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Antitumor effect of 22-oxa-1α,25-dihydroxyvitamin D_3, a potent angiogenesis inhibitor, on rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene

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The effect of 22-oxa-1α,25-dihydroxyvitamin D_3 (22-oxa-1,25$(OH)_2D_3$) on the growth of autochthonous rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) was examined on the basis of our previous finding that this synthetic vitamin D_3 analog has a potent angiogenesis inhibitory effect. Two doses of 22-oxa-1,25$(OH)_2D_3$, 0.1 and 1 μg/kg of body weight, due to the limited amount of the compound available, were used. The daily administration of 22-oxa-1,25$(OH)_2D_3$ at the dose of 1 μg/kg/day resulted in significant inhibition of the growth of these mammary tumors at 1, 2 and 3 weeks after the administration of this agent, although the agent caused little or no regression of the tumors. After daily administration for 3 weeks, a significant antitumor effect was also observed in the group treated with 0.1 μg/kg/day. Treatment with 22-oxa-1,25$(OH)_2D_3$ did not affect the serum calcium levels in the treated rats. The lower dose of 22-oxa-1,25$(OH)_2D_3$ neither affected weight gain nor caused a decrease in body weight, while the higher dose, although having some effect on weight gain, did not induce a decrease in body weight. There were no significant differences in the weights of adrenals, uteri and ovaries between the treated groups and controls. These results suggest that 22-oxa-1,25$(OH)_2D_3$ has a significant growth inhibitory effect on DMBA-induced autochthonous mammary tumors in rats, without producing severe side effects, including hypercalcemic activity. Additionally, it might be possible that the antitumor effect of 22-oxa-1,25$(OH)_2D_3$ is due, in part, to the antiangiogenic activity of this agent, since tumor growth depends on an angiogenic response by the host tissues and since the agent exhibits remarkable antiangiogenic activity.

Key words: Angiogenesis inhibitor, antitumor effect, mammary tumor, 22-oxa-1α,25-dihydroxyvitamin D_3.

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Introduction

It is widely accepted that solid tumors induce an angiogenic response by the host blood vessels to form a new vascular network for the supply of fresh nutrients and oxygen and for the elimination of metabolic waste, which are indispensable for progressive growth. This might be supported by the evidence that almost all, if not all, tumors exhibit angiogenic activity in an *in vivo* assay system involving rabbit cornea or the chorioallantoic membrane (CAM) of chick embryo, and that tumors, when implanted into organs maintained by isolated perfusion in glass chambers, do not grow beyond a mass of 2–3 mm in diameter.[1,2] Thus it is reasonable to assume that inhibition of angiogenesis results in the suppression of tumor growth. Treatment involving an angiogenesis inhibitor would be a new strategy for cancer therapy.

Studies on angiogenesis inhibitors have become a focus of increasing attention. Several steroids, when co-administered with exogenous heparin, have been found to inhibit angiogenic activity, as determined with bioassay systems involving rabbit cornea or the CAM of chick embryo.[3–5] Some of these angiostatic steroids have been shown to exert a growth-inhibitory effect on experimental tumors.[3,5] Recent studies showed that several substances exhibit antiangiogenic activity, including angiostatic antibiotics, angiostatic vitamins, a sulfated polysaccharide–peptidoglycan complex and a synthetic laminin peptide.[6–11] On the other hand, we have shown that medroxyprogesterone acetate

(MPA) alone remarkably suppresses the growth of rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA), probably by inhibiting angiogenic activity triggered by these tumors.[12] MPA also inhibited embryonic angiogenesis in the CAM assay system.[6]

Differing from quiescent vascular endothelial cells, angiogenic endothelial cells are thought to migrate and proliferate, and to subsequently differentiate into vessel-forming quiescent phenotypes. We have hypothesized that agents, which modulate the differentiation activity of angiogenic endothelial cells, could affect angiogenic responses which are triggered by a variety of stimulants, including tumor-derived factors or which occur in the process of embryogenic development. To verify this hypothesis, we firstly examined the antiangiogenic activity of several natural and synthetic retinoids, because these substances are well known to exhibit differentiation-modifying activity toward several cell types. All four retinoids tested have been found to induce an antiangiogenic action.[8] The order of the potency of antiangiogenic activity of the retinoids well agreed with the order of their abilities to cause cell differentiation in *in vitro* systems. Additional evidence for our hypothesis has been provided by experiments involving vitamin D_3 analogs which, like retinoids, induce the differentiation of several transformed cell lines into normal phenotypes. Similar to the results obtained using retinoids, the order of the antiangiogenic activity of vitamin D_3 analogs tested shows good agreement with the order of their abilities to induce the differentiation of several cultured cells, including that of human acute promyelocyte leukemia cells HL-60; 22-oxa-1α,25-dihydroxyvitamin D_3 (22-oxa-1,25$(OH)_2D_3$) exhibits a stronger antiangiogenic effect than 1,25$(OH)_2D_3$. The aim of the present study is to determine whether or not 22-oxa-1,25$(OH)_2D_3$ suppresses the growth of DMBA-induced autochthonous mammary tumors in rats. This was found to have a significant antitumor effect on such a mammary tumor, without inducing hypercalcemia, a critical side effect of the active forms of vitamin D_3, including 1,25$(OH)_2D_3$. The present results strongly suggest that 22-oxa-1,25$(OH)_2D_3$ is a promising agent which induces antitumor activity through the inhibition of angiogenic activity.

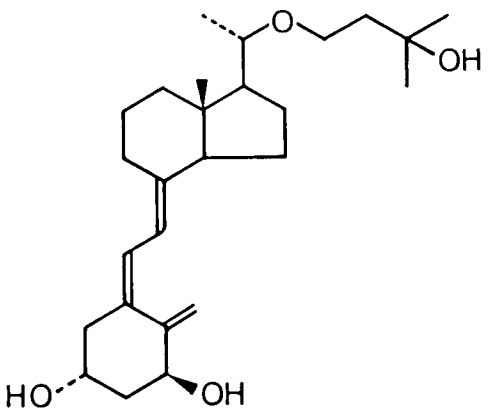

Figure 1. Structure of 22-oxa-1,25$(OH)_2D_3$.

Materials and methods

Chemicals

22-Oxa-1,25$(OH)_2D_3$ was generously provided by Chugai Pharmaceuticals (Tokyo, Japan). Its structure is shown in Figure 1. DMBA was obtained from Wako Chemicals (Tokyo, Japan).

Treatment of mammary tumor-bearing rats with 22-oxa-1,25$(OH)_2D_3$

Mammary tumors were induced in female Sprague-Dawley (SD) rats (Charles River Japan Inc., Kanagawa, Japan) at 8 weeks of age by intragastric administration of DMBA (100 mg/kg), as described previously.[12,13] After 8–12 weeks, rats bearing mammary tumors of about 10 mm diameter were divided into three groups and given intramuscular injections of 22-oxa-1,25$(OH)_2D_3$ (0.1 or 1 μg/kg) or the vehicle (sesame oil) once a day for 21 consecutive days. The tumor size and body weight were measured before treatment and weekly thereafter. The stock solution of 22-oxa-1,25$(OH)_2D_3$ was dissolved in absolute ethanol (1 mg/ml) and kept at −20°C. Immediately before use, the stock solution was diluted with sesame oil.

The tumor size (mm^2) was defined as the product of the widest diameter and the greatest diameter perpendicular to it, and was expressed as the percentage of the initial size, i.e. on day 0.

At the end of the experiment (3 weeks), the animals were killed, and their adrenals, uteri and ovaries were removed and weighed. At the same time, the serum calcium level in treated rats was determined by the method of Connerty and Briggs.[14]

Statistical analyses

Data as to antitumor experiments and body weight were analysed by means of the Mann–Whitney *U*-test. The results of other assays were analysed by means of Student's *t*-test.

Results

Effect of 22-oxa-1,25(OH)$_2$D$_3$ on autochthonous mammary tumor growth in rats

22-Oxa-1,25(OH)$_2$D$_3$, a synthetic analog of vitamin D$_3$, is a potent angiogenesis inhibitor. Experiments were therefore conducted to determine whether or not this angiostatic vitamin affects the tumor growth in the DMBA-induced autochthonous rat mammary tumor model system. Rats bearing mammary tumors received consecutive injections of two different doses of 22-oxa-1,25(OH)$_2$D$_3$ or the vehicle (sesame oil) for 3 weeks. The results are shown in Figure 2. The values for the tumor size (mean ± SE; $n = 17$) of the control group treated with the vehicle were 131 ± 7, 145 ± 13 and 174 ± 16% at 1, 2 and 3 weeks, respectively, of the control size at the start of the experiment (day 0).

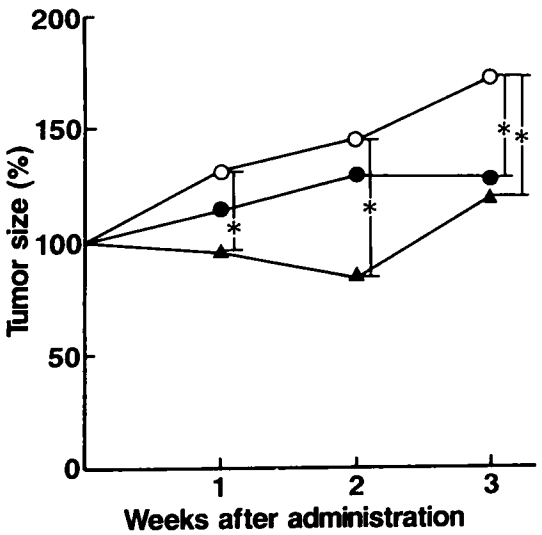

Figure 2. Effect of 22-oxa-1,25(OH)$_2$D$_3$ on autochthonous rat mammary tumors induced by DMBA. Values represent means for groups treated with 22-oxa-1,25(OH)$_2$D$_3$: the numbers of tumors used were 17, 19 and 19 for the doses of 0 (○), 0.1 (●) and 1 (▲) μg/kg 22-oxa-1,25(OH)$_2$D$_3$, respectively. *Significant differences between groups ($p < 0.05$).

The values for the tumor size (mean ± SE; $n = 19$) of the group treated with 0.1 μg/kg of 22-oxa-1,25(OH)$_2$D$_3$ were 115 ± 4, 130 ± 6 and 128 ± 6% at 1, 2 and 3 weeks, respectively, of the initial size on day 0. At this dose, 22-oxa-1,25(OH)$_2$D$_3$ had significantly inhibited the growth of the rat mammary tumors after 3 weeks of consecutive administration ($p < 0.05$). More effective inhibition was observed in the group treated with the dose of 1 μg/kg/day. After consecutive administration for 1, 2, and 3 weeks, 22-oxa-1,25(OH)$_2$D$_3$ showed a significant antitumor effect on the mammary tumors ($p < 0.05$); significant growth inhibition was found, although little or no regression was induced. The values for the tumor size ($n = 19$) were 74, 59 and 69% at 1, 2 and 3 weeks, respectively, of the respective controls.

Effect of 22-oxa-1,25(OH)$_2$D$_3$ treatment on the serum calcium level

Active metabolites of vitamin D$_3$, including 1,25(OH)$_2$D$_3$, have been shown to induce severe hypercalcemia in treated animals. Thus, experiments were performed to determine whether or not such activity was also induced by 22-oxa-1,25(OH)$_2$D$_3$. There were no significant differences in the serum calcium level between the control and 1,25(OH)$_2$D$_3$-treated groups, suggesting that 22-oxa-1,25(OH)$_2$D$_3$, unlike other active vitamin D$_3$ derivatives such as 1,25(OH)$_2$D$_3$ does not exhibit hypercalcemia-inducing activity (Table 1).

Effect of 22-oxa-1,25(OH)$_2$D$_3$ treatment on body and organ weights

A decrease in body weight was not observed in the two treated groups compared to the initial body weight on day 0, although weight gain was slightly retarded in the groups treated with 1 μg/kg of 22-oxa-1,25(OH)$_2$D$_3$ for 2 or 3 weeks. The agent,

Table 1. Serum calcium levels in 22-oxa-1,25(OH)$_2$D$_3$-treated rats

Dose (μg/kg)	Serum calcium levels (mg/dl)
0	10.96 ± 0.64[a]
0.1	11.76 ± 0.78
1	10.62 ± 0.38

[a] Values are the means ± SE for six animals.

at the dose of 0.1 μg/kg, neither caused a body weight decrease nor affected weight gain (Figure 3).

The ovaries, uteri and adrenals of the control and treated groups were also weighed at the end of the experiment to determine the toxicity of 22-oxa-1,25$(OH)_2D_3$. No significant differences were found in the weights of the ovaries, uterus and adrenals between the control and 22-oxa-1,25$(OH)_2D_3$-treated groups (Table 2).

Table 2. Wet weights of organs of 22-oxa-1,25$(OH)_2D_3$-treated rats

Organs	22-Oxa-1,25$(OH)_2D_3$-treated groups (μg/kg)		
	0	0.1	1
Uterus (mg)	464.0 ± 49.6[a]	535.0 ± 80.1	427.0 ± 36.4
Ovary (mg)	112.0 ± 6.7	106.5 ± 9.1	103.3 ± 2.5
Adrenal (mg)	73.2 ± 3.1	67.3 ± 2.7	71.3 ± 1.8

[a] Values are the means ± SE for six animals.

Discussion

The progressive growth of solid tumors depends on an angiogenic response by the host blood tissues. It is, therefore, expected that angiogenesis inhibition would be a novel strategy for cancer treatment. Our previous study showed that the antitumor effect of MPA, a synthetic derivative of progesterone, on DMBA-induced autochthonous rat mammary tumors was probably due to the inhibitory activity of the agent toward angiogenesis triggered by these tumors.[12] We also found that 22-oxa-1,25$(OH)_2D_3$, an analog of 1,25$(OH)_2D_3$, exhibited more potent angiogenesis-inhibitory activity than 1,25$(OH)_2D_3$ in the CAM assay system and exerted antiangiogenic activity comparable to that of Ch 55, a synthetic retinoid, which is the most effective angiogenesis inhibitor identified so far.[8,9] Based on these findings, the present study was performed to determine whether or not 22-oxa-1,25$(OH)_2D_3$ shows antitumor activity toward DMBA-induced autochthonous mammary tumors in rats. Treatment with two doses (0.1 and 1 μg/kg) of 22-oxa-1,25$(OH)_2D_3$ produced, although it did not induce tumor regression, significant suppression of the tumor growth. Additionally, the higher dose caused an interesting phenomenon: the tumor size throughout the treatment remained roughly constant and approximately similar to the initial size before treatment. It is conceivable that the primary action of an angiogenesis inhibitor is on any step(s) of the angiogenic process to prevent new vascular network formation, resulting in the suppression of tumor growth, and not to influence the pre-existing vascular network in the tumor tissues and thereby induce tumor regression. The inhibitory effect of 22-oxa-1,25$(OH)_2D_3$ on the growth of a DMBA-induced rat mammary tumor observed in this study appears to be along these lines. Thus, the present results indicate the possibility that 22-oxa-1,25$(OH)_2D_3$ exerts its antitumor activity toward autochthonous mammary tumors by inhibiting angiogenic activity induced by these tumors. Abe *et al.* have shown the growth inhibition, but not the regression, by 22-oxa-1,25$(OH)_2D_3$ of a transplantable human breast carcinoma implanted into athymic mice.[15] Since 22-oxa-1,25$(OH)_2D_3$, like 1,25$(OH)_2D_3$, exhibits differentiation activity or growth-inhibitory activity toward several tumor cell lines,[16,17] it is also possible that these effects might be involved in the antitumor effect of the agent on DMBA-induced rat mammary tumors. By contrast, some angiogenesis inhibitors such as cortisone (in the presence of exogenous heparin) and MPA alone exhibited tumor-regressive activity. Thus, it would be interesting to determine whether 22-oxa-1,25$(OH)_2D_3$, at higher doses than those used in this study, induces the regression of a DMBA-induced rat mammary tumor, or whether the agent exhibits a cytotoxic effect on mammary tumor cells or affects the differentiation of angiogenic endothelial cells.

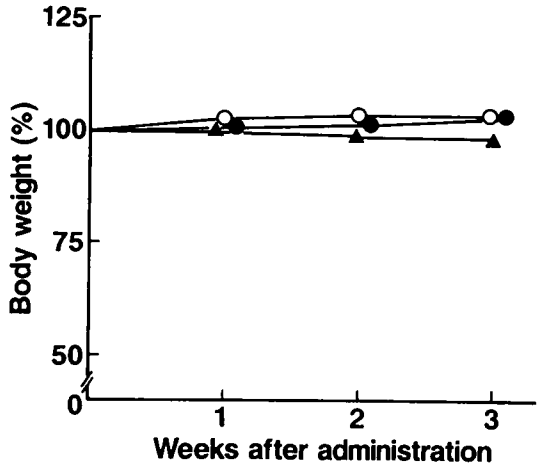

Figure 3. Effect of 22-oxa-1,25$(OH)_2D_3$ treatment on weight gain. Values represent means for six rats with SE of 0.01–0.03 for all values. ○, Vehicle only; ●, 0.1 μg/kg 22-oxa-1,25$(OH)_2D_3$; ▲, 1 μg/kg 22-oxa-1,25$(OH)_2D_3$.

The active forms and derivatives of vitamin D_3, such as $1,25(OH)_2D_3$, and its hydroxylated or fluorinated analogs, have been assessed as to their effects on the growth or the development of solid tumors as well as non-solid tumors *in vivo* on the basis of previous observation that these active vitamin D_3 derivatives affected the proliferation of several tumor cell lines *in vitro*.[18–20] Treatment with these agents, however, produced significant or severe hypercalcemia in the treated animals, with or without the induction of antitumor activity. This critical side effect is a limiting factor for application of these active analogs to the treatment of cancers. Therefore, it has been demanded that a novel, effective analog with little or no hypercalcemic activity be developed or that a new treatment system in which an increase in serum calcium level does not occur at all or is within acceptable limits be established. Eisman *et al.* found that $1,25(OH)_2D_3$ at a high dose (0.1 μg/mouse) induced severe hypercalcemia in mice maintained on a normal laboratory diet but did not cause unacceptable hypercalcemic activity in mice maintained on a low calcium diet.[19] On the basis of this observation they showed that $1,25(OH)_2D_3$ exhibited a growth-inhibitory effect or a regressive effect on some solid tumors in mice maintained on a low calcium diet. This suppressive effect was limited to xenografts derived from human cancer cells having a detectable level of $1,25(OH)_2D_3$ receptor. In this study we demonstrated that 22-oxa-$1,25(OH)_2D_3$ significantly suppresses the growth of DMBA-induced mammary tumors without the production of critical hypercalcemia in rats maintained on a normal laboratory diet. In addition, this agent seemed not to induce significant toxicity in the treated rats, as judged from data concerning the weights of their uteri, ovaries and adrenals, as well as their body weights, although further study is necessary to determine whether or not there are other side effects, including the weight of the lower limb bone or kidneys, the calcium content of kidneys, and the contents of host cells such as erythrocytes, neutrophils and platelets. It is, therefore, highly likely that 22-oxa-$1,25(OH)_2D_3$ is a highly promising antitumor agent for treating human cancers.

The exact mechanism of the antitumor activity of 22-oxa-$1,25(OH)_2D_3$ toward a DMBA-induced rat mammary tumor remains to be determined. The biological activities of $1,25(OH)_2D_3$, including its differentiation-inducing activity toward HL-60 cells, are believed to be mediated by a mechanism involving a receptor. Thus, it appears that a vitamin D_3 analog having higher binding affinity for the receptor exhibits more potent biological activity than one having lower affinity for it. However, although 22-oxa-$1,25(OH)_2D_3$ shows lower binding affinity for the $1,25(OH)_2D_3$ receptor than $1,25(OH)_2D_3$, the former exhibits greater activity than the latter in inducing antiangiogenic activity in the CAM assay system,[9] as well as the differentiation of HL-60 cells in *in vitro*[16] and *in vivo* immunoregulating activity.[17] Therefore, it is reasonable that the antitumor activity of $1,25(OH)_2D_3$ in the *in vivo* model system employed in this study cannot be explained only by a receptor-mediated mechanism. The stability, cellular uptake and intracellular metabolism of vitamin D_3 compounds have been suggested to be factors other than the binding affinity for the receptor.[16,17]

Until now, most angiogenesis inhibitors have been examined as to their antitumor activity in experimental systems involving a transplantable tumor. Additionally, in most of the experiments the angiogenesis inhibitor was injected into the animals immediately after implantation of the tumors or when the implanted tumors were not yet fully established, and their antitumor effects were assessed. Accordingly, if there is a positive antitumor effect, careful interpretation is required, because its effect might include an inhibitory effect on the anchoring of the tumor implanted into the host tissues. For the first time, to our best knowledge, we demonstrated the antitumor effect of MPA on DMBA-induced autochthonous mammary tumors in rats, in which the tumor size diameter was at least 10 mm. In this study we obtained additional evidence by using 22-oxa-$1,25(OH)_2D_3$, a potent angiostatic vitamin. Thus, the DMBA-induced mammary tumor is supposed to be a useful model system in which to assess the antitumor effects of certain angiogenesis inhibitor(s).

Conclusion

22-Oxa-$1,25(OH)_2D_3$, a potent angiogenesis inhibitor, exhibited significant antitumor activity in an *in-vivo* model system involving a DMBA-induced autochthonous mammary tumor in rats. The agent at the doses used in this study did not induce unacceptable toxicity, including hypercalcemia. These results strongly suggest that 22-oxa-$1,25(OH)_2D_3$ is a promising antitumor agent which exerts its effect by inhibiting tumor angiogenesis.

References

1. Gullino PM. Angiogenesis factor(s). In: Baserga R, ed. *Handbook of Experimental Pharmacology*, Vol. 57. Berlin: Springer-Verlag 1981: 427–49.
2. Folkman J. Tumor angiogenesis. *Adv Cancer Res* 1985; **43**: 175–203.
3. Folkman J, Langer R, Linhardt RJ, *et al.* Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* 1983; **221**: 719–25.
4. Crum R, Szabo S, Folkman J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* 1985; **230**: 1375–8.
5. Sakamoto N, Tanaka NG, Tohgo A, *et al.* Inhibitory effects of heparin plus cortisone acetate on endothelial cell growth both in cultures and in tumor masses. *J Natl Cancer Inst* 1987; **78**: 581–6.
6. Oikawa T, Hirotani K, Shimamura M, *et al.* Powerful antiangiogenic activity of herbimycin A (named angiostatic antibiotic). *J Antibiotics* 1989; **42**: 1202–4.
7. Ingber D, Fujita T, Kishimoto S, *et al.* Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. *Nature* 1990; **348**: 555–7.
8. Oikawa T, Hirotani K, Nakamura O, *et al.* A highly potent antiangiogenic activity of retinoids. *Cancer Lett* 1989; **48**: 157–62.
9. Oikawa T, Hirotani K, Ogasawara H, *et al.* Inhibition of angiogenesis by vitamin D_3 analogues. *Eur J Pharmacol* 1990; **178**: 247–50.
10. Tanaka NG, Sakamoto N, Inoue K, *et al.* Antitumor effects of an antiangiogenic polysaccharide from an *Arthrobacter* species with or without a steroid. *Cancer Res* 1989; **49**: 6727–30.
11. Sakamoto N, Iwahana M, Tanaka NG, Osada Y. Inhibition of angiogenesis and tumor growth by a synthetic laminin peptide, CDPGYIGSR-NH_2. *Cancer Res* 1991; **51**: 903–6.
12. Oikawa T, Hiragun A, Yoshida Y, *et al.* Angiogenic activity of rat mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene and its inhibition by medroxyprogesterone acetate: possible involvement of antiangiogenic action of medroxyprogesterone acetate in its tumor growth inhibition. *Cancer Lett* 1988; **43**: 85–92.
13. Tominaga T, Yoshida Y, Shimozuma K, *et al.* Effect of CGS 16949A plus tamoxifen on induced mammary tumours in rats. *Eur J Cancer* 1990; **26**: 600–3.
14. Connerty HV, Briggs AR. Determination of serum calcium by means of orthocresolphthalein complexone. *Am J Clin Pathol* 1966; **45**: 290–6.
15. Abe J, Nakano T, Nishii Y, *et al.* A novel vitamin D_3 analogue, 22-oxa-1,25-dihydroxyvitamin D_3, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology* 1991; **129**: 832–7.
16. Abe J, Morikawa M, Miyamoto K, *et al.* Synthetic analogues of vitamin D_3 with an oxygen atom in the side chain skeleton. *FEBS Lett* 1987; **226**: 58–62.
17. Abe J, Takita Y, Nakano T, *et al.* A synthetic analogue of vitamin D_3, 22-oxa-1α,25-dihydroxyvitamin D_3, is a potent modulator of *in vivo* immunoregulating activity without inducing hypercalcemia in mice. *Endocrinology* 1989; **124**: 2645–7.
18. Honma Y, Hozumi M, Abe E, *et al.* 1α,25-Dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 prolong survival time of mice inoculated with myeloid leukemia cells. *Proc Natl Acad Sci USA* 1983; **80**: 201–4.
19. Eisman JA, Barkla DH, Tuton PJ. Suppression of *in vivo* growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D_3. *Cancer Res* 1987; **47**: 21–5.
20. Zhou J-Y, Norman AW, Chen D-L, *et al.* 1,25-Dihydroxy-16-ene-23-yne-vitamin D_3 prolongs survival time of leukemic mice. *Proc Natl Acad Sci USA* 1990; **87**: 3929–32.

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