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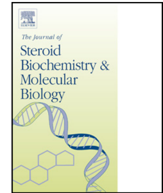

Review

Eldecalcitol (ED-71), an analog of 1α,25-dihydroxyvitamin D3 as a potential anti-cancer agent for oral squamous cell carcinomas☆

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ABSTRACT

We have previously reported that $1,25(OH)_2D_3$ inhibits NF-κB activity and thus inhibits growth of OSCC cells in serum-free culture and down-regulates HBp17/FGFBP-1 expression, which is important for cancer cell growth and angiogenesis. Here, we have investigated the effects of ED-71, an analog of vitamin D3 (VD) on OSCC cell lines in serum-free culture. It is known that ED-71 has a stronger inhibitory effect on bone resorption compared to VD and other VD analogs. To the best of our knowledge, there was no report examining the potential of ED-71 as an anti-cancer agent for OSCC. We found that ED-71 is able to inhibit the growth of cancer cell lines at a concentration of hundred times lower than calcitriol. As Cyp24A1 was reportedly induced in cancer cells, we measured the expression of CYP24A1 in OSCC cell lines (NA and UE), A431 epidermoid carcinoma and normal fibroblast cell (gfi) in serum-free culture. As a result, CYP24A1 mRNA and the protein expression in the OSCC cells treated with ED-71 increased in a dose-dependent manner. However, *in vivo* experiment, in which the A431 cells were implanted in mice, tumor formation was reduced by the ED-71 treatment with no significant difference between Cyp24A1 expression in the tumors of ED-71-treated and control group, as analyzed by western blotting and immunohistochemistry. These results suggest that ED-71 is a potential anti-cancer agent for OSCC.
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Contents

1. Introduction . . . 00
2. Materials and methods . . . 00
   2.1. Cell culture . . . 00
   2.2. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) . . . 00
   2.3. Animal study . . . 00
   2.4. Western blotting . . . 00
   2.5. siRNA transfection . . . 00
   2.6. Immunohistochemistry . . . 00
   2.7. Statistical analyses . . . 00
3. Results . . . 00
   3.1. ED-71 (0.4 nM) inhibited the growth of OSCC cell lines *in vitro* . . . 00

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3.2. ED-71 (0.4 nM) further induced highly expressed CYP24A1 mRNA in OSCC cell lines 00
3.3. Up-regulation of CYP24A1 is parallel with ED-71 concentration 00
3.4. ED-71 (0.4 nM) induced expression of CYP24A1 mediated by VDR 00
3.5. Growth inhibition of A431 cells in athymic mice by administration of ED-71 00
4. Discussion 00
5. Conclusion 00
Conflict of interest 00
Acknowledgements 00
References 00

1. Introduction

1α,25-Dihydroxyvitamin D3 (1α,25$(OH)_2D_3$ or calcitriol) is a multifunctional hormone renowned for its role in the regulation of calcium and skeletal homeostasis [1]. Since 1980, the potential of this compound has been expanded, and it is recognized as a potential anti-cancer agent. Recently, we reported the possibility of calcitriol as a molecular-targeted anti-cancer drug for oral squamous cell carcinomas (OSCC) [2].

24-Hydroxylase (encoded by Cyp24A1) is strongly induced by calcitriol to produce less active vitamin D metabolites in the kidney. Cyp24A1 is thus considered to be an essential factor determining the biological half-life of calcitriol. Cyp24A1 mRNA expression was found to be up-regulated in many tumors, and it may counteract with the anti-proliferative activity of calcitriol, presumably by decreasing calcitriol levels [3]. It has been speculated that calcitriol metabolism could hamper the use of vitamin D3 in cancer therapy. Therefore, it is very important to overcome the enzymatic activity of Cyp24A1 to achieve the maximal efficacy of vitamin D3 and its analogs in cancer therapy.

It is well known that the use of high concentration of vitamin D3 may cause hypercalcemia. To avoid the risk of hypercalcemia, eldecalcitol (ED-71) has been approved recently for the use in osteoporosis treatment in Japan [4].

ED-71 has a lower affinity for VDR, thus making it 1/3–1/8 as active as calcitriol in stimulating target genes [4]. However, this limitation is compensated by its high affinity for the vitamin D binding protein (DBP), which expands its half-life in comparison to other analogs and calcitriol [5]. This particular feature of ED-71 prompted us to study its anti-cancer properties. In order to use ED-71 in oral cancer therapy, it is important to see how this substance may affect Cyp24A1 expression in OSCC cell lines. Cyp24A1's response in ED-71 treatment may assist us to foresee the efficacy of ED-71 in oral cancer treatment.

2. Materials and methods

2.1. Cell culture

The cell lines used were OSCC cell lines (UE: HO-1-*u*-1 [6], NA: HO-1-*n*-1 [7], established from a patient with oral cancer in our laboratory, and gingival fibroblast (gfi) and epidermoid carcinoma cell line, A431 [8]. All cell lines were cultured in serum-free medium as described previously. The total of 2.5×10^4 cells/well in 6-well plates (non-collagen coated) (BD Falcon™, CA, USA) in DF6F medium [9,10] which was composed of DMEM/Ham F-12 medium (DF) supplemented with 6 factors, *i.e.* insulin (10 μg/ml), transferrin (5 μg/ml), 2-aminoethanol (10 μM), sodium selenite (10 nM), 2-mercaptoethanol (10 μM), and oleic acid conjugated with acid-free bovine serum albumin (9.4 μg/ml) (All chemicals were from Sigma–Aldrich, St. Louis, USA).

The cells were cultured at 37 °C in a humidified 95% air/5% CO_2 condition in CO_2 incubator (Thermo Fisher Scientific™, Massachusetts, USA) until they reached 60–70% confluency. Subsequently, 40 nM 1α,25$(OH)_2D_3$ (Enzo Life Sciences, Inc.©, NY, US) or 0.4–40 nM ED-71 (Kindly provided from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was added and further cultured.

We examined the optimum concentration of ED-71 in which the cell growth is significantly suppressed *in vitro*. A431, UE, and NA cell (1×10^4 cell/well) were cultivated in 24-well dish and treated with ED-71. The experiment was repeated using ED-71 and 1,25 $(OH)_2D_3$ at the concentrations ranging from 0.004 to 40 nM, followed by cell count on day 6.

To examine the expression of CYP24A1 by different concentrations of ED-71, NA cells were cultured and treated with various concentrations of ED-71 ranging from 0 to 40 nM. After 24 h of treatment, the mRNA and protein were extracted for further analysis.

2.2. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the RNeasy total RNA spin Mini RNA Isolation Kit (GE Healthcare UK, Ltd., Buckinghamshire, England). Reverse transcription (RT) was performed using the super script first-strand synthesis system (Life Technologies™, NY, USA). Quantitative RT-PCR analyses for Cyp24A1 and VDR was performed using the Stratagene Mx3000P™ system (Stratagene, Agilent Technologies, USA) with GAPDH as the internal control. The sequences of primers and TaqMan™ fluorogenic probes for Cyp24A1 (NM-000782) and VDR (NM-001017536) was designed according to the ProbeFinder™ software of Roche Universal Probe Library system (Roche Applied Science, N.J., USA): 5′-tcatcatggccatcaaaaca-3′, 5′-gcagctcgactggagtgac-3′ and #88 fluorescence probe (Roche Diagnostics) for Cyp24A1 and 5′-cttctctggggactcctcct-3′,5′-tggacgagtccatcatgtct-3′ and #15 fluorescence probe (Roche Diagnostics) for VDR [1].

2.3. Animal study

Eight-week-old male athymic nude BALB/c mice (Charles River Japan, Tokyo, Japan) were fed with normal rodent chow and free access to tap water. They were maintained under specific pathogen-free conditions. After 3 weeks until the animals acclimated to the conditions described above, the experiment was carried out. Each mouse was injected with 1×10^6 A431 cells in PBS, subcutaneously at the dorsal skin. The mice were then divided into two groups (5 mice per group). The treatment group, which were fed with ED-71 (0.1 or 0.5 μg/kg/0.5%Tween 80/MQ), and the other is the control group, in which were fed with 0.5%Tween80/MQ. Feeding was performed using oral gavage and performed in the frequency of once for every 4 days. On day 28, mice were sacrificed and the tumors were extracted for analysis.

The animal study was carried out with the granted permission (Permission #A13-1) by the Committee on Animal Experimentation of Hiroshima University.

2.4. Western blotting

The cells were cultivated at 2.5×10^4 cells/well in 6-well plates (BD Falcon™) in serum-free medium for 48 h, then treated with

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