# Inhibitory Effect of 22-Oxa-1,25-Dihydroxyvitamin $D_3$, Maxacalcitol, on the Proliferation of Pancreatic Cancer Cell Lines

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**Abstract**

Effective chemotherapy for pancreatic cancer is urgently needed. The aim of this study was to compare the anti-proliferative activity on pancreatic cancer cell lines of the vitamin $D_3$ analog, 22-oxa-1,25-dihydroxyvitamin $D_3$, maxacalcitol, with that of 1,25-dihydroxyvitamin $D_3$, calcitriol, with analysis of vitamin D receptor status and the $G_1$-phase cell cycle-regulating factors. Antiproliferative effects of both agents were compared using the 3-(4,5-dimethyl thiazol-2-yl)--2,5-diphenyltetrazolium bromide method and by measuring the tumor size of xenografts inoculated into athymic mice. Scatchard analysis of vitamin D receptor contents, and mutational analysis of receptor complementary DNA were performed. Levels of expression of cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors, p21 and p27, were analysed by western blotting. *In vitro*, maxacalcitol and calcitriol markedly inhibited the proliferation and caused a $G_1$ phase cell cycle arrest with the appearance of numerous domes. *In vivo*, maxacalcitol inhibited the growth of BxPC-3 xenografts more significantly than calcitriol, without inducing hypercalcemia. Responsive cells had abundant functional vitamin D receptors. However, Hs 766T, showing no response to either agent, had the second highest receptor contents with no abnormalities in its primary structure deduced by receptor complementary DNA. In the responsive cells, p21 and p27 were markedly up-regulated after 24 hours of treatment with both agents. In non-responsive cells, no such changes were observed. In conclusion, maxacalcitol and calcitriol up-regulate p21 and p27 as an early event, which in turn could block the $G_1$/S transition and induce growth inhibition in responsive cells, and maxacalcitol may provide a more useful tool for the chemotherapy of pancreatic cancer than calcitriol because of its low toxicity.

**Key Words:** maxacalcitol, 22-oxa-1,25-dihydroxyvitamin $D_3$, calcitriol, 1,25-dihydroxyvitamin $D_3$, pancreatic cancer.

## 1. Introduction

Pancreatic cancer is the fifth leading cause of cancer death in Japan. The number of deaths caused by pancreatic cancer has recently been increasing in Japan, likely because of a shift to a more western lifestyle and diet, and to progress in diagnostic procedures identifying more patients. Similarly, in the United States, pancreatic cancer is the fourth leading cause of cancer death, and has the worst 1- and 5-year prognoses of any cancer. The major reason for its poor prognosis is the difficulty of early diagnosis and the lack of effective therapeutic agents for advanced stage cancer. Until 1997, the key drug for pancreatic cancer treatment had been 5-FU. This changed to gemcitabine after its efficacy was reported [1], and various combinations with gemcitabine have been tried since then to improve its effect. Despite the many cytotoxic therapeutic agents that have been combined with gemcitabine however, there has been no definitive increase in survival with any of these regimens compared with single-agent gemcitabine. Accordingly, it is urgent to search for a new drug other than a cytotoxic one to support gemcitabine's effect on pancreatic cancer. 1,25-dihydroxyvitamin $D_3$, calcitriol, an active form of vitamin $D_3$, has been reported to induce differentiation and inhibit the proliferation of various types of cancer cells in addition to its well-known biological activities in enhancing intestinal calcium transport and bone and mineral mobilization [2-5]. However, clinical application of this compound is limited because of its hypercalcemia-inducing activity. On the other hand, 22-oxa-1,25-dihydroxyvitamin $D_3$, maxacalcitol, had been synthesized to reduce the hypercalcemia-inducing activity and enhance its differentiation-inducing and antiproliferative effects [6]. Based on these premises, we sought to clarify the growth inhibitory effects of maxacalcitol on pancreatic cancer cell lines, compared with calcitriol. In addition, we investigated the vitamin D receptor status and cell cycle analysis to determine the mechanisms of growth inhibitory effects of these vitamin D analogs.

## 2. Inhibitory effect *in vitro*

We used nine human pancreatic cancer cell lines obtained from ATCC or established in our laboratory. Maxacalcitol was kindly provided by Chugai Pharmaceuticals, and calcitriol was purchased from Philip Duphar. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for *in vitro* growth inhibition experiments.

Marked dose-dependent antiproliferative effects of both agents were observed in three of the nine cell lines: Hs 700T, BxPC-3 and SUP-1. Weak suppression was observed in two cell lines: Capan-2 and AsPC-1. No effect was found in the remaining four cell lines at any concentration

tested [7]. At the concentration of $1x10^{-7}$ M, proliferation of responsive cells was suppressed to about 10-40% of the control, and the effect of maxacalcitol was comparable to that of calcitriol (Table 1).

Flow cytometry showed that a significant increase in the $G_1$ population and decreases in S and G2/M populations were seen in BxPC-3 cells after maxacalcitol treatment and no changes were seen in non-responsive Hs 766T cells, indicating that this agent induced the blockage of $G_1$/S transition or $G_1$ arrest. A characteristic morphological change, the appearance of numerous domes, was observed in maxacalcitol-treated cells, while few such domes were seen in non-treated cells [7]. Calcitriol had the same effect and the same results were obtained in Hs700T cells. No dome formation was observed in the remaining highly responsive cell line SUP-1. In other poorly or non-responsive cell lines, $G_1$ arrest and morphological changes were not found except for Capan-1 which showed dome formation regardless of treatment.

**3. Inhibitory effect *in vivo***

*In vivo* growth inhibition assays were performed by measuring tumor size of the responsive cell line, BxPC-3, inoculated in athymic mice. Oral administration of maxacalcitol significantly delayed the growth of BxPC-3 tumors after 10 days of treatment compared with controls, and the tumor volume of the treated group was 38 % of controls at the end of the experiment, on Day 31 [7]. On the other hand, no significant growth inhibition was observed using calcitriol. Additionally, maxacalcitol exerted no hypercalcemic effect, whereas calcitriol induced significant elevation of serum calcium. Furthermore, maxacalcitol had no inhibitory effect on body weight gain as compared with the other two groups, indicating the low toxicity of this substance [7].

The discrepancies between *in vitro* and *in vivo* effects of both agents may have been the result of pharmacokinetic or metabolic differences. Because maxacalcitol is most likely metabolized more rapidly than calcitriol [8], it is possible that the antiproliferative activity of maxacalcitol *in vitro* may be underestimated, and that maxacalcitol may offer a new strategy for therapy of pancreatic cancer.

**4. Inhibitory effect and vitamin D receptor status**

The growth inhibition by maxacalcitol and calcitriol was selectively seen in some cell lines, so it is important to clarify the factors contributing to this selectivity before clinical use. Various effects of calcitriol, including inhibition of proliferation and induction of differentiation, have been reported to depend on quantitative or qualitative differences in vitamin D receptor (VDR).

Accordingly, we next investigated the correlation between the growth inhibitory effects of vitamin D analogs and VDR status.

Immunostaining of VDR showed that all pancreatic cancer cells expressed VDR. So we next examined the differences in VDR content among the cell lines tested, and quantification of VDR was performed by Scatchard plot analysis [9]. Scatchard plot analysis was available for seven cell lines, showing a single class of specific and high affinity receptors. However, for two cell lines, MIA PaCA-2 and Panc-1, VDR content was not determined because reliable plots could not be obtained due to the small contents of functional VDR in samples [7]. In general, highly responsive cell lines, BxPC-3, Hs 700T and SUP-1, have greater amounts of VDR as compared with non-responsive cell lines (Table 1). For example, BxPC-3 showed the highest VDR content, and MIA PaCa-2 and Panc-1 contained scarcely any VDR. Accordingly, sufficient VDR content was considered to be a prerequisite for such biological effects. However, two non-responsive cell lines, Hs 766T and Capan-1, also showed abundant VDR (Table 1). These cell lines had higher levels of VDR than one highly responsive cell line, SUP-1. A particularly prominent example of this was Hs 766T, which had the second highest VDR content among the lines examined. Accordingly, other factors besides VDR content may regulate the selectivity of the effects of maxacalcitol or calcitriol.

Various mutations of the VDR gene have been reported in patients with hereditary vitamin D-resistant rickets [10]. These mutations were mainly found in the DNA-binding domain, and in some cases were found in the hormone-binding domain. Therefore, we next investigated whether the unresponsiveness of Hs 766T to maxacalcitol and calcitriol was due to an abnormality in the VDR structure, and sequenced the coding region of the VDR cDNA in comparison with that of a responsive cell line, BxPC-3.

No nucleotide point mutations were found in the coding region of the VDR cDNA from BxPC-3. In Hs 766T, there was a silent mutation at codon 349 with no exchange of isoleucine at this position [11]. Accordingly, structural changes of VDR have no relation to the unresponsiveness of Hs 766T cells to maxacalcitol and calcitriol treatment. These results suggest that other factors are also regulating the selectivity of these agents.

**5. Inhibitory effect and cell cycle**

Maxacalcitol, as well as calcitriol, inhibited the proliferation of some pancreatic cancer cell lines, which was linked to $G_1$ phase cell cycle arrest and cell differentiation. This cellular differentiation is thought to be closely associated with factors regulating the $G_1$-phase of the cell cycle, and the cascades of these cell cycle signals may be induced by cyclin-dependent kinase

inhibitors such as p16, p21 and p27 [12-13]. The anti-proliferative and differentiation-inducing effects of vitamin D analogs might be mediated by molecules that regulate the cell cycle of $G_1$. Accordingly, we studied the differences in levels of expression of cyclins, CDKs and CDKIs between two types of pancreatic cancer cell lines, responsive and non-responsive, to both maxacalcitol and calcitriol.

Western blotting analysis for cell cycle agents was performed by ECL methods, and showed marked increases in p21 and p27 content after 24 hours of treatment in BxPC-3 (Fig. 1) but not in Hs 766T, suggesting that the induction of p21 and p27 is an early event provoked by vitamin D analogs [11]. Densitometric analysis showed increases of p21 and p27 proteins after treatment over double those of controls [11].

We also studied whether the induction of CDK inhibitor, p21, inhibits CDK2 activity. Protein extracts from BxPC-3 cells were incubated with anti-CDK2 antibody, and precipitated by the addition of protein G agarose. Kinase activity of the immunopellets was assayed with the use of histone H1 and [$\gamma$-$^{32}$P] ATP as substrates. After electrophoresis with SDS-PAGE, the gel was imaged by image plate autoradiography. In proportion to p21 change, we found decreased CDK2 kinase activity after maxacalcitol and calcitriol treatment [14].

Concomitant with marked induction of p21 and p27, an increase in level of hypophosphory-lated-form Rb protein was observed in responsive cells after 24 hours of treatment, in which the change was more prominent in the maxacalcitol group (Fig.) [11].

Marked changes in the cellular contents of PCNA, cyclins, and CDKs were not seen after one or three days in both treatment groups. On the seventh day of treatment, when prominent growth inhibition was observed in treated groups, cellular contents of PCNA and other antigens decreased markedly in responsive cells. On the other hand, in non-responsive cell lines, no marked changes in expression of any of the antigens examined were observed after treatment [11].

The present study shows that p21 and p27 play a major role in the growth inhibition of pancreatic cancer cells which respond to vitamin D analogs. In leukemic cells, up-regulation of p21 and/or p27 has been reported after treatment with calcitriol [15-18]. For cells to proceed through the $G_1$/S transition, $G_1$ cyclin/CDK complexes promote the phosphorylation of Rb protein, which releases the transcription factor E2F, resulting in the expression of various genes whose products mediate cell cycle progression. Under these conditions, $G_1$ cyclins/CDK activities exceed the threshold of inhibition set of by p21 and p27 [19]. In vitamin D responsive cells, elevated level of p21 and p27 overcome the activities of $G_1$ cyclin/CDK complex, which would in turn block G1/S transition and induce exit from cell cycle or growth arrest. In contrast, P16 and

p53 are also considered to be a negative regulator of restriction point control of $G_1$, but frequent somatic mutations and homozygous deletions have been observed in the p16 and p53 gene in pancreatic cancer cells [20], suggesting that the growth inhibitory effects of vitamin D analogues can not be mediated by these. Vitamin D analogs can use the normal acting machinery of p21 and p27 for $G_1$-phase cell cycle arrest by inhibiting the $G_1$-cyclin/CDK complex and inducing the hypophosphorylation of Rb protein.

The same results were reported in the treatment of 25-hydroxyvitamin $D_3$, the prohormone of calcitriol, on pancreatic cancer cell lines *in vitro* [21]. In *in vivo* experiments, however, 25-hydroxyvitamin $D_3$ is not selectively taken into tumor tissue, and will be also distributed to the kidney resulting in 1,25-dihydroxyvitamin $D_3$ and inducing hypercalcemia. Accordingly, 25-hydroxyvitamin $D_3$ has no benefit in *in vivo* treatment compared with maxacalcitol.

A Phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer has been reported [22]. Seocalcitol is well tolerated in patients with pancreatic cancer but has no objective anti-tumour activity in advanced disease, and the most frequent toxicity was the anticipated dose-dependent hypercalcaemia. In this context, we should find non-calcemic and more potent vitamin D analogs, and maxacalcitol will be a promising candidate.

## Conclusion

This study establishes that maxacalcitol and calcitriol showed marked growth inhibitory effects on three out of nine pancreatic cell lines with appearance of cell differentiation and $G_1$ phase cell cycle arrest. Maxacalcitol showed a growth inhibitory effect on BxPC-3 cells inoculated in athymic mice without causing hypercalcemia. Growth inhibitory effects of vitamin D analogs are in part linked to vitamin D receptor content and structure and the induction of CDK inhibitors, p21 and p27. These results suggest that maxacalcitol may provide a useful agent for the chemotherapy of pancreatic cancer.

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**References**

[1] H.A. Burris 3rd, M.J. Moore, J. Andersen, M.R. Green, M.L. Rothenberg, M,R, Modiano, M.C. Cripps, R.K. Portenoy, A.M. Storniolo, P. Tarassoff, R Nelson, F.A. Dorr, C.D. Stephens, D.D. Von Hoff. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 15 (1997) 2403-13.

[2] E. Abe, C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, T. Suda. Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin $D_3$. Proc Natl Acad Sci USA. 78 (1981) 4990-4994.

[3] Y. Honma, M. Hozumi, E. Abe, K. Konno, M. Fukushima, S. Hata, Y. Nishi, H.F. DeLuca, T. Suda. 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. 80 (1983) 201-204.

[4] J.A. Eisman, D.H. Barkla, P.J. Tutton. Suppression of in vivo growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin $D_3$. Cancer Res. 47 (1987) 21-25.

[5] D.M. Peehl, R.J. Skowronski, G.K. Leung, S.T. Wong, T.A. Stamey, D. Feldman. Antiproliferative effects of 1,25-dihydroxyvitamin $D_3$ on primary cultures of human prostatic cells. Cancer Res. 54 (1994) 805-810.

[6] J. Abe, T. Nakano, Y. Nishii, T. Matsumoto, E. Ogata, K. Ikeda. A novel vitamin $D_3$ analog, 22-oxa-1,25-dihydroxyvitamin $D_3$, inhibits the growth of human breast cancer in vitro and in vivo without causing hypercalcemia. Endocrinology. 129 (1991) 832-837.

[7] S. Kawa, K. Yoshizawa, M. Tokoo, H. Imai, H. Oguchi, K. Kiyosawa, T. Homma, T. Nikaido, K. Furihata. Inhibitory effect of 22-oxa-1,25-dihydroxyvitamin D3 on the proliferation of pancreatic cancer cell lines. Gastroenterology. 110(5) (1996) 1605-13.

[8] S. Kamimura, M. Gallieni, N. Kubodera, Y. Nishii, A.J. Brown, E. Slatopolsky, A. Dusso. Differential catabolism of 22-oxacalcitriol and 1,25-dihydroxyvitamin $D_3$ by normal human peripheral monocytes. Endocrinology. 133 (1993) 2719-2723.

[9] K. Endo, F. Ichikawa, Y. Uchiyama, K. Katsumata, H. Ohkawa, K. Kumaki, E. Ogata, K. Ikeda. Evidence for the uptake of a vitamin D analogue (OCT) by a human carcinoma and its effect of suppressing the transcription of parathyroid hormone-related peptide gene in vivo. J Biol Chem. 269 (1994) 32693-32699.

[10] M.R. Hughes, P.J. Malloy, D.G. Kieback, R.A. Kesterson, J.W. Pike, D. Feldman, B.W.

O'Malley. Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. Science. 242 (1988) 1702-1705.

[11] S. Kawa, T. Nikaido, Y. Aoki, Y. Zhai, T. Kumagai, K. Furihata, S. Fujii, K. Kiyosawa. Vitamin D analogues up-regulate p21 and p27 during growth inhibition of pancreatic cancer cell lines. Br J Cancer. 76(7) (1997) 884-9.

[12] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarsi, S.J. Elledge The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 75(4) (1993) 805-16.

[13] K. Polyak, J.Y. Kato, M.J. Solomon, C.J. Sherr, J. Massague, J.M. Roberts, A. Koff. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. 8(1) (1994) 9-22.

[14] S. Kawa, K Kiyosawa. Growth inhibition of pancreatic cancer cell lines by ligand activation of nuclear hormon receptors through up-regulation of WAF1/CIP1/p21, in H. Asakura, Y. Aoyagi, S. Nakazawa (Eds.). Trends in gastroenterology and hepatology, Springer-Verlag, Tokyo, 2001, pp147-152

[15] H. Jiang, J. Lin, Z.Z. Su, F.R. Collart, E. Huberman, P.B. Fisher. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene. 9(11) (1994) 3397-406.

[16] R.A. Steinman, B. Hoffman, A. Iro, C. Guillouf, D.A. Liebermann, M.E. el-Houseini. Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene. 9(11)(1994)3389-96.

[17] Q.M. Wang, J.B. Jones, G.P. Studzinski. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. Cancer Res. 56(2) (1996) 264-7.

[18] M. Liu, M.H. Lee, M. Cohen, M. Bommakanti, L.P. Freedman. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev. 10(2) (1996) 142-53.

[19] M. Peter, I. Herskowitz. Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. Cell. 79(2) (1994) 181-4.

[20] C. Caldas, S.A. Hahn, L.T. da Costa, M.S. Redston, M. Schutte, A.B. Seymour, C.L. Weinstein, R.H. Hruban, C.J. Yeo, S.E. Kern. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nat Genet. 8(1) (1994) 27-32.

[21] G.G. Schwartz, D. Eads, Rao A, S.D. Cramer, M.C. Willingham, T.C. Chen, D.P. Jamieson, L. Wang, K.L. Burnstein, M.F. Holick, C. Koumenis. Pancreatic cancer cells express

25-hydroxyvitamin D-1 alpha-hydroxylase and their proliferation is inhibited by the prohormone 25-hydroxyvitamin D3. Carcinogenesis. 25(6) (2004) 1015-26.

[22] T.R. Evans, K.W. Colston, F.J. Lofts, D. Cunningham, D.A. Anthoney, H. Gogas, J.S. de Bono, K.J. Hamberg, T. Skov, J.L. Mansi. A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. Br J Cancer. 86(5) (2002) 680-5.

**Table 1**

Growth inhibition after vitamin D analog treatment, and vitamin D receptor content for each cell line.

| Cell Line | Growth Inhibition % of control at 100 nm | | VDR content* (fmol/mg of DNA) |
|---|---|---|---|
| | Maxacalcitol | Calcitriol | |
| Responsive cells | | | |
| BxPC-3 | 23 | 27 | 2,743 |
| Hs 700T | 16 | 18 | 800 |
| SUP-1 | 42 | 41 | 670 |
| Non-responsive cells | | | |
| Hs 766T | 95 | 93 | 1,397 |
| Capan-1 | 97 | 108 | 695 |
| Capan-2 | 82 | 78 | 344 |
| As PC-1 | 92 | 86 | 242 |
| MIA PaCa-2 | 102 | 107 | ND** |
| Panc-1 | 95 | 101 | ND** |

*from ref. [7]. **not determined

**Fig. 1 legend**

Fig. 1. Immunoblotting analysis of p27, p21 and Rb protein in BxPC-3 cells treated with calcitriol and maxacalcitol for the indicated times in hours (hr). C, untreated cells exposed to ethanol vehicle; D and O, cells treated with $1x10^{-7}$ mol $l^{-1}$ 1,25-dihydroxyvitamin $D_3$, calcitriol, and 22-oxa-1,25-dihydroxyvitamin $D_3$, maxacalcitol, respectively.