg^{-1} i.p. ($n=11$), paricalcitol 240 ng kg^{-1} i.p. ($n=10$), AMG 641 1.5 mg kg^{-1} subcutaneously ($n=7$), the combination calcitriol 80 ng kg^{-1} and AMG 641 1.5 mg kg^{-1} ($n=10$), or the combination paricalcitol 240 ng kg^{-1} and AMG 641 1.5 mg kg^{-1} ($n=10$).

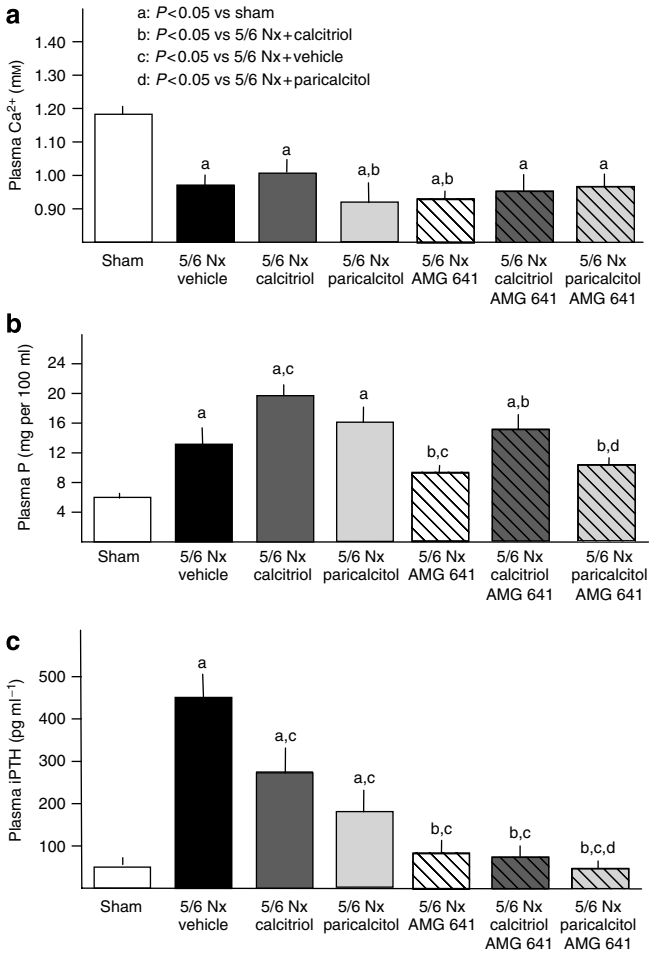

Figure 2 | Blood biochemistry. Plasma levels of (**a**) ionized calcium, (**b**) phosphorus, and (**c**) PTH in sham-operated rats ($n=7$), or 5/6 Nx rats treated for 14 days (qod) with vehicle ($n=18$), calcitriol (80 ng kg^{-1}) ($n=20$), paricalcitol (240 ng kg^{-1}) ($n=12$), AMG 641 1.5 mg kg^{-1} ($n=16$), calcitriol + AMG 641 ($n=15$), or paricalcitol + AMG 641 ($n=10$).

(191.1 ± 41.5 pg ml^{-1}, $P = 0.01$) than in sham-operated animals (Figure 2c).

The examination of the plasma biochemical parameters at day 28 was limited by mortality in the vitamin D-treated groups. Plasma creatinine in 5/6 Nx rats treated with vehicle (1.10 ± 0.06 mg per 100 ml), AMG 641(1.19 ± 0.13 mg per 100 ml), and paricalcitol (1.74 ± 0.24 mg per 100 ml) was similar to the values recorded at day 14. However, rats treated with calcitriol, alone (3.19 ± 0.79 mg per 100 ml) or in combination with AMG 641 (2.25 ± 0.59 mg per 100 ml), showed a further increase in creatinine. Plasma PTH concentrations were increased in all treatment groups at day 28 when compared with the values obtained at day 14. Again, lower PTH levels were recorded in rats treated with the combination of AMG 641 plus either calcitriol (165.2 ± 82.3 pg ml^{-1}) or paricalcitol (236.6 ± 90.5 pg ml^{-1}). When used alone, AMG 641 achieved a better control of plasma PTH (433.5 ± 150.1 pg ml^{-1}) than paricalcitol (617.9 ± 142 pg ml^{-1}) or calcitriol (1132.6 ± 947.4 pg ml^{-1}). Calcium and phosphorus values were similar to the values observed at day 14. Since vitamin D-treated rats were underrepresented, the differences between groups were less evident.

Aortic and soft-tissue mineral content

Induction of renal failure and secondary HPT (5/6 Nx group) resulted in a modest and nonsignificant increase in aortic Ca content (3.5 ± 0.3 vs 2.3 ± 0.2 mg g^{-1} of tissue in sham-operated rats). Aortic Ca increased in rats treated with calcitriol (15.5 ± 1.5 mg g^{-1} of tissue, $P < 0.001$ vs 5/6 Nx). Animals treated with AMG 641 had lower ($P < 0.05$) aortic calcium levels (3.0 ± 0.3 mg g^{-1} of tissue) than rats treated with paricalcitol (6.4 ± 1.3 mg g^{-1} of tissue). Addition of AMG 641 decreased aortic calcium in rats treated with vitamin D analogs to 8.7 ± 1.4 mg g^{-1} (calcitriol + AMG 641) and 3.2 ± 0.7 mg g^{-1} (paricalcitol + AMG 641) (Figure 3a). Aortic Ca values recorded at day 28 were similar to those recorded at day 14 (Figure 4a).

Aortic phosphorus content was increased in rats treated with calcitriol (11.5 ± 1.4 mg g^{-1} of tissue, $P < 0.001$), paricalcitol (5.3 ± 1.7 mg g^{-1} of tissue, $P < 0.01$), and the combination calcitriol + AMG 641 (6.6 ± 1.9 mg g^{-1} of tissue, $P < 0.001$) when compared with 5/6 Nx rats that did not receive treatment (0.5 ± 0.1 mg g^{-1}). Treatment with AMG 641 did not increase aortic phosphorus, neither when used alone (0.4 ± 0.1 mg g^{-1}) nor in combination with paricalcitol (0.8 ± 1.7 mg g^{-1}). When compared with day 14, aortic phosphorus values were significantly ($P < 0.05$) increased in all groups at day 28, except in rats treated with AMG 641 (0.2 ± 0.1 mg g^{-1} of tissue) (Figures 3b and 4b).

In situ aortic mineralization was examined by means of the von Kossa staining method (Figure 5). No obvious mineral deposits in the aorta (zero semiquantitative score) were observed in either the sham-operated (not shown), vehicle (Figure 5a), or AMG 641-treated 5/6 Nx groups (Figure 5d). However, marked von Kossa staining (3.8 ± 0.2

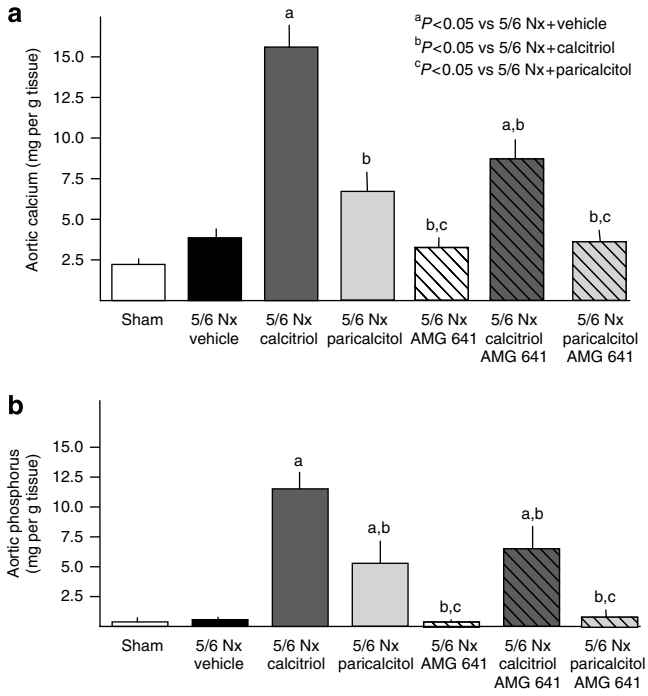

Figure 3 | Aortic mineral content. (**a**) Calcium and (**b**) phosphorus content of the aorta in sham-operated rats ($n = 7$), or 5/6 Nx rats treated for 14 days (qod) with vehicle ($n = 18$), calcitriol (80 ng kg^{-1}) ($n = 20$), paricalcitol (240 ng kg^{-1}) ($n = 12$), AMG 641 1.5 mg kg^{-1} ($n = 16$), calcitriol + AMG 641 ($n = 15$), or paricalcitol + AMG 641 ($n = 10$).

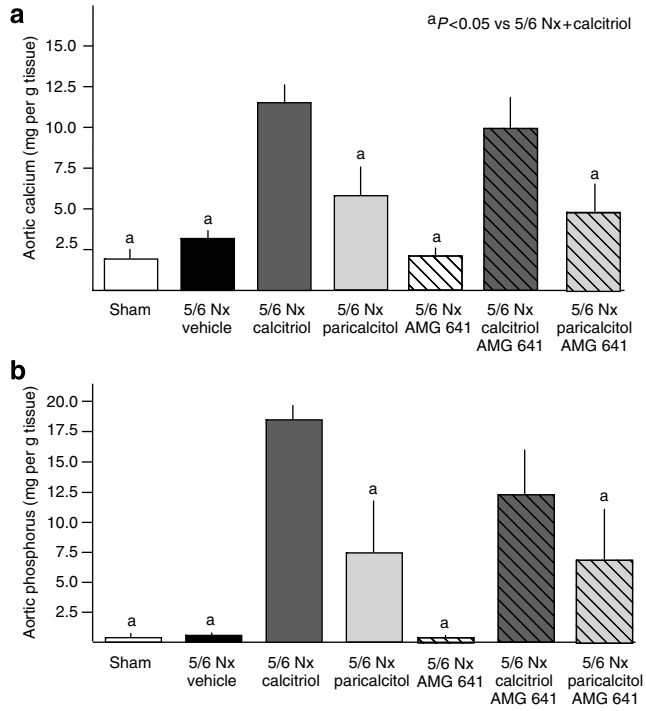

Figure 4 | Aortic mineral content. (**a**) Calcium and (**b**) phosphorus content of the aorta in sham-operated rats ($n = 6$), or 5/6 Nx rats treated for 28 days (qod) with vehicle ($n = 8$), calcitriol (80 ng kg^{-1}) ($n = 10$), paricalcitol (240 ng kg^{-1}) ($n = 9$), AMG 641 1.5 mg kg^{-1} ($n = 7$), calcitriol + AMG 641 ($n = 10$), or paricalcitol + AMG 641 ($n = 10$).

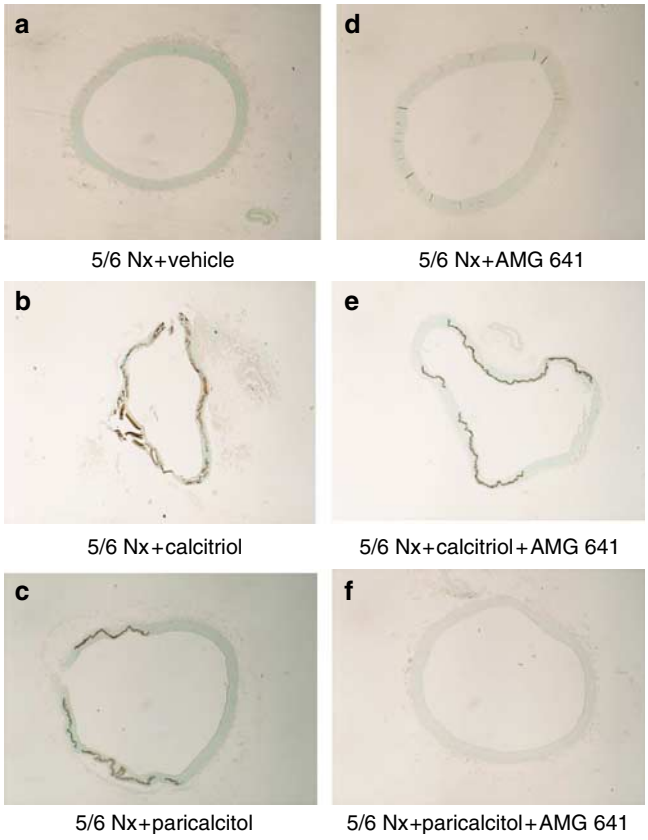

Figure 5 | *In situ* aortic mineralization. Von Kossa stained sections of the aorta in 5/6 Nx rats treated for 14 days (qod) with (**a**) vehicle, (**b**) calcitriol (80 ng kg^{-1}), (**c**) paricalcitol (240 ng kg^{-1}), (**d**) AMG 641 1.5 mg kg^{-1}, (**e**) calcitriol + AMG 641, or (**f**) paricalcitol + AMG 641.

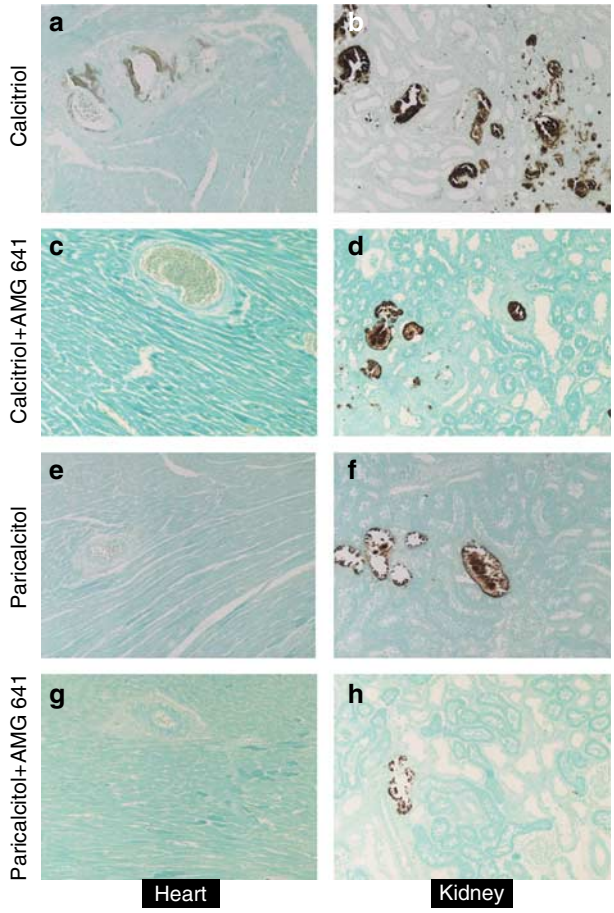

Figure 6 | Von Kossa-stained sections of the heart and kidney from rats treated with calcitriol (80 ng kg^{-1}, qod) showing severe calcification of (**a**) heart arteries and (**b**) renal tubules and glomeruli. Rats treated with paricalcitol alone (240 ng kg^{-1}, qod) did not show (**e**) cardiac calcifications but showed abundant calcium deposits in (**f**) some renal tubules. Addition of AMG 641 (1.5 mg kg^{-1}, qod) significantly decreased calcification in both (**c** and **d**) calcitriol- and (**g** and **h**) paricalcitol-treated rats. Original magnification = × 100.

score) was detected in the aortic media of 5/6 Nx rats treated with calcitriol alone (Figure 5b). Rats treated with paricalcitol showed aortic mineralization to a lesser extent (2.4 ± 0.3 score) compared with calcitriol (Figure 5c). The addition of AMG 641 to the vitamin D treatment regimen reduced mineralizations in calcitriol-treated rats (3 ± 0.4 score) (Figure 5e) and, in rats receiving the combination paricalcitol + AMG 641, prevented (0.4 ± 0.2 score) the development of aortic calcification (Figure 5f).

Calcification was also demonstrated in soft tissues by histology (heart and remnant kidney) and measurement of mineral content (lung and stomach). Severe calcification of heart arteries and renal tubules and glomeruli was observed in rats treated with calcitriol (Figure 6a and b). Rats treated with paricalcitol alone did not show cardiac calcifications but showed abundant calcium deposits in some renal tubules (Figure 6e and f). Addition of AMG 641 significantly decreased calcification in both calcitriol (Figure 6c and d)- and paricalcitol (Figure 6g and h)-treated rats.

Measurements of tissue calcium and phosphorus content in the stomach and in the lung at days 14 and 28 are shown in Tables 1 and 2. Calcitriol-treated rats had a higher calcium and phosphorus content in both stomach and lung than vehicle-treated rats ($P < 0.05$). Although the differences were not statistically significant, rats treated with AMG 641 had lower mineral content in the stomach and the lung. Moreover, in general, addition of AMG 641 tended to reduce the mineral organ content in rats treated with vitamin D derivatives at 14 days.

DISCUSSION

In this study, in uremic rats with severe secondary HPT, the calcimimetic AMG 641 was more effective in reducing serum PTH levels and induced less extraskeletal calcifications than calcitriol or paricalcitol. When used in combination with paricalcitol, AMG 641 provided excellent control of secondary HPT without inducing significant calcifications and prevented mortality associated with the use of vitamin D derivatives.

Cardiovascular disease is the leading cause of mortality in patients receiving dialysis.[23] Management of patients receiving dialysis requires control of secondary HPT and associated

Table 1 | Mineral content (mg per g of tissue) of the stomach and lung after 14 days of treatment in sham-operated (Sham) rats and 5/6 nephrectomized rats (5/6 Nx) treated on alternate days with vehicle (saline solution), calcitriol (80 ng kg^{-1} i.p.), paricalcitol (240 ng kg^{-1} i.p.), the calcimimetic AMG 641 (1.5 mg kg^{-1} s.c.), the combination calcitriol+AMG 641, or paricalcitol+AMG 641

	Stomach		Lung	
	Calcium (mg g^{-1})	Phosphorus (mg g^{-1})	Calcium (mg g^{-1})	Phosphorus (mg g^{-1})
Sham (n=7)	0.17 ± 0.01	0.31 ± 0.05	0.24 ± 0.05	0.29 ± 0.05
5/6 Nx+vehicle (n=18)	0.28 ± 0.05	0.36 ± 0.05	0.31 ± 0.07	0.29 ± 0.03
5/6 Nx+calcitriol (n=20)	1.78 ± 0.34[a]	1.50 ± 0.37[a]	1.45 ± 0.46[a]	1.26 ± 0.36[a]
5/6 Nx+paricalcitol (n=11)	0.70 ± 0.21	0.96 ± 0.38	0.78 ± 0.36	0.63 ± 0.34
5/6 Nx+AMG 641 (n=16)	0.09 ± 0.02	0.25 ± 0.02	0.28 ± 0.11	0.20 ± 0.02
5/6 Nx+calcitriol+AMG 641 (n=15)	1.33 ± 0.26[a]	1.18 ± 0.36[a]	1.14 ± 0.26[a]	0.89 ± 0.34
5/6 Nx+paricalcitol+AMG 641 (n=9)	0.65 ± 0.24	0.42 ± 0.15	0.49 ± 0.18	0.53 ± 0.27

i.p., intraperitoneally; s.c., subcutaneously.
[a]$P<0.05$ vs 5/6 Nx+vehicle.

Table 2 | Mineral content (mg per g of tissue) of the stomach and lung after 28 days of treatment in sham-operated (Sham) rats and 5/6 nephrectomized rats (5/6 Nx) treated on alternate days with vehicle (saline solution), calcitriol (80 ng kg^{-1} i.p.), paricalcitol (240 ng kg^{-1} i.p.), AMG 641 (1.5 mg kg^{-1} s.c.), the combination calcitriol+AMG 641, or paricalcitol+AMG 641

	Stomach		Lung	
	Calcium (mg g^{-1})	Phosphorus (mg g^{-1})	Calcium (mg g^{-1})	Phosphorus (mg g^{-1})
Sham (n=6)	0.14 ± 0.01	0.35 ± 0.05	0.20 ± 0.05	0.27 ± 0.05
5/6 Nx+vehicle (n=8)	0.13 ± 0.02	0.33 ± 0.04	0.19 ± 0.08	0.28 ± 0.06
5/6 Nx+calcitriol (n=10)	2.18 ± 0.33[a]	2.35 ± 0.39[a]	0.63 ± 0.39[a]	0.59 ± 0.16[a]
5/6 Nx+paricalcitol (n=9)	0.87 ± 0.25	0.96 ± 0.20	0.17 ± 0.06	0.14 ± 0.03
5/6 Nx+AMG 641 (n=7)	0.25 ± 0.09	0.39 ± 0.03	0.15 ± 0.04	0.19 ± .0.04
5/6 Nx+calcitriol+AMG 641 (n=10)	0.89 ± 0.35	1.07 ± 0.26	0.12 ± 0.04	0.18 ± 0.04
5/6 Nx+paricalcitol+AMG 641 (n=10)	0.25 ± 0.06	0.54 ± 0.07	0.19 ± 0.02	0.18 ± 0.02

i.p., intraperitoneally; s.c., subcutaneously.
[a]$P<0.05$ vs 5/6 Nx+vehicle.

extraosseous calcification. Vitamin D derivatives, which decrease the serum levels of PTH, are part of the traditional treatment for secondary HPT. However, recent evidence suggests that treatment with the vitamin D derivatives may be associated with the development of VC in patients receiving dialysis,[15,22] and in animal-models of uremia,[24,25] although not all reports agree with this.[26] The calcifying effects of vitamin D may be related to elevations in blood calcium and phosphorus, and it may also have a direct effect on VSMC.[12,14,15] To overcome the side effects of calcitriol on serum calcium and phosphorus, vitamin D derivatives with less reported calcemic actions, like paricalcitol, have been developed.[16,17]

A different approach for treatment of secondary HPT in dialysis patients is the use of calcimimetics, which increase the sensitivity of the calcium-sensing receptor to extracellular calcium, thereby suppressing PTH synthesis[18] and secretion[19] without inducing hypercalcemia.[20,21] In a recent study, we have shown that the calcimimetic R-568 reduced serum PTH and calcium and phosphorus levels without causing aortic and soft-tissue calcification, and attenuated the mineralizing effect of calcitriol on vascular and extravascular tissue.[22]

Currently, there is little clinical evidence supporting a positive effect of a pharmacologic intervention for secondary HPT on patient survival. Based on results of a retrospective analysis of an observational database, it has been suggested that vitamin D may increase patient survival,[27] although additional evidence suggests that this may not be the case.[28] In this trial, rats treated with either calcitriol or paricalcitol demonstrated a significantly greater mortality than those treated with AMG 641. It is difficult to compare human retrospective studies (in which patients may be receiving vitamin D replacement therapy to account for the lack of production of endogenous calcitriol) with this prospective study (in which calcitriol was administered to control secondary HPT). Thus, it would be necessary to conduct human trials to compare the influence of the different treatments for secondary HPT on survival.

The reasons for the higher survival rate in the non-vitamin D-treated rats, although they have not been fully elucidated, may be related to the vitamin D-associated reduction in renal function and increase in VC, or with a calcimimetic-mediated positive effect on blood pressure[29] or renoprotection.[30] When uremic survivors and non-survivors were compared in this study, mineralization was found to be more accentuated in non-survivors, aortic calcium (5.5 ± 0.8 vs 11.5 ± 0.6 mg g^{-1} of tissue, $P<0.001$), aortic phosphorus (6.7 ± 1.7 vs 19.7 ± 1.1 mg g^{-1} of tissue, $P<0.001$), gastric calcium (0.5 ± 01 vs 1.6 ± 0.3 mg g^{-1} of tissue, $P<0.001$), and gastric phosphorus (0.6 ± 0.1 vs 1.8 ± 0.3 mg g^{-1} of tissue,

$P<0.001$). Thus, mineral deposition and its associated cardiovascular consequences seem to play a pivotal role as the cause of death. In addition, a significant decrease in body weight (202 ± 10 vs 251 ± 16 g, $P<0.05$) was also detected in the rats that died. Of interest, in this study, while treatment with a calcimimetic did not improve renal function when used alone, it did abrogate the calcitriol- and paricalcitol-induced increases in serum creatinine. The renoprotective effect of AMG 641 is in agreement with previous studies with calcimimetics R-568 and R-467, which have been shown to attenuate vitamin D- and furosemide-mediated nephrocalcinosis in rats.[22,31]

Moreover, treatment with the calcimimetic reduced the extraosseus calcifications observed with both calcitriol and paricalcitol. Recent evidence has suggested that uremia-associated VC results from a myriad of changes in the expression of both mediators and inhibitors of calcification, and the transformation of the vascular smooth muscle cells to a more osteo/chondrocytic cell type. Of particular interest to the authors is *in vitro* evidence has shown that both paricalcitol and calcitriol induce significant amounts of mineralization in vascular smooth muscle cells, while AMG 641 does not.[15] Thus, vitamin D derivatives seem to have direct procalcific effects beyond their influence on parameters of mineral metabolism. In previous studies, we have shown that the anticalcifying effect of calcimimetics is due, in part, to the reductions in the serum $Ca \times P$ product.[22] This mechanism may also be responsible for the decreased calcification found in this study since all groups treated with AMG 641 had lower Ca and, particularly, lower P. However, other mechanisms may also be implicated since VSMC express calcium-sensing receptor[32] and may directly respond to calcimimetics.

A frequent critique to the *in vivo* studies of vitamin D-induced VC is that the doses of vitamin D derivatives used are very high. In this study, the doses of calcitriol and paricalcitol cannot be considered too high since they did not achieve control of secondary HPT (as demonstrated by the elevated PTH levels in the calcitriol- and paricalcitol-treated rats). In fact, to achieve complete control of secondary HPT using only vitamin D derivatives, higher doses of calcitriol or paricalcitol would have been necessary, potentially resulting in more severe calcifications. However, it should also be noted that the relationship between calcitriol dose and degree of PTH reduction is not perfectly linear. When high doses of calcitriol are administered to rats fed a high phosphorus diet, plasma P may increase to a point in which it is not possible to suppress PTH secretion. Thus, even though it may seem paradoxical, a high dosage of calcitriol can result in an increase in PTH secretion in uremic rats, as demonstrated by recent studies.[25] In addition, very high doses of calcitriol cause massive calcifications,[24,25] which may not prevented by calcimimetic treatment.[24]

In contrast with a previous study in which R-568 was used,[22] the dosing regime of AMG 641 was similar to the vitamin D analogs (every 48 h) thus excluding any influence of the more frequent dosing required with R-568 on the anticalcifying effects of the calcimimetic.

In conclusion, this study shows that, when administered to uremic rats, the calcimimetic AMG 641 effectively reduces PTH without inducing extraskeletal calcifications under conditions in which the vitamin D derivatives did. Calcitriol and paricalcitol are both less effective than AMG 641 in controlling secondary HPT and in avoiding VCs. When used in combination with paricalcitol, AMG 641 provided excellent control of secondary HPT and diminished paricalcitol-mediated VC.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250 g were purchased from the Animal Breeding Facility of the University of Cordoba (Spain). Rats were housed with a 12/12 h light/dark cycle and given *ad libitum* access to normal diet (calcium: 0.9%, phosphorus: 0.6%, vitamin D: 500 IU kg^{-1}). The experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba, and all animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science.

5/6 Nephrectomy

The uremia in the rodent model of CKD used in these studies was induced by 5/6 nephrectomy (5/6 Nx), a two-step procedure that reduces the original functional renal mass by 5/6. In the first step, animals were anesthetized using xylazine (5 mg kg^{-1}, intraperitoneally (i.p.)) and ketamine (80 mg kg^{-1}, i.p.), an 8-mm incision was made on the left medio-lateral surface of the abdomen, and the left kidney was exposed. The two renal poles were tightly ligated and ablated, thus leaving 1/3 of the original renal mass. After 1 week of recovery, the animal was reanesthetized and an 8-mm incision was made on the right medio-lateral surface of the abdomen. The right kidney was exposed and unencapsulated, the renal pedicle clamped and ligated, and the kidney was removed. Sham-operated animals underwent the same procedures without renal manipulation.

Experimental design

The experimental schedule is shown in Figure 7. After the second surgery, the diet was changed to lower calcium (0.6%) and increased phosphorus (1.2%) content. The rats were randomly assigned (based on the normal distribution of baseline body weights) into seven experimental groups: sham-operated ($n=13$) (used as a control), 5/6 Nx + vehicle (saline) ($n=26$), 5/6 Nx + calcitriol 80 ng kg^{-1} i.p. (Calcijex, Abbot, Madrid, Spain) ($n=31$), 5/6 Nx + paricalcitol 240 ng kg^{-1} i.p. (Zemplar, Abbot, Madrid, Spain) ($n=22$), 5/6 Nx + AMG 641 1.5 mg kg^{-1} subcutaneously (Amgen, Thousand Oaks, CA, USA) ($n=23$), 5/6 Nx + combination calcitriol 80 ng kg^{-1} and AMG 641 1.5 mg kg^{-1} ($n=25$) dosed as above, or 5/6 Nx + combination paricalcitol 240 ng kg^{-1} and AMG 641 1.5 mg kg^{-1} ($n=20$) dosed as above. All treatments were administered every 48 h. Preliminary experiments with AMG 641 in rats indicated that the drug maintained its pharmacological effect through a 48-h period when administered either i.p. or subcutaneously (data not shown). The subcutaneous route was chosen to avoid any potential drug interference between AMG 641 and

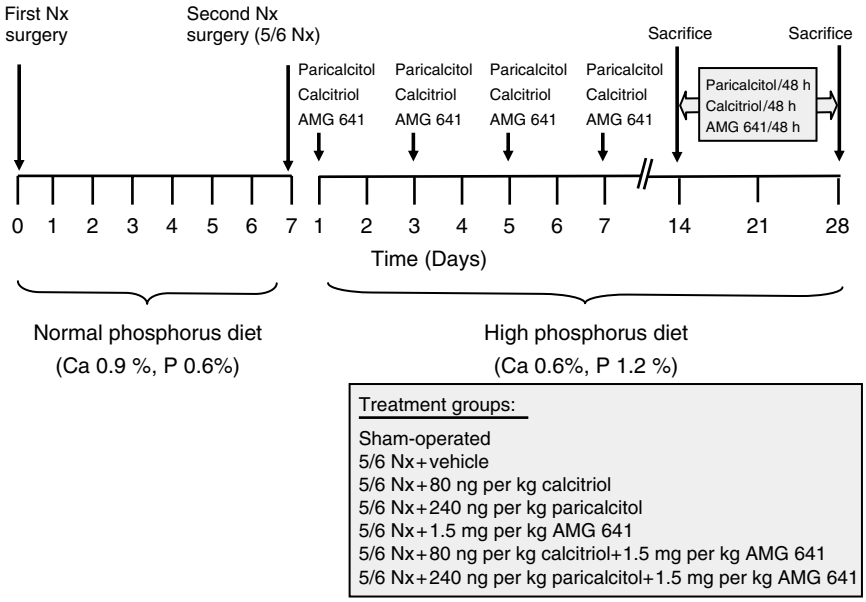

Figure 7 | Experimental design.

vitamin D analogs (administered i.p.) in rats receiving both drugs. At day 14, 2/3 of the animals were killed, sham-operated rats ($n=7$), 5/6 Nx + vehicle ($n=18$), 5/6 Nx + calcitriol ($n=20$), 5/6 Nx + paricalcitol ($n=12$), 5/6 Nx + AMG 641 ($n=16$), 5/6 Nx + calcitriol + AMG 641 ($n=15$), and 5/6 Nx + paricalcitol + AMG 641 ($n=10$). Treatments were maintained for the remaining animals for 28 days. At day 28, all surviving rats were killed by aortic puncture and exsanguination under general anesthesia (i.p. sodium thiopental). Euthanasia was performed 24 h after receiving the last dose of study drugs. Following killing, the thoracic aorta, the heart, the lungs, the stomach, and the remnant kidney were dissected.

In the 28-day experiments, some rats died before the completion of the study. Deaths were recorded to assess the difference in survival rate between treatments. In dead rats, blood samples could not be obtained; however, the aortas and organs from these rats were retrieved shortly after death and were used for assessment of calcification.

Assessment of vascular and soft-tissue calcification

Vascular and soft-tissue calcification was studied by histology and by measuring the tissue calcium and phosphorus content. To avoid sampling bias, the same section of the aorta from every animal was selected for each study: cranial third for histology and middle third for calcium and phosphorus quantification. The cranial part of the thoracic aorta, the heart, and the remnant kidney were fixed in 10% buffered formalin and subsequently sectioned and stained for mineralization by the von Kossa method. The extent of calcification (von Kossa staining) was blindly evaluated by three independent observers using a semiquantitave score (range 0–4). The middle part of the aorta was demineralized in 10% formic acid, and the arterial tissue calcium and phosphorous content was measured in the supernatant according to the method described by Price *et al.*[33] Based on the results of a previous study in which significant gastric and pulmonary calcifications were found in this model of uremia,[22] the stomach and the lung were chosen for quantification of tissue mineral content. The stomach and the left lung from each animal were placed into separate 30 ml tubes. Twenty milliliter of 150 mM HCl was added to each tube. The tubes were mixed by inversion for 24 h at room temperature and calcium and phosphorus were measured in the acid extract.[34]

Blood chemistries

Blood for chemistry analyses was obtained from the abdominal aorta at the time of killing. Blood for measurements of ionized calcium levels was collected in heparinized syringes and immediately analyzed using a Ciba-Corning 634 ISE Ca^{2+}/pH Analyzer (Ciba-Corning, Essex, UK). Afterward, plasma was separated by centrifugation and stored at $-20^{\circ}C$ until assayed. PTH levels were quantified according to the vendor's instructions using a rat $PTH_{(1-34)}$ immunoradiometric assay kit (Immunotopics, San Clemente, CA, USA). Plasma creatinine, phosphorous, and total calcium were measured by spectrophotometry (Sigma Diagnostics, St Louis, MO, USA).

Statistics

Values are expressed as the mean ± s.e. The difference between means for two different groups was determined by *t*-test; the difference between means for three or more groups was assessed by analysis of variance followed by an least significant difference *post hoc* test. $P<0.05$ was considered significant. Survival was studied by Kaplan–Meier curves followed by log-rank tests.

DISCLOSURE

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