Pergamon

Biochemical Pharmacology, Vol. 50, No. 5, pp. 577–583, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00121-2

COMMENTARY

NON-HYPERCALCEMIC PHARMACOLOGICAL ASPECTS OF VITAMIN D ANALOGS

ROGER BOUILLON,* ANNEMIEKE VERSTUYF, LIEVE VERLINDEN, KATRIEN ALLEWAERT, DUMITRI BRANISTEANU, CHANTAL MATHIEU and HUGO VAN BAELEN

Laboratorium voor Experimentele Geneeskunde en Endocrinologie (LEGENDO), Onderwijs en Navorsing, Gasthuisberg, B-3000 Leuven, Belgium

Key words: vitamin D; vitamin D analogs; psoriasis; cancer; autoimmune diseases

Vitamin D was recognized as a substance different from vitamin A in the beginning of this century [1], although it soon became the odd man out among the vitamins as it could also be produced in the skin from provitamin D (7-dehydrocholesterol) if the appropriate short UV irradiation, normally provided by sunlight, was regularly available. This soon resulted in the widespread use of vitamin D supplementation and the virtual eradication of endemic rickets in most Western countries. A new impetus to the vitamin D field started with the study of the lag time between the administration and the start of activity of vitamin D, resulting in the discovery of a complex metabolic pathway of vitamin D_3.

1α,25-$(OH)_2D_3$ AS A SYSTEMIC CALCIOTROPIC HORMONE

About two decades ago, vitamin D was promoted from a single vitamin to a steroid hormone [2] since (a) its further metabolism from liver-produced 25-OHD_3 into 1,25-$(OH)_2D_3$ in the kidney tubules is tightly feedback controlled by hormones (e.g. PTH†) and ions (e.g. phosphate and calcium), and (b) its mode of action is similar to that of other steroid/thyroid hormones. Indeed, 1α,25-$(OH)_2D_3$ acts by binding to a specific high-affinity intracellular binding protein shuttling between the cytoplasm and the nucleus. This VDR belongs to a class of steroid/thyroid/retinoid receptors that are *cis*-acting trans-activation factors. In fact, the VDR, both by its structure and by its heterodimerization with RXR, belongs to the subclass of thyroid/retinoid and PPAR/orphan receptor subgroups [3]. This VDR is present, of course, in the classical target tissues responsible for calcium/bone homeostasis (Table 1) where the vitamin D hormone [1,25-$(OH)_2D_3$] receptor complex regulates the gene expression of numerous proteins involved in calcium homeostasis. These proteins include: (a) calbindin D-9K and the ATPase-dependent membrane calcium pump to permit active calcium transport in the intestine; and (b) numerous osteoblast proteins that have a yet largely unknown role in bone mineralization and resorption. Moreover, 1α,25-$(OH)_2D_3$ also stimulates osteoclast progenitors, probably by a prostaglandin and cytokine-dependent mechanism, into mature osteoclasts, which lose their VDR in the final maturation process. Vitamin D also regulates several enzymes involved in its own metabolism (down-regulation of 25-OHD-1α-hydroxylase and up-regulation of its 24-hydroxylase), and it inhibits the synthesis of PTH, thereby creating a complex mechanism for regulating calcium homeostasis by a double-loop regulation of PTH secretion via serum calcium and 1α,25-$(OH)_2D_3$ [4–6]. For this reason, there is no doubt that 1α,25-$(OH)_2D_3$ fulfills all the criteria of a systemic (calciotropic) hormone (Table 1).

1α,25-$(OH)_2D_3$ AS A PARACRINE FACTOR

Its presence in other tissues that are involved in important calcium transport (mammary gland, placenta, egg shell) and in endocrine cells that heavily depend on intracellular calcium for their secretion (e.g. pancreatic B-cells, pituitary cells) could still be related to a further extension of calcium homeostasis in a broader sense, but the presence of VDR in fibroblasts, immune cells, keratinocytes, pneumocytes, brain glial cells and, in fact, in most cells could no longer be explained merely on the basis of calcium homeostasis. Moreover, these VDRs are usually functional, since physiologic or supraphysiologic concentrations (10- to 100-fold the normal plasma level) markedly affect most cells (*vide infra*) and their activation can generally be described as inhibition of cell proliferation and induction of a cell-type specific differentiation [4–6]. At the same time, it became evident that the kidney

* Corresponding author: Dr. R. Bouillon, LEGENDO, Onderwijs en Navorsing, Gasthuisberg, B-3000 Leuven, Belgium. Tel. (32)16/345971; FAX (32)16/345934.

† Abbreviations: PTH, parathyroid hormone; VDR, vitamin D receptor; RXR, receptor for 9-*cis*-retinoic acid; PPAR, peroxisomal-proliferator-activated receptor; IL, interleukin; IFN, interferon; Th, T helper cell; VDRE, vitamin D responsive elements; hGH, human growth hormone; IGF-BP, insulin-like growth factor-binding protein; EGF, epidermal growth factor; TGFβ, transforming growth factor β; M-CSF, macrophage-colony stimulating factor; PSA, prostatic specific antigen; and DBP, vitamin D binding protein.

Table 1. Role of vitamin D metabolites as systemic calciotropic hormone

$1\alpha,25\text{-}(OH)_2D_3$ is a classic secosteroid calciotropic hormone.
(1) Synthesis regulated by a feedback control system via serum calcium/phosphate, via PTH synthesis, and via regulation of 1α- and 24-hydroxylase activity.
(2) Secretion in plasma from a single tissue (renal tubules).
(3) Mode of action via a genomic (VDR as *cis*-acting transcription factor) and possible non-genomic action.
(4) Tissue-specific gene regulation.

	Gene/Protein	Overall effect
Intestine	Calbindin D-9K*	Ca/P absorption
	Plasma membrane Ca group	
Bone	Osteocalcin*	Bone resorption
	Matrix gla protein*	Bone mineralization
	Osteopontin*	
Kidney	Calbindin D-28K*	Vitamin D metabolism
	24-Hydroxylase*	Ca/P reabsorption
Parathyroid gland	PTH†	PTH secretion

* VDRE identified as hexanucleotide direct repeats [7, 8].
† VDRE identified: NNNNNN TGAACC [9].

does not have a monopoly on functional 25-OHD-1α-hydroxylase activity. Indeed, whereas the kidney is undoubtedly the main or sole origin of systemic $1\alpha,25\text{-}(OH)_2D_3$, numerous other cells can synthesize or can be induced to synthesize $1\alpha,25\text{-}(OH)_2D_3$. These cells include keratinocytes, monocytes/macrophages, bone cells, pneumocytes, brain glial cells and placental cells. When these data are combined, a possible paracrine function of $1\alpha,25\text{-}(OH)_2D_3$ can be suspected, although direct proof of such a function is much more difficult to obtain, beyond doubt, for $1\alpha,25(OH)_2D_3$ than for a systemic hormone. Two specific examples for a paracrine role for $1\alpha,25\text{-}(OH)_2D_3$ have received special attention.

(1) The keratinocytes of the basal layer of the epidermis proliferate at a massive rate to form the spinous and the granular layers, which later, by an apoptotic-like mechanism, are transformed into a cornified envelope. The lower layers of keratinocytes also possess 25-OHD-1α-hydroxylase activity, constitutively express VDR, and respond to $1\alpha,25\text{-}(OH)_2D_3$ by decreased proliferation and enhanced differentiation (induction of involucrin, transglutaminase and mature keratins) [10]. During this differentiation process, the 1α-hydroxylase, VDR and vitamin D responsiveness are lost.
(2) A second example for a possible paracrine role is found in the immune system. Monocytes constitutively express the VDR but not the 1α-hydroxylase activity. Stimulated monocytes (e.g. by foreign antigens) will, by direct cell contact and cytokine (IL-1) secretion, stimulate T-cell activation and proliferation, thereby creating the start of a positive feedback cascade of increasing T-cell proliferation (by IL-2 production and induction of IL-2 receptors) and B cell activation. However, the T-cell cytokine, INF-γ, will switch on the monocytic 1α-hydroxylase, and this locally produced $1\alpha,25\text{-}(OH)_2D_3$ can then inhibit the further proliferative and functional cascade of T-cell activation. Indeed, the VDR-deficient resting T-cells acquire the VDR during their activation, and $1\alpha,25\text{-}(OH)_2D_3$ inhibits IL-2 and INF-γ secretion and T-cell proliferation even at nanomolar concentrations [11–13]. Moreover, $1\alpha,25\text{-}(OH)_2D_3$ potently inhibits monocyte or B-cell IL-12 production, a cytokine that stimulates the early Th_0 cell differentiation towards functional Th_1 cells (Fig. 1).

A similar paracrine system may be functional in the bone marrow, placenta, lung and brain (Table 2).

$1\alpha,25\text{-}(OH)_2D_3$ AND ITS ANALOGS AS NEW PHARMACEUTICAL AGENTS

A double systemic/paracrine role of a hormone is certainly not an exception, but it creates the opportunity for therapeutic applications when the hormone involved can be selectively delivered to a target tissue by pharmacological manipulation of its structure and/or of its transport. Moreover, the vitamin D hormone has unique chemical properties in comparison with other steroid hormones. Indeed, it has maintained its long cholesterol-type side-chain, a characteristic shared only with the insect hormone ecdysone. As the only secosteroid hormone yet discovered, it is characterized by additional flexibility of the A-B ring segment. Despite the chemical difficulties involved in the synthesis of a complex molecule with a conjugated triene system, numerous analogs of vitamin D have been synthesized, motivated by academic interest in resolving the structure/function relation of vitamin D but probably even more by pharmaceutical interest in developing (super)agonists or antagonists with dissociated effects, i.e. an activity profile that allows a specific action on non-classical tissues (e.g. the immune system) without the calcemic effects. An analog count in early 1994 [6] supplemented by new information after the 9th Vitamin D Workshop in May 1994 [14] revealed more than 400 analogs described in the non-patent literature, mainly

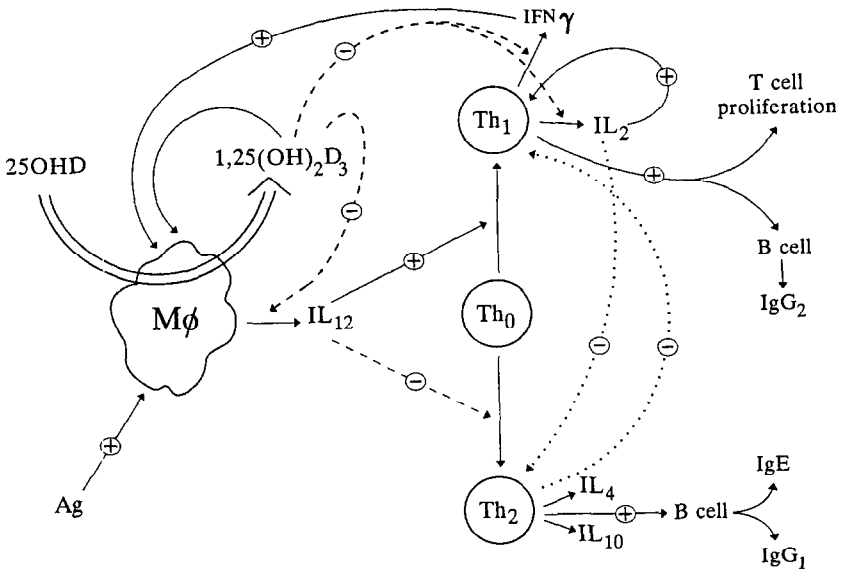

Fig. 1. Paracrine role of 1α,25-$(OH)_2D_3$ in the immune system.

Table 2. Possible paracrine function of vitamin D metabolite(s)

Several tissues combine vitamin D production and responsiveness:

1. Presence of 25-OHD-1α-hydroxylase for local production of 1α,25-$(OH)_2D_3$, e.g.
   - keratinocytes
   - immune system
   - bone cells
   - pneumocytes
   - glia cells
2. Presence of vitamin D receptors
   - especially in undifferentiated cells
   - lost during differentiation (e.g. keratinocytes, osteoclasts)
3. Responsiveness to 1α,25-$(OH)_2D_3$
   - inhibition of proliferation
   - induction of differentiation

involving the side chain but followed by an increasing list of CD, B and A ring modifications. An extensive review of the biological activity profile of these analogs [6] revealed that some general rules regarding the biological consequences of structural modifications can be drawn, but no clear picture of the steroid–receptor interaction is yet possible. A large number of analogs have superagonistic activities when tested *in vitro* on cell proliferation/differentiation using either normal cells (e.g. keratinocytes) or malignant cell lines [e.g. leukemia cells such as HL-60 and U937, breast cancer (e.g. MCF-7) or osteosarcoma cells (e.g. MG63)] or on immune interactions *in vitro* (e.g. mixed lymphocyte reaction or cytokine secretion). At least 25 analogs are 10-fold more active than the natural hormone, and the most potent ones have a more than 100-fold increased activity [6]. On the contrary, similar or other chemical modifications decrease the effects of such analogs on serum calcium or bone homeostasis. The biochemical background for these superagonist

Table 3. Mode of action of 1α,25-$(OH)_2D_3$ superanalogs with dissociated effects on cell differentiation versus calcemic activity

	Potential mode of action of superanalogs	
1.	Differences in intestinal absorption	Not demonstrated
2.	Differences in plasma transport	Yes [15]
	Low DBP affinity and high metabolic clearance rate	[16]
3.	Differences in membrane transport	Not demonstrated
4.	Differences in activation	
	– of non-genomic pathway	[17]
	– via non-genomic receptor	[18]
5.	Differences in genomic receptor	
	– binding affinity	Not demonstrated
	– transactivation	[19, 20]
	– VDR-RX receptor interaction	[21]
	– multiple VDR	Not demonstrated
6.	Differences in intracellular	
	– activation/degradation	[22, 23]

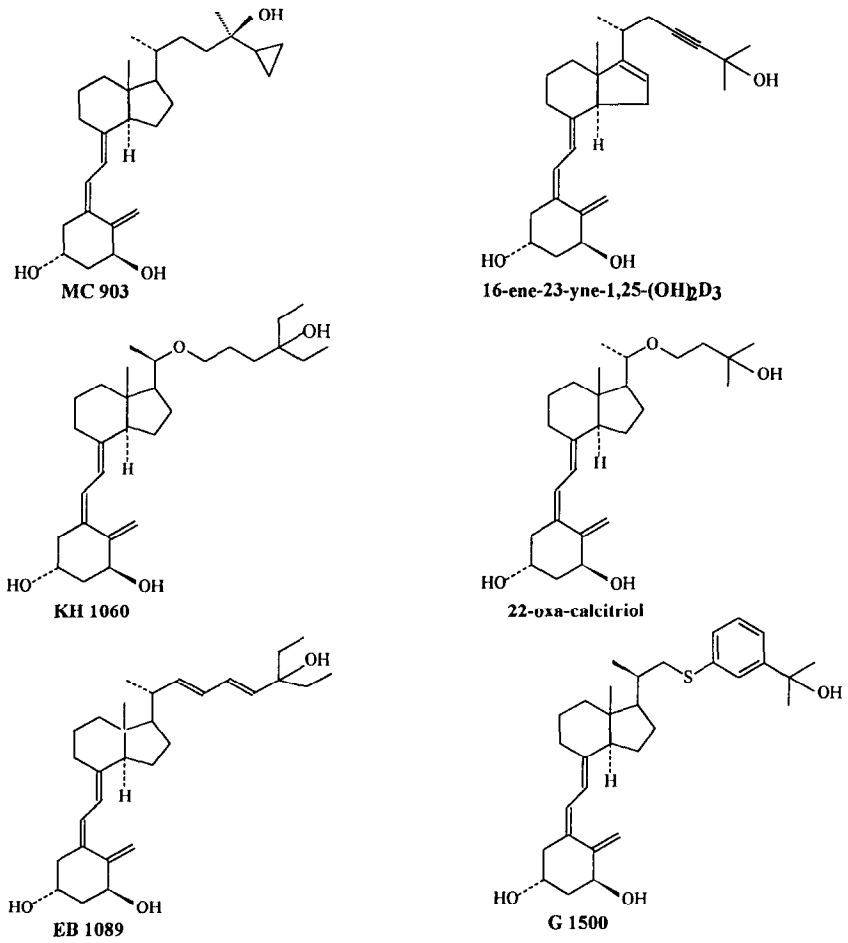

Fig. 2. Selected 1α,25-$(OH)_2D_3$ analogs with superagonist activities profile: Hit parade 1994.

properties with dissociated effects on cell differentiation versus calcium/bone homeostasis is far from being completely understood but several mechanisms are probably involved (Table 3). First, the vitamin D analogs interact with several proteins and lipid membranes during their life-span, and structural modifications of the parent 1α,25-$(OH)_2D_3$ structure can cause differences in such ligand-protein/lipid interaction. The available superagonists have clearly no increased affinity for the VDR monomer as present in cell or nuclear homogenates. All interesting analogs, however, have a clearly decreased or absent affinity for the plasma vitamin D-binding protein, which belongs to the albumin-α-fetoprotein family of proteins. This low plasma binding can explain their rapid cellular uptake and short extracellular $T_{1/2}$ [15, 16]. Their further intracellular metabolism is, however, both analog and cell-type specific. Indeed, ketoconazole, a metabolic inhibitor of the vitamin D side-chain degradation, can enhance the effect of all superanalogs tested in breast cancer but not in osteosarcoma or leukemia cells. Second, the enhancement of the biological activity varied between 1.3 and 25 according to the configuration of the side-chain [22]. Finally, all superanalogs show increased transactivation activity when tested in cells transfected with a VDR and a rat osteocalcin-VDRE-hGH construct [19–21]. Both gel shift analysis and sensitivity to proteolysis suggest that superagonists enhance the VDR–RXR interaction necessary for further activation of the transcription complex and gene activation [21]. A selection of the most relevant superagonists is presented in Fig. 2.

The possible therapeutic application of the new vitamin D analogs is diverse (Fig. 3) and based on *in vitro* and *in vivo* animal studies, whereas human trials (except for psoriasis, *vide infra*) are still absent or in their early phases. 1α,25-$(OH)_2D_3$ and its analogs can inhibit cell proliferation and induce cell differentiation of non-transformed keratinocytes. A serendipitous observation of improvement of psoriatic lesions of a patient who was treated with 1α,25-$(OH)_2D_3$ for other reasons initiated several trials of oral and topical use of 1α,25-$(OH)_2D_3$ and several analogs for this hyperproliferative skin disorder [10]. Both 1α,25-$(OH)_2D_3$ and calcipotriol are at least equivalent to glucocorticoids when applied topically to psoriatic plaques [24] without the risk of skin atrophy. The systemic effects on

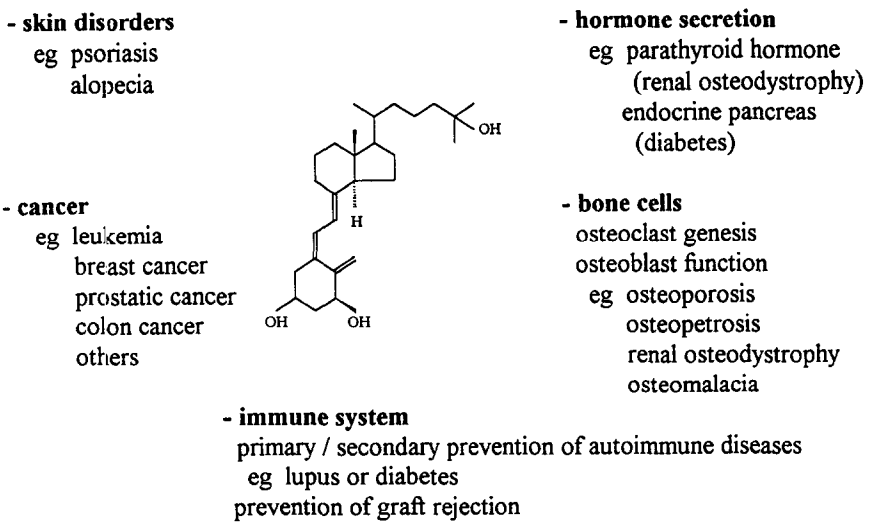

Fig. 3. Potential clinical applications for $1\alpha,25\text{-}(OH)_2D_3$ (analogs).

serum or urinary calcium are minimal, although a few cases of hypercalcemia after too generous use of calcipotriol have been reported. Other similar analogs with either a higher intrinsic potency or a higher safety margin are being explored. Other potential applications for skin lesions (e.g. skin atrophy or alopecia) are being considered.

Vitamin D analogs also potently inhibit proliferation of most cancer cells, at least when they constitutively express the VDR. Most human breast cancers (±90%) possess VDR, thus far exceeding the prevalence of estrogen or progesterone receptor positivity. A high number of leukemias, prostate, colon or squamous carcinomas and osteosarcomas are also VDR positive. $1\alpha,25\text{-}(OH)_2D_3$ can decrease their proliferation with an ED_{50} of 10^{-8} to 10^{-9} M (i.e. 10- to 100-fold higher than the normal plasma concentration). This therefore excludes the therapeutic use of the natural hormone, but several superanalogs have a potency ratio (differentiation versus calcemic effects) exceeding 100 and, therefore, become valuable candidate drugs for cancer treatment. Indeed, animals with either carcinogen-induced or transplanted tumors (in either syngeneic or immune compromised animals) show a decreased tumor occurrence or growth when treated with potent vitamin D analogs [25, 26]. Their safety margin is still limited and no complete tumor regression has yet been obtained, but recent data show clear additive or cooperative effects of vitamin D analogs when administered in combination with anticancer drugs (antihormones, retinoids, cytokines or cytotoxic drugs). The major advantage of vitamin D analogs is their potency to induce cell differentiation rather than causing cell toxicity, whereas their intrinsic toxicity is limited to calcium and bone homeostasis. The mode of action of $1\alpha,25\text{-}(OH)_2D_3$ and its analogs on cell proliferation and differentiation is complex. The c-*myc* mRNA concentration decreases rapidly after exposure to $1\alpha,25\text{-}(OH)_2D_3$ due not only to decreased transcription but especially to increased instability. The phosphorylation of the retinoblastoma gene product also decreases in many cell types after exposure to $1\alpha,25\text{-}(OH)_2D_3$. This could then explain a retardation of G_0/G_1 transition into the S phase of the cell cycle. However, the effect on the retinoblastoma gene may be secondary to an autocrine effect by $1\alpha,25\text{-}(OH)_2D_3$-induced TGF$\beta$ secretion. The effect of $1\alpha,25\text{-}(OH)_2D_3$ on apoptosis is probably cell-type specific, as it can induce apoptosis in MCF-7 cells [27] but causes resistance to other apoptotic stimuli in HL-60 cells [28]. This contrasts with a $1\alpha,25\text{-}(OH)_2D_3$-induced reduction in bcl-2, a protein that by itself normally increases resistance to apoptosis. Moreover, part of the $1\alpha,25\text{-}(OH)_2D_3$ effects may be due to local changes in the secretion of prostaglandins (e.g. for generation of pro-osteoclasts), or growth factors (e.g. IGF-BP, EGF, TGFβ, IL-2), or growth factor receptor (e.g. M-CSF or c-*fms*) expression. The reduction in cell proliferation is usually associated by a simultaneous induction of cell differentiation with a nearly identical ED_{50} for both phenomena, although the biochemical background for these phenomena is unclear. This differentiation can be documented morphologically, or biochemically as revealed by the synthesis of proteins typical for mature cells (either secreted proteins such as osteocalcin for osteoblasts, or PSA for prostate cells or typical cell surface markers such as CD14 and class II antigens in monocytes or $\alpha v\beta 3$ integrins in osteoclasts) or by induction of functional characteristics (e.g. phagocytosis in macrophages).

The differentiation induction can be irreversible (e.g. keratinocyte transformation in cornified envelope) or reversible (e.g. in HL-60 cells). Combined exposure to both $1\alpha,25\text{-}(OH)_2D_3$ and retinoids, however, can induce a prolonged state of full differentiation of such leukemia HL-60 cells without resumption of cell proliferation even after withdrawal of the ligands for 14 days [29].

The clinical applicability of these pharmacological

Table 4. Effects of vitamin D on autoimmunity or transplantation

Prevention of graft rejection
Skin [30]
Heart [31]
Pancreatic islets [32]
Prevention of spontaneous autoimmune diseases
Thyroiditis [33]
Lupus [34]
Diabetes mellitus [35]
Prevention of induced autoimmune disorders
Experimental allergic encephalitis [36]
$HgCl_2$ and Heymann nephritis [37]
Streptozotocin-induced diabetes [38]

effects of vitamin D analogs is clearly demonstrated by the successful introduction of calcipotriol for topical use on psoriatic plaques, but its use for cancer treatment is still in its experimental phase. The paracrine or cytokine-like effects of $1\alpha,25\text{-}(OH)_2D_3$ on the immune system could also have some therapeutic value in humans. Indeed, several animal models of spontaneous or induced ("allergic") autoimmune diseases have shown a remarkably good response to $1\alpha,25\text{-}(OH)_2D_3$ (analogs) treatment (Table 4). This includes the prevention of experimental allergic encephalitis (= multiple sclerosis model) or glomerulonephritis, or the prevention of skin and kidney lesions (lupus mouse model) or insulitis and diabetes in the NOD mouse [35–37, 39]. Moreover, some vitamin D superanalogs could prevent the rejection of syngeneic islet transplantation in diabetic NOD mice [32]. This primary or secondary diabetes prevention is probably due to the restoration of a deficient suppressor cell activity of the NOD mouse [35]. In transplantation models (heart, skin, islets), some vitamin D superanalogs [e.g. KH 1060 or 16-ene-23-yne-$1,25\text{-}(OH)_2D_3$] could prolong the graft survival at least equally well as cyclosporin A [13, 30]. Moreover, a combination of $1\alpha,25\text{-}(OH)_2D_3$ with cyclosporin A or rapamycin showed clear cooperative effects, allowing better results with lower doses and reduced side-effects [35]. These vitamin D analogs do not severely immunocompromize the treated animals since resting T-cells do not possess VDRs. It is therefore quite realistic to foresee the use of carefully selected vitamin D analogs alone or as dose-reducing agents for more toxic immunosuppressive agents in (auto)immune disorders.

CONCLUSION

Vitamin D has evolved from a single nutritional factor discovered in the beginning of the 20th century into a secosteroid hormone with a complex metabolic pathway leading to at least one steroid hormone, $1\alpha,25\text{-}(OH)_2D_3$, and some candidate hormone metabolites [e.g. $24,25\text{-}(OH)_2D_3$ and $1\alpha,25\text{-}(OH)_2$-26,23-lactone]. The tissue originally thought to be the exclusive origin of vitamin D (skin) is now only the starting point for the photochemical synthesis of the basic substrate that can then be further metabolized in many tissues (liver, kidney, placenta, keratinocytes, bone, etc.) to create at least 37 natural metabolites. Moreover, $1\alpha,25\text{-}(OH)_2D_3$ proved to be not only a systemic calciotropic hormone but also a paracrine factor in many tissues, especially the skin and the immune system. Chemists have been able to modify the very flexible vitamin D molecule and have created an unusually large number of superagonists with a clearly dissociated action profile. Therefore, a new class of therapeutic agents may become available soon (Fig. 3) and has already found a first application in the treatment of hyperproliferative skin disorders.

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