Antiproliferative and Calcemic Actions of *Trans*-Decalin CD-Ring Analogs of 1,25-Dihydroxyvitamin D_3

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Abstract. *Background: 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] has potent antiproliferative actions but calcemic effects obstruct its application in the treatment of hyperproliferative disorders. Therefore, analogs of 1,25-$(OH)_2D_3$ are designed with a clear dissociation between both effects. Here the biological activity of the trans-decalin CD-ring analog CY10012 is discussed. Materials and Methods: Proliferation/differentiation/transactivation assays as well as mouse models were used to determine the activity of CY10012 in vitro and in vivo. Results: CY10012, has ten-fold higher antiproliferative actions than 1,25$(OH)_2D_3$ but is also twice as calcemic. To determine the role of the Vitamin D Receptor (VDR) in mediating the calcemic actions of CY10012, the analog was daily administered to VDR^{wt} and VDR^{ko} mice. This treatment caused drastic weight loss and death in VDR^{wt} mice but not in VDR^{ko} mice. Conclusion: Analog CY10012 has greater antiproliferative action but also two-fold higher calcemic effects which depended entirely on VDR-mediated signalling pathways.*

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is a major regulator of calcium and phosphate homeostasis and plays a crucial role in bone maintenance. At the molecular level, 1,25$(OH)_2D_3$ binds to its receptor, the vitamin D receptor (VDR), a member of the superfamily of nuclear receptors, which can heterodimerize with the retinoid X receptor (RXR). In turn, VDR-RXR heterodimers can bind to vitamin D response elements (VDREs) in the promoter of target genes and alter their transcription (1). During the last decades it became obvious that, beside its action on bone, 1,25$(OH)_2D_3$ has a strong antiproliferative and pro-differentiating effect on various cell types including cancer cells. This antiproliferative action is mostly characterized by a blocked transition from the G_1- to the S-phase of the cell cycle (2). Extensive research on the molecular mechanism underlying this growth-inhibition has shown that different cell types probably use different (combinations of) pathways to establish this 1,25$(OH)_2D_3$-mediated growth arrest. However, most of the pathways join at the level of important cell cycle regulators such as cyclins, cyclin-dependent kinases and their inhibitors, E2F transcription factors and the retinoblastoma family of tumor suppressors (3, 4). Theoretically, its antiproliferative action would make 1,25$(OH)_2D_3$ an ideal drug to treat hyperproliferative disorders. However, at the doses required for the antiproliferative action, the calcemic effects of 1,25$(OH)_2D_3$ cause severe hypercalcemia, hypercalciuria and bone resorption. Therefore, structural analogs of 1,25$(OH)_2D_3$ have been designed with increased antiproliferative/pro-differentiating action and/or reduced calcemic effects. As far as analog development is concerned, three target regions in the structure of 1,25$(OH)_2D_3$ can be distinguished: the side chain, the central rigid CD-ring system and the seco-B,A-ring system. To determine how important the less accessible CD-ring system is for the different biological actions of 1,25$(OH)_2D_3$, different series of CD-ring modified 1,25$(OH)_2D_3$ analogs have been designed, developed and screened. Here, the biological activities of the *trans*-decalin CD-ring analog CY10012, which features a six-membered D-ring instead of the natural five-membered ring (Figure 1), are discussed. In different cell differentiation and cell proliferation assays, the CY10012 analog proved to be at least ten-fold more potent than the parent compound 1,25$(OH)_2D_3$. However, CY10012 is also twice as calcemic as 1,25$(OH)_2D_3$. To determine the role of the VDR in mediating the increased calcemic actions of CY10012, *VDR* wild-type (VDR^{wt}) and *VDR* knock out (VDR^{ko}) mice were administered 0.5 μg CY10012/kg daily over 19 days. In VDR^{wt} mice, this treatment led to significant weight loss and eventual death whereas VDR^{ko} mice were not affected at all

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Key Words: Vitamin D analogs, CD-ring, antiproliferative, calcemic.

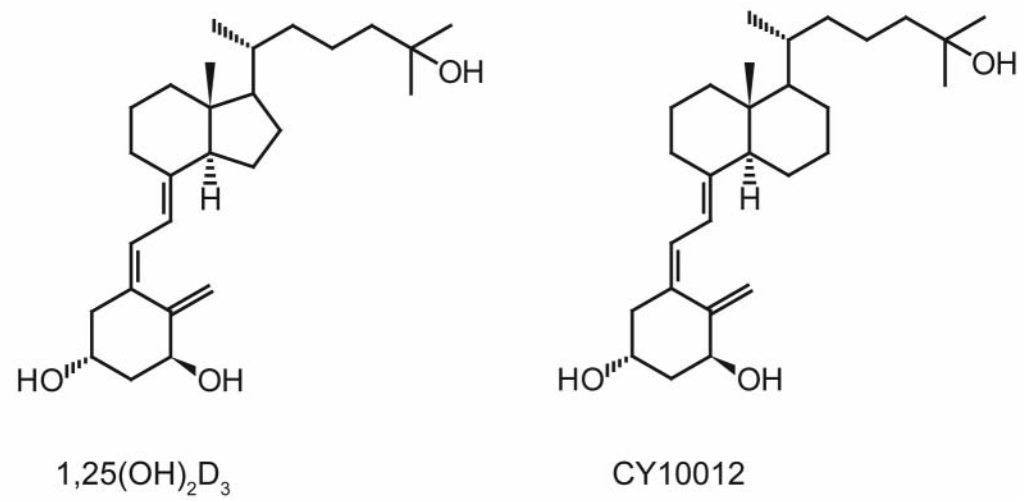

Figure 1. *Chemical structure of 1,25(OH)$_2$D$_3$ and the trans-decalin CD-ring analog CY10012.*

when administered the same dose. The data show that the *trans*-decalin CD-ring analog CY10012 had much higher antiproliferative action in comparison with the parent compound 1,25(OH)$_2$D$_3$ but also greater calcemic effects which were proven to be mainly VDR-mediated.

Materials and Methods

Reagents and cell culture. 1,25(OH)$_2$D$_3$ was obtained from JP van de Velde (Solvay, Weesp, the Netherlands). Human breast cancer MCF-7 cells (ATCC) and African green monkey kidney COS-7 cells (ATCC) were cultured in DMEM with 2 mmol/L glutaMAX-I containing 10% heat-inactivated FCS, 100 units/mL penicillin and 100 μg/mL streptomycin. Human HL-60 promyelocytic leukemia cells were maintained in RPMI 1640 medium supplemented with 20% FCS and gentamicin (50 μg/mL).

Cell proliferation and differentiation assay. [^{3}H]-Thymidine incorporation in human breast cancer MCF-7 cells was determined after a 72 h incubation period with different doses of 1,25(OH)$_2$D$_3$, CY10012, or vehicle and was performed as previously described (5). At four days after treatment with different doses of 1,25(OH)$_2$D$_3$, CY10012, or vehicle, differentiation of human promyelocytic HL-60 leukemia cells was determined with the nitro blue tetrazolium (NBT) reduction assay as previously described (6).

Transactivation assay. COS-7 cells were grown in 6-well plates in DMEM with 10% FCS until 40–60% confluency. After 24 h, the serum was removed and replaced by culture medium containing 2% dextran-coated charcoal-treated FCS. The cells were then co-transfected with a pSG5-h*VDR* expression plasmid (1.5 μg) (7) and a human growth hormone reporter construct, (CT4)$_4$TKGH, with four copies of the rat osteocalcin vitamin D–responsive element CT4 (8). The cells were exposed for 24 h to different concentrations of 1,25(OH)$_2$D$_3$ or analog CY10012. The medium was assayed for the expression of human growth hormone using an in-house radioimmunoassay as described previously (6).

In vivo activity of 1,25(OH)$_2$D$_3$ and CY10012. Calcemic effects were tested in vitamin D-replete NMRI mice (obtained from the Animal house facility of the Katholieke Universiteit Leuven, Belgium) by daily intraperitoneal injections of 1,25(OH)$_2$D$_3$, CY10012, or vehicle during seven consecutive days. Serum calcium concentration (Sigma; atomic absorptiometry) and body weight were used as parameters. The importance of the VDR in mediating the increased calcemic action of analog CY10012 was determined by daily intraperitoneal injections of CY10012 in VDR^{wt} and VDR^{ko} mice over 19 consecutive days. VDR^{ko} mice were obtained from Dr. S. Kato (Tokyo, Japan).

Binding studies. The binding affinity of 1,25(OH)$_2$D$_3$ and CY10012 for the VDR was determined by their ability to compete with [^{3}H]-1,25(OH)$_2$D$_3$ for binding to high speed supernatant from pig intestinal mucosa homogenates performed as previously described (5). Binding studies to determine the affinity of 1,25(OH)$_2$D$_3$ and CY10012 for human vitamin D-binding protein (DBP) were also performed as previously described (5).

Statistical analysis. Student's *t*-tests were performed with the software program Statistica (StatSoft Inc.). Significant differences were determined at $p<0.05$ and $p<0.01$.

Results

To determine the impact of the introduction of a *trans*-decalin CD-ring system in analog CY10012 on its affinity for DBP and VDR, the ability of this analog to compete with [^{3}H]-1,25(OH)$_2$D$_3$ for binding to human DBP and pig mucosal VDR was tested. In comparison with the parent compound 1,25(OH)$_2$D$_3$, analog CY10012 had a significantly lower affinity for DBP (Kd=2.54×10^{-6} M *vs*. Kd=7.46×10^{-8} M for 1,25(OH)$_2$D$_3$) (Figure 2A). In contrast, the affinity of CY10012 for VDR was similar to the affinity of 1,25(OH)$_2$D$_3$ (Kd=1.70×10^{-11} M and Kd=1.67×10^{-11} M respectively) (Figure 2B).

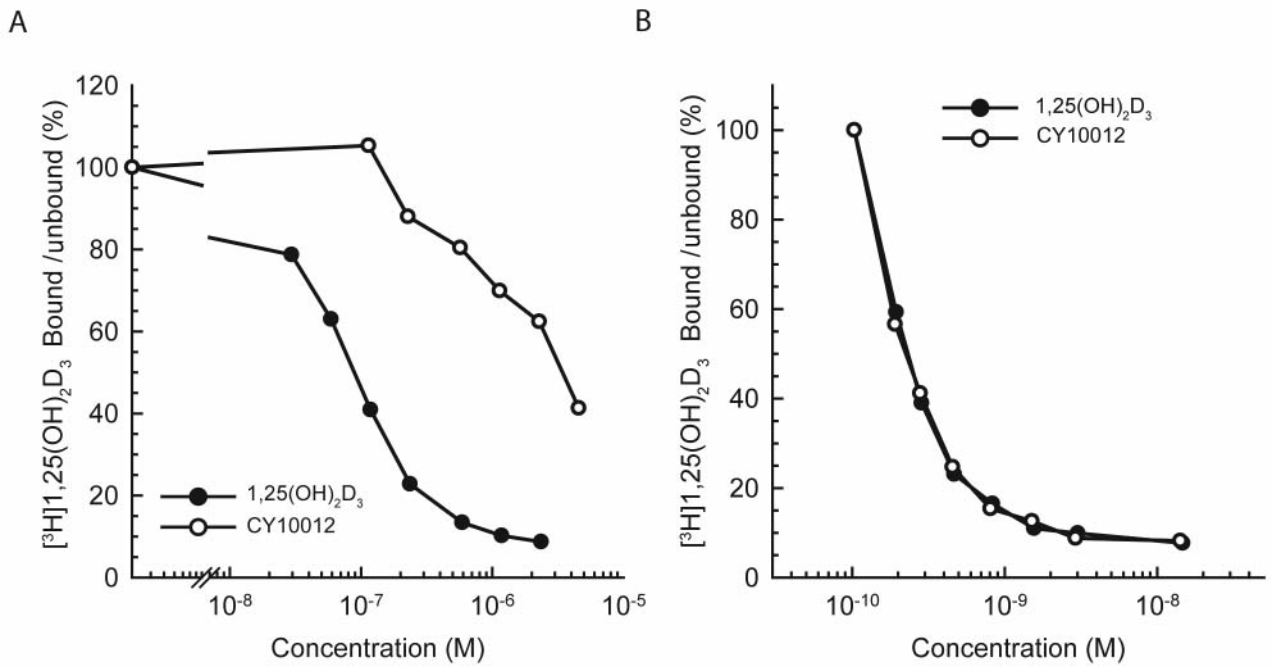

Figure 2. *Binding affinities of 1,25(OH)$_2$D$_3$ and CY10012 for the VDR and human DBP. Affinity of 1,25(OH)$_2$D$_3$ and the trans-decalin CD-ring analog CY10012 for hDBP (A) and for pig mucosal VDR (B). For details see 'Materials and Methods' section. Data shown are the mean of duplicate samples from a representative experiment.*

[^{3}H]-Thymidine incorporation assays on human breast cancer MCF-7 cells treated with different doses of 1,25(OH)$_2$D$_3$ or analog CY10012 revealed that the *trans*-decalin analog had an at least 20-fold stronger antiproliferative action than 1,25(OH)$_2$D$_3$ (Figure 3A). Likewise, CY10012 showed a ten-fold stronger pro-differentiating activity on human HL-60 promyelocytic leukemia cells (Figure 3B) in comparison with 1,25(OH)$_2$D$_3$. A possible explanation for this increase in antiproliferative and pro-differentiating potency might be found at the level of target gene transcription. Co-transfection assays in COS-7 cells showed that CY10012 had a marked increase in transactivation potency in comparison with 1,25(OH)$_2$D$_3$. At 10^{-10} M for example, CY10012 displayed a ten-fold stronger transactivation potency than 1,25(OH)$_2$D$_3$. To equal the transactivation induced by 10^{-11} M CY10012, approximately 20-fold higher doses of the parent compound were required (Figure 3C).

To test whether the *trans*-decalin CD-ring analog CY10012 has a good dissociation between antiproliferative/pro-differentiating action and calcemic effects, NMRI mice were daily injected with 1,25(OH)$_2$D$_3$, CY10012 or vehicle for seven consecutive days, after which serum calcium levels and body weight were measured. As expected, administration of 0.1 μg/kg/day of 1,25(OH)$_2$D$_3$ resulted in a significant increase in serum calcium levels from 10.24 mg/dL (control) to 12.23 mg/dL (1,25(OH)$_2$D$_3$) (Figure 4A). When 0.2 μg/kg/day of 1,25(OH)$_2$D$_3$ was administered, serum calcium levels further increased to 14.17 mg/dL. When 0.1 μg/kg/day CY10012 was administered, serum calcium levels rose to 14.3 mg/dL and a two-fold higher dose made calcium serum levels rise further to 16.0 mg/dL. Because serum calcium levels in mice administered 0.1 μg/kg/day CY10012 equalled those of mice administered 0.2 μg/kg/day 1,25(OH)$_2$D$_3$, it can be concluded that the *trans*-decalin CD-ring analog CY10012 is approximately twice as calcemic as 1,25(OH)$_2$D$_3$. Likewise, CY10012 had a more drastic impact on body weight than 1,25(OH)$_2$D$_3$. Administration of either 0.1 or 0.2 μg/kg/day CY10012 resulted in a significantly stronger reduction in body weight than administration of the same doses of 1,25(OH)$_2$D$_3$ (Figure 4B). To determine if this CY10012-induced body weight loss was entirely dependent on VDR-mediated calcemic effects, VDR^{wt} and VDR^{ko} mice were daily administered a high dose (0.5 μg/kg/day) of CY10012 for 19 consecutive days and their body weights were measured daily. VDR^{wt} mice rapidly began losing weight and continued to lose weight gradually during the 19 day period (Figure 5A). In contrast, the VDR^{ko} mice did not show any significant weight loss at any point in the time course. Moreover, all VDR^{ko} mice survived the 19-day period, whereas none of the VDR^{wt} mice survived beyond day 17 (Figure 5B).

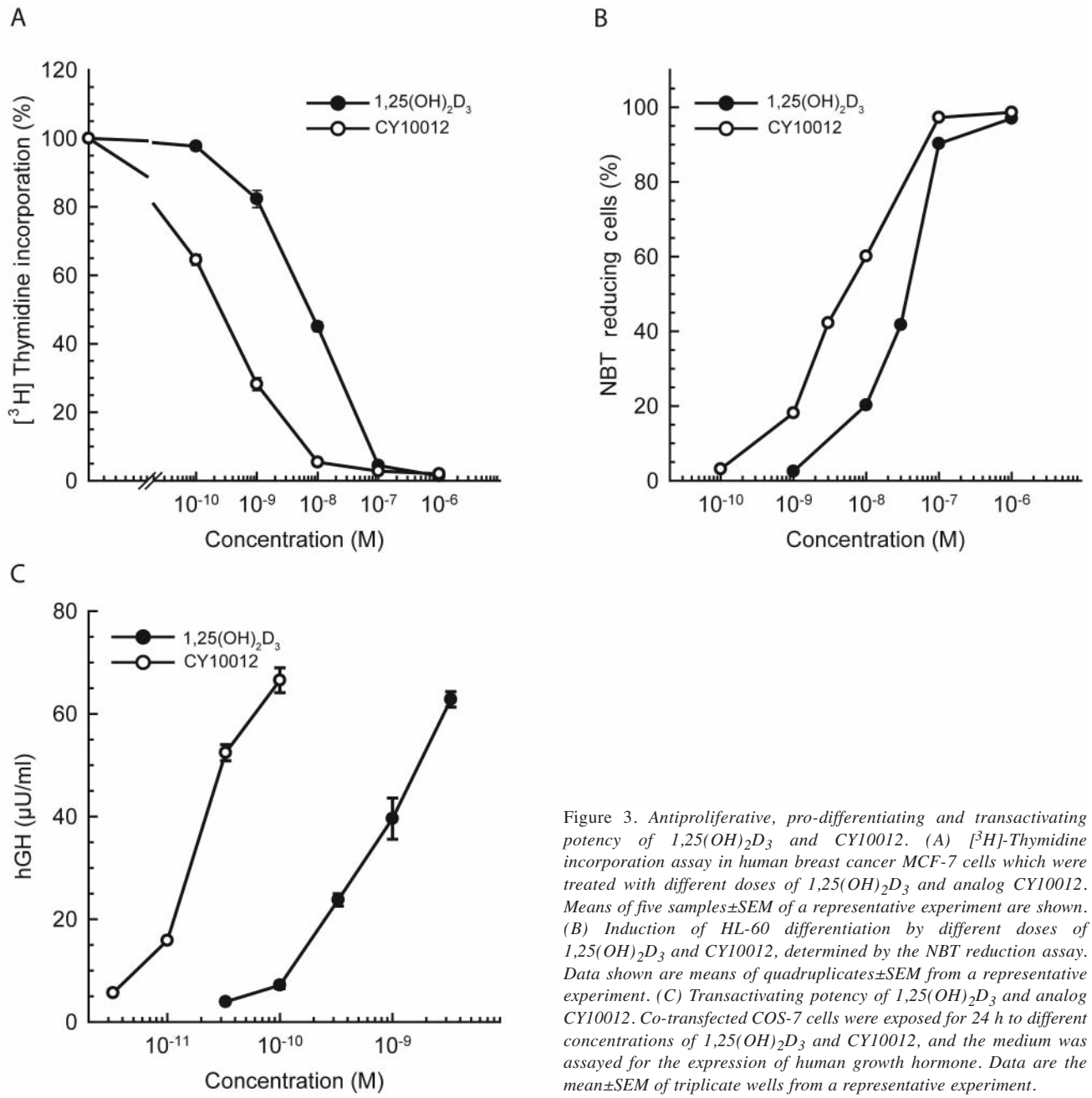

Figure 3. *Antiproliferative, pro-differentiating and transactivating potency of 1,25(OH)$_2$D$_3$ and CY10012. (A) [^{3}H]-Thymidine incorporation assay in human breast cancer MCF-7 cells which were treated with different doses of 1,25(OH)$_2$D$_3$ and analog CY10012. Means of five samples±SEM of a representative experiment are shown. (B) Induction of HL-60 differentiation by different doses of 1,25(OH)$_2$D$_3$ and CY10012, determined by the NBT reduction assay. Data shown are means of quadruplicates±SEM from a representative experiment. (C) Transactivating potency of 1,25(OH)$_2$D$_3$ and analog CY10012. Co-transfected COS-7 cells were exposed for 24 h to different concentrations of 1,25(OH)$_2$D$_3$ and CY10012, and the medium was assayed for the expression of human growth hormone. Data are the mean±SEM of triplicate wells from a representative experiment.*

Discussion

In the development of 1,25(OH)$_2$D$_3$ analogs there are in general three target sites: the side chain, the seco-B,A-ring system and the central CD-bicyclic ring structure. Although the latter is synthetically less accessible, there has been a focus on the development of analogs harbouring structural changes in this area. As such, non-steroidal analogs were developed containing only the six-membered C-ring or only the five-membered D-ring (6), as well as analogs containing an unnatural five-membered E-ring (9). Analogs with less dramatic structural changes at the CD-ring system are the *trans*-decalin CD-ring analogs in which the natural five-membered D-ring is replaced by a six-membered ring (10). The analog CY10012 is the most basic representative of this class of *trans*-decalin CD-ring analogs containing no additional modifications to either the side chain or the seco-B,A-ring system. Binding studies revealed that CY10012 had significantly lower affinity for hDBP in comparison with 1,25(OH)$_2$D$_3$, whereas the affinity for

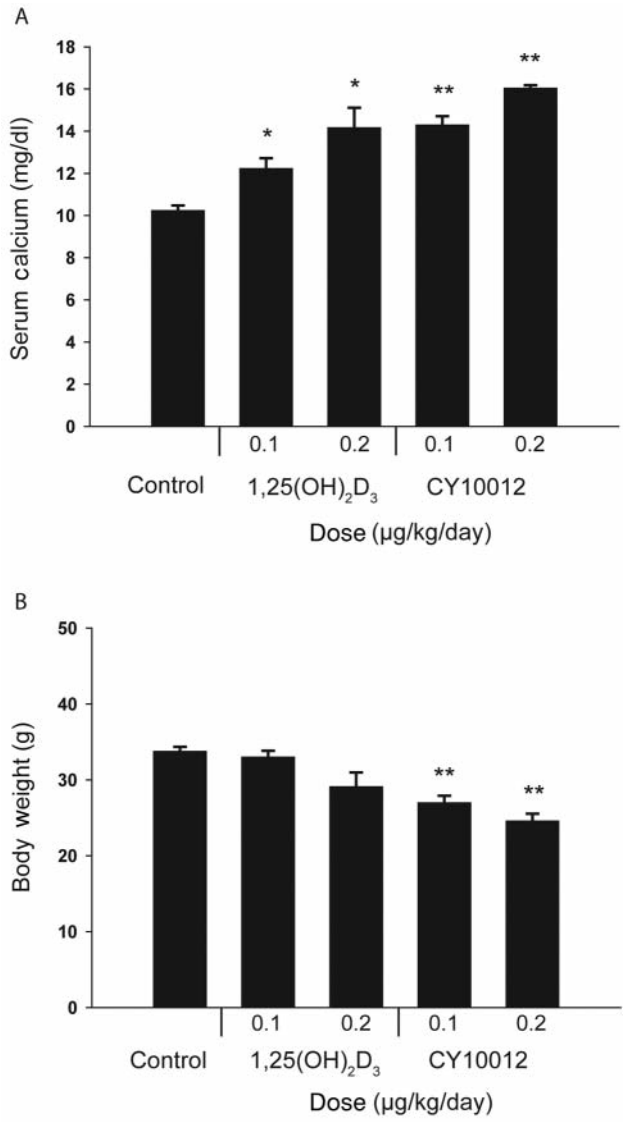

Figure 4. *In vivo calcemic actions of 1,25(OH)$_2$D$_3$ and CY10012. In vivo biological effects of 1,25(OH)$_2$D$_3$ and CY10012 determined by measuring serum calcium levels (A) and body weight (B) in NMRI mice (n=6) after intraperitoneal injections for seven consecutive days. Data shown are mean±SEM from a representative experiment. * and ** indicate significant differences from control condition at p<0.05 and p<0.01 respectively according to Student's t-test.*

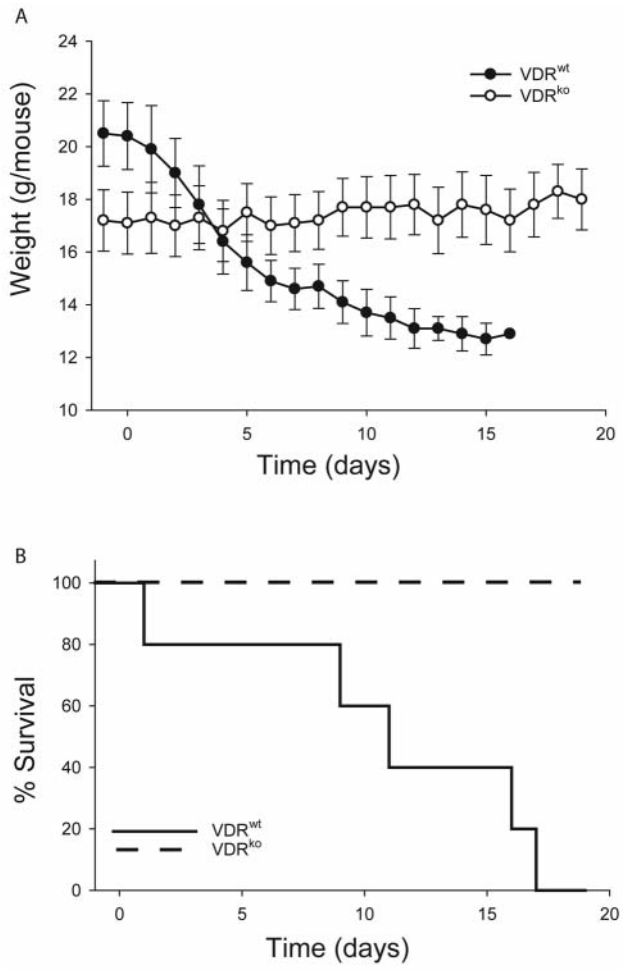

Figure 5. *Effect of treatment with 1,25(OH)$_2$D$_3$ or CY10012 on VDR^{wt} and VDR^{ko} mice. (A) VDR^{wt} (n=5) and VDRko (n=6) mice were injected daily with 0.5 µg/kg 1,25(OH)$_2$D$_3$ or CY10012 for 19 days and weighed daily. Data shown are mean±SEM. (B) Survival curve of VDR^{wt} (n=5) and VDR^{ko} (n=6) mice daily injected with 0.5 µg/kg 1,25(OH)$_2$D$_3$ or CY10012.*

VDR was similar to the parent compound. Cell proliferation and differentiation assays on MCF-7 and HL-60 cells, respectively, demonstrated that CY10012 is at least ten-fold more potent than 1,25(OH)$_2$D$_3$. This strongly increased antiproliferative action was clearly reflected by the analog's transactivating potency as shown by co-transfection assays. Increased transactivating potency and, in particular, increased coactivator recruitment to the analog-bound VDR has previously been shown to be a possible mechanism underlying increased antiproliferative action (11-13). However, *in vivo* studies proved that CY10012 is also twice as calcemic as the parent compound. Removal of the 19-exo-methylene group on the A-ring of CY10012 (19-*nor* analog CY10010) resulted in lower calcemic effects, comparable to those of 1,25(OH)$_2$D$_3$ whereas the pro-differentiating and antiproliferative activity did not significantly change (14). Additional introduction of a 23-yne structure (analog CY10048), further reduced calcemic effects at least 30-fold (data not shown), whereas antiproliferative and pro-differentiating effects were comparable to those of CY10012. Analog CY943, the 20-*epi* counterpart of CY10012, has lower antiproliferative and pro-differentiating action than 1,25(OH)$_2$D$_3$ and is one of

the examples of a 20-*epi* analog with lower biological activity than the corresponding analog with the natural configuration (15). It is interesting to note that CY10012 has significantly lower binding affinity for hDBP than $1,25(OH)_2D_3$. Decreased affinity of an analog for DBP could result in a shorter circulating half-life *in vivo* and a higher clearance rate and consequently in decreased calcemic effects (1). CY10012, however, showed a two-fold increase in calcemic actions notwithstanding lower DBP affinity. Daily administration of only 0.1 μg/kg/day of CY10012 for seven consecutive days caused mice to significantly lose body weight, an effect that was not seen for $1,25(OH)_2D_3$. Since body weight of a mouse is indicative of its overall health, it was investigated whether this weight loss was entirely due to VDR-mediated calcemic effects. For this matter, VDR^{wt} and VDR^{ko} mice were treated with a high dose (0.5 μg/kg/day) of CY10012 for 19-consecutive days and body weights were measured daily. VDR^{wt} mice rapidly began losing weight and none survived the 19 day treatment period. In contrast, VDR^{ko} mice hardly showed any change in body weight and all survived the treatment period. These data clearly show that the dramatic impact of CY10012 administration on body weight and survival entirely depended on VDR-mediated calcemic effects.

In conclusion, this study shows that the *trans*-decalin CD-ring analog CY10012 displays strongly increased antiproliferative and pro-differentiating action in comparison with the parent compound $1,25(OH)_2D_3$. However, the analog has an overall increased biological activity which is reflected by its two-fold higher calcemic actions. The negative impact of analog CY10012 on body weight was shown to depend entirely on these increased VDR-mediated calcemic effects.

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