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# Analogs of 1α,25-Dihydroxyvitamin $D_3$ as Pluripotent Immunomodulators

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**Abstract** The active form of vitamin $D_3$, $1,25(OH)_2D_3$, is known, besides its classical effects on calcium and bone, for its pronounced immunomodulatory effects that are exerted both on the antigen-presenting cell level as well as directly on the T lymphocyte level. In animal models, these immune effects of $1,25(OH)_2D_3$ are reflected by a strong potency to prevent onset and even recurrence of autoimmune diseases. A major limitation in using $1,25(OH)_2D_3$ in clinical immune therapy are the adverse side effects on calcium and on bone. TX527 (19-nor-14,20-bisepi-23-yne-$1,25(OH)_2D_3$) is a structural $1,25(OH)_2D_3$ analog showing reduced calcemic activity associated with enhanced in vitro and in vivo immunomodulating capacity compared to the mother-molecule. Indeed, in vitro TX527 is more potent that $1,25(OH)_2D_3$ in redirecting differentiation and maturation of dendritic cells and in inhibiting phytohemagglutinin-stimulated T lymphocyte proliferation. In vivo, this enhanced potency of TX527 is confirmed by a stronger potential to prevent type 1 diabetes in nonobese diabetic (NOD) mice and to prolong the survival of syngeneic islets grafts, both alone and in combination with cyclosporine A, in overtly diabetic NOD mice. Moreover, these in vivo effects of TX527 are obtained without the adverse side effects observed for $1,25(OH)_2D_3$ itself. We believe therefore that TX527 is a potentially interesting candidate to be considered for clinical intervention trails in autoimmune diseases. J. Cell. Biochem. 88: 223–226, 2003. 

**Key words:** structural analogs of $1,25(OH)_2D_3$; dendritic cell differentiation; T lymphocyte proliferation; NOD mouse; type 1 diabetes

Receptors for the active form of vitamin $D_3$, 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), are present in monocytes, monocyte-derived cells and (T- and B-) lymphocytes [Veldman et al., 2000]. Moreover, activated macrophages themselves are able to produce $1,25(OH)_2D_3$ in a regulated fashion. Regulation of the enzyme responsible for the final and rate-limiting step in the synthesis of the active $1,25(OH)_2D_3$ ($25(OH)D_3$-1-α-hydroxylase) is, however, completely different in macrophages than in kidney cells [Overbergh et al., 2000]. In vivo, in animal models, administration of $1,25(OH)_2D_3$ has important immune effects, such as prolongation of graft survival (of for example pancreatic islets, heart, liver and skin) and prevention of autoimmune diseases (in experimental models such as autoimmune diabetes, experimental autoimmune encephalomyelitis and collagen-induced arthritis) [Mathieu and Adorini, 2002]. This effect is achieved through its actions on dendritic cells (DC; inhibition of antigen presentation and modulation of cytokine production), being the central antigen-presenting cells in the immune system, as well as directly on T lymphocytes (inhibition of proliferation and cytokine production) [Mathieu and Adorini, 2002]. Clinical applications of $1,25(OH)_2D_3$ in immune therapy can only be considered when the adverse side effects on calcium and bone can be avoided. This has been achieved through the design of structural analogs of $1,25(OH)_2D_3$ [Bouillon et al., 1995; Verstuyf et al., 2000] showing a reduced calcemic activity often associated with an enhanced capacity, when compared to the mother-molecule, to modulate the immune system in vitro, on DC and T lymphocytes, and in vivo.

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We describe here the effects of a structural analog of $1,25(OH)_2D_3$, TX527 (19-nor-14,20-bisepi-23-yne-$1,25(OH)_2D_3$) [Verlinden et al., 2000], on proliferation of human T lymphocytes, on differentiation of human DC and on development and recurrence of type 1 diabetes in nonobese diabetic (NOD) mice, an animal model for the human disease.

## TX527, A STRUCTURAL ANALOG OF $1,25(OH)_2D_3$

The analog TX527 demonstrates decreased affinity to the vitamin $D_3$ receptor (VDR) and the vitamin $D_3$ binding protein (DBP), compared with $1,25(OH)_2D_3$, in combination with markedly diminished in vivo calcemic effects (Table I) [Verlinden et al., 2000]. The maximal dose of $1,25(OH)_2D_3$ that could be administered intraperitoneally during 7 consecutive days in NMRI mice, without inducing hypercalcemia, was 0.1 μg/kg/day whereas TX527 could be injected at a dose up to 10 μg/kg/day without inducing hypercalcemia. The toxic effect of this analog was further tested by daily intraperitoneal injections in SJL mice [van Etten et al., 2000]. The dose that induced a 10% loss of bodyweight over a period of 2 weeks was calculated by linear regression and was referred to as the 10% toxic dose (TD10). This TD10 is 7.2 μg/kg/day for TX527 compared to 0.1 μg/kg/day for $1,25(OH)_2D_3$.

Besides its decreased calcemic effects in vivo, the anti-proliferative and pro-differentiating capacity of TX527 was strongly (2–50 times) enhanced on different normal and malignant cell types as compared to $1,25(OH)_2D_3$. Breast cancer cells treated in vitro with TX527 accumulated in the G1 phase of the cell cycle [Verlinden et al., 2000]. Protein levels of cell cycle regulatory proteins cyclin C and cyclin D1 were down-regulated and protein levels of the cyclin dependent kinase-inhibitors p21 and p27 were up-regulated after in vitro treatment of these breast cancer cells with TX527. Diminished phosphorylation of the retinoblastoma protein contributed also to this TX527-mediated growth inhibition.

Based on its interesting profile of enhanced in vitro anti-proliferative activity and decreased in vivo calcemic effect, this analog was tested in an in vivo model of MCF-7 breast cancer cells established in nude mice and retardation of tumor progression was proven [Verlinden et al., 2000].

To gain more insight in the mechanism of action of this analog, the interaction of both $1,25(OH)_2D_3$ and TX527 with VDR-retinoid X receptor (RXR) heterodimers on vitamin $D_3$ responsive elements (VDREs) was investigated [Verlinden et al., 2001]. The results indicated that the superagonistic activity of TX527 starts beyond the binding of the ligand-heterodimer (VDR-RXR) complex to VDRE and thus probably involves co-activator/co-repressor molecules (currently under investigation).

## TX527 INHIBITS HUMAN T CELLS PROLIFERATION AND REDIRECTS HUMAN DC DIFFERENTIATION

When human peripheral blood mononuclear cells (PBMC) are incubated for 72 hours with TX527 in a dose range of $10^{-12}$ M to $10^{-6}$ M, a clear dose dependent inhibition of phytohemagglutin-induced T lymphocyte proliferation can be observed as demonstrated in Figure 1. When compared to natural $1,25(OH)_2D_3$ in the same dose range, the effect of TX527 is about 5 times more potent.

Moreover, not only T cells are affected by TX527, also the central antigen-presenting cell in the immune system, the DC, carries receptors for $1,25(OH)_2D_3$ and responds dramatically to

**TABLE I. Biological Activity of the 14-epi Analog TX 527**

| | Bindings studies | | In vitro studies | | | In vivo study |
|---|---|---|---|---|---|---|
| Compound | VDR | DBP | HL60 | MCF-7 | KERAT | Calcium serum |
| $1,25(OH)_2D_3$ | 100 | 100 | 100 | 100 | 100 | 100 |
| TX527 | 25 | 0.1 | 2,200 | 5,300 | 4,400 | 1 |

Summary of the in vitro effects of TX527 on vitamin $D_3$ receptor (VDR)- and vitamin $D_3$ binding protein (DBP)-binding, HL60 differentiation and MCF-7 and keratinocyte proliferation. The in vitro effects are expressed as percentage activity (at $ED_{50}$) in comparison with $1,25(OH)_2D_3$ (expressed as 100% activity). The in vivo activity in mice is determined by intraperitoneal injections during seven consecutive days. A dose–response curve for TX527 is compared with the dose–response curve for $1,25(OH)_2D_3$ (expressed as 100% activity).

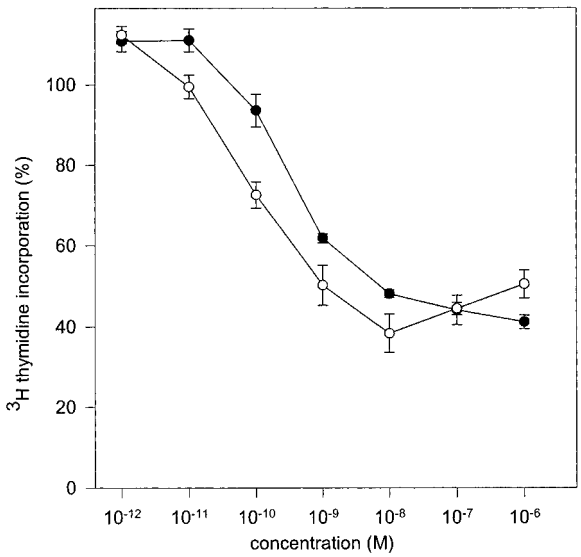


**Fig. 1.** Inhibition of phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) proliferation by serial dilutions of $1,25(OH)_2D_3$ (white symbols) and TX527 (black symbols). Human PBMC were isolated and cultured as described previously [van Etten et al., 2000]. Briefly, human PBMC were isolated from freshly collected, heparinized venous blood of healthy volunteers by Ficoll-density gradient centrifugation. Cells were cultured at $10^6$ per milliliter and stimulated with phytohemagglutinin. $1,25(OH)_2D_3$ or TX527 were added at a final concentration of $10^{-6}$ to $10^{-12}$ M. After 72 h of incubation, proliferation was measured by [$^3$H]thymidine incorporation. The results are expressed as mean proliferation compared to positive controls ± SD.

incubation with TX527 [van Halteren et al., 2002]. Addition of TX527 to human CD14-positive PBMC impairs IL-4 + GM-CSF driven DC-differentiation as well as LPS + IFN-γ induced DC-maturation, as characterized by marked changes in DC morphology (generation of spindle shaped cells), profound alterations in surface marker expression and abrogation of IL-12 release upon CD40-ligation. These changes mean the generation of a new type of cell and not the maintenance of the original cell (DC) in an immature state. A differentiation-shift of the original CD14-positive monocytes towards macrophages is not induced by this TX527-incubation since phagocytosis capacity remains very low in these new cells as compared to true macrophages. Interestingly, when an autoreactive T cell clone is exposed to these antigen-presenting cells, the modifications induced by TX527 on the DC-level result in a profound change in behavior of the T cell clone, which by itself has not been exposed to TX527. Indeed, exposing the autoreactive T cell clone to TX527-treated DC results in almost complete abrogation of Th1-specific IFN-γ production, while leaving IL-13 production unaffected. Proliferation of the T cell clone was also inhibited. This suggests a dramatic, long-lasting effect of the $1,25(OH)_2D_3$ analog on the DC at the level of the cell surface marker expression but especially at the level of the cytokine profile secreted.

This major immune potential in vitro combined with decreased side effects on calcium and bone metabolism, as demonstrated in in vivo screening systems, makes this TX527 a promising $1,25(OH)_2D_3$ analog for in vivo applications in autoimmune diseases and transplantation.

## TX527 AS IMMUNOMODULATOR IN IN VIVO MODELS OF TYPE 1 DIABETES

Administration of high doses of $1,25(OH)_2D_3$ prevents diabetes and also the histological lesion of diabetes, insulitis, in animal models of type 1 diabetes [Mathieu et al., 1992; Mathieu et al., 1994]. This protective effect has also been described for structural analogues of $1,25(OH)_2D_3$ [Mathieu et al., 1995] and is mediated both through immunomodulation, and through direct beta-cell protective effects [Riachy et al., 2001].

Chronic administration of TX527, from weaning until 200 days of age, prevents diabetes in female NOD mice. Indeed, whereas vehicle-treated mice developed diabetes in 58% of cases (n = 137), only 9 out of 28 mice (32%) treated intraperitoneally with TX527 (12.5 μg/kg every other day) developed the disease ($P < 0.025$). This effect is clearly dose-dependent, since TX527 at only 5 μg/kg every other day conferred no protection against diabetes (14/33 mice, 42%, NS vs. vehicle-treated mice). Protection with the high dose of the analog was achieved without induction of hypercalcemia (8.5 ± 0.9 vs. 8.4 ± 0.5 mg/dl in controls, NS) or bone decalcification (5.1 ± 0.6 vs. 4.8 ± 0.8 mg calcium per tibia in controls, NS). In addition, no increased bone turnover was observed (94 ± 28 vs. 79 ± 24 ng/ml osteocalcin in serum of controls, NS).

Also, tertiary prevention (after diabetes is diagnosed and the immune system fully activated) of type 1 diabetes can be achieved with TX527. When overtly diabetic NOD mice are transplanted with syngeneic islets, these are unequivocally destroyed in controls (mean

survival time of 10 days) by recurrence of the original autoimmune disease. Treatment with high doses of cyclosporine A (15 mg/kg/day), a well known immunosuppressant that acts mainly as an inhibitor of IL-2 secretion and T lymphocyte proliferation, can prolong islet survival until 24 days ($P < 0.05$), but is not able to completely prevent diabetes recurrence. A combination of the structural $1,25(OH)_2D_3$ analog KH1060 with a subtherapeutical dose of cyclosporine A (7.5 mg/kg/day), however, was able to significantly prolong islet survival and even to prevent recurrence in 4 of 7 mice [Casteels et al., 1998]. Monotherapy with TX527 at 10 μg/kg every other day or 5 μg/kg per day prolonged islet graft survival until 28 and 15 days after transplantation, respectively ($P < 0.05$ and $< 0.01$ vs. controls, respectively). Again, a combination of these doses of TX527 with the subtherapeutical dose of cyclosporine A (7.5 mg/kg/day) further prolonged islet graft survival to 74 and 31 days, respectively (both $P < 0.001$ vs. controls). Diabetes protection with both treatment regimens of TX527 was achieved without inducing hypercalcemia nor increased bone turnover, either with or without cyclosporine A co-administration (data not shown). Furthermore, combinations of TX527 with other immunomodulators such as IFN-β, also have synergistic effects [Gysemans et al., 2002].

## CONCLUSIONS

TX527, a structural analog of $1,25(OH)_2D_3$, is a pluripotent immunomodulator that in vitro redirects differentiation and maturation of dendritic cells and also directly inhibits proliferation of T lymphocytes. In vivo, these immune effects of TX527 are reflected by a potential to prevent autoimmune diseases, such as type 1 diabetes in the NOD mouse, and even to prevent the recurrence of these autoimmune diseases. Moreover, these in vivo effects are obtained without the adverse side effects observed for $1,25(OH)_2D_3$ itself. We believe therefore that this agent is a potentially interesting candidate to be considered for clinical intervention trials in autoimmune disease.

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