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COMMENTARIES

New Nonsteroidal Vitamin D Receptor Modulators: Are These Ligands the Real Deal?

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Commentary on: Ma Y, Khalifa B, Yee YK, Lu J, Memezawa A, Savkur RS, Yamamoto Y, Chintalacharuvu SR, Yamaoka K, Stayrook KR, Bramlett KS, Zeng QQ, Chandrasekhar S, Yu XP, Linebarger JH, Iturria SJ, Burris TP, Kato S, Chin WW, Nagpal S. Identification and characterization of noncalcemic, tissue-selective, nonsecosteroidal vitamin D receptor modulators. *J Clin Invest*. 2006 Apr;116(4):892-904.

Ma and coworkers (1) report on the recent identification and characterization of several nonsteroidal vitamin D receptor modulators that appear to manifest noncalcemic, tissue-selective properties in vivo. The compounds themselves are derived from synthetic work by Lilly chemists on a bis-phenyl template originally identified as a vitamin D receptor activator by Allegretto and coworkers (2) at Ligand Pharmaceuticals, Inc. The present authors perform an extensive series of both in vitro, ex vivo and in vivo studies, all of which support the capacity of at least one of these ligands to be noncalcemic and tissue-selective in its actions in vivo. Additional molecular studies in a variety of cultured cells support the idea that these compounds may well represent selective vitamin D receptor modulators.

Background

The classical function of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is to regulate calcium and phosphorus homeostasis in vertebrate organisms. This activity is achieved by the hormone through direct actions on intestine, kidney and bone, and through an ability to regulate negatively the production of parathyroid hormone (PTH) from the parathyroid glands (3). Intestinal absorption of calcium from the diet and conservation of calcium at the level of the kidney are considered paramount in maintaining blood levels of calcium and phosphorus in the long-term, although bone provides an immediate and readily available emergency source, particularly when the dietary mineral content is deficient. These fundamental actions provided the early impetus for the use of vitamin D and its metabolites in a variety of clinical settings of deranged mineral metabolism. More recently, it has become clear that $1,25(OH)_2D_3$ also exerts additional biologic actions in a wide range of cell and tissue types, primarily as a regulator of growth, differentiation, and general cell function (4-6). These activities appear to be manifested on such target tissues as hematopoietic cells, keratinocytes and the immune system, suggesting potential therapeutic roles for vitamin D in hematopoietic syndromes, skin diseases such as psoriasis and in various autoimmune diseases (7). The ability of vitamin D to induce differentiation in a wide variety of tumors and tumor cells both in culture and *in vivo* (8) also suggests the possibility that the hormone or derivatives thereof might be useful in anticancer therapy.

It is universally accepted, however, that the primary debilitating "side effect" of $1,25(OH)_2D_3$ is its tendency to raise serum calcium levels and to cause hypercalciuria and hypercalcemia. This effect leads to soft tissue calcification and renal stones, and can even be lethal when extreme. This toxic side effect has narrowed the use of natural

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vitamin D metabolites for both mineral-related as well as novel indications and has prompted a search for synthetic vitamin D analogues lacking this particular trait. Surprisingly, many vitamin D analogs active in the above therapeutic indications were found to manifest weak intestinal calcium absorbing activity and/or enhanced bone-sparing calcium activity (9;10). The identification of these compounds supported the idea that the hypercalcemic effects of the natural vitamin D hormone $1,25(OH)_2D_3$ could be dissociated from those affecting cellular growth and differentiation, and spurred the quest for newer versions with even greater selectivity. Currently, a number of vitamin D analogs with this property(s) are under investigation for a wide variety of therapeutic indications (11).

Interestingly, the molecular mechanism(s) whereby vitamin D analogs with low calcemic potential achieve their apparent tissue- and/or gene-selective activity remains unclear. This is due largely to the fact that calcemic activity can be determined only in animal models, and surrogate measures of this activity have not been recapitulated adequately in cellular models *in vitro*. A working hypothesis, nevertheless, is that novel vitamin D analogs induce unusual structural conformations within the vitamin D receptor such that the protein's regulatory potential is altered in certain tissues and perhaps on specific genes. This idea stems from the Selective Receptor Modulator Hypothesis, a hypothesis that is strongly supported through studies of estrogen, androgen, and glucocorticoid receptors as well as other receptors belonging to this transcription factor family (12). The hypothesis postulates that the direct interaction between a nuclear receptor and downstream coregulators that are essential for appropriate transcriptional output can be uniquely modulated by synthetic ligands (13). Importantly, some evidence exists to support this concept for ligands that modulate the vitamin D receptor (14). Unfortunately, the activity of such ligands on this receptor have not been directly linked to the molecular events associated with hypercalciuria and hypercalcemia. The absence of this direct link opens the possibility that the tissue-selective actions of at least some vitamin D analogs are based upon largely pharmacokinetic or pharmacodynamic mechanisms that only occur *in vivo*. Thus, the Selective Receptor Modulator Hypothesis remains to be proven for vitamin D ligands and the vitamin D receptor.

Commentary

In typical pharmaceutical company fashion, the studies by Ma *et al*. (1) are initiated at the cellular level and then advance to the *in vivo* level. In this *Commentary*, however, we will discuss the *in vivo* results first, and then return to the observations made *in vitro*.

In Vivo Studies

A series of experiments outline the biological activities of LY2108491 and LY2109866 (LY compounds) following treatment both *in vivo* and as measured *ex vivo*. The focus is largely on skin and the route of administration is topical. LY compounds manifest a clear capacity to stimulate epidermal thickness in the hairless mouse, a surrogate model for human psoriasis. This activity in normal mice was vitamin D receptor-dependent, as it was not seen in a VDR-knockout strain of mice. Importantly, the concentrations used did not stimulate an increase in blood ionized calcium, unlike that observed with $1,25(OH)_2D_3$. These data and others lead the authors to conclude that the LY compounds exhibit an improved therapeutic index relative to $1,25(OH)_2D_3$. Thus, these data support a potential utility for the LY compounds as topical treatments for psoriasis.

The real question in these studies, however, is whether these compounds are orally active at the level of the epidermis. Unfortunately, that question remains unanswered, although short term, oral administration of the LY compounds at concentrations as high as 3 mg/kg body weight for 6 days did not lead to an increase in serum calcium levels. The investigators conduct a series of additional *in vivo* studies wherein they treat immunized mice with each of the LY compounds and assess the Th1 cytokine response in isolated splenocytes *ex vivo*. Although $1,25(OH)_2D_3$

as a comparator was not examined in these experiments and the method of administration was unclear, the activity of the LY compounds at the level of IFN-γ and IL-2 suppression was promising, particularly at the higher dose of 100 ug/kg body weight. These studies collectively suggest that the LY compounds are biologically active *in vivo*, yet display a reduced capacity to stimulate hypercalcemia. Whether these compounds are biologically active when put to a challenging test in a systemic application remains unclear.

In Vitro Studies

In experiments leading up to the studies described above, the authors explored three aspects of LY activity *in vitro*. The first relates to the agonist actions of the LY compounds on gene expression in target cells such as keratinocytes and PBMCs. The second relates to why the LY compounds fail to induce hypercalcemia *in vivo*. A third relates to the mechanism thereof.

1. Agonist activity of LY: As might be expected of compounds that interact with the vitamin D receptor, the LY compounds manifest significant agonist activity in keratinocytes both on cell proliferation and on CYP24A1 gene expression. They appear equally active in regulating the expression of transcripts for the cytokines IL-2, IL-4 and IL-10 as well as for the transcription factor GATA3 in TPA- and PHA-activated PBMCs. The EC_{50} profiles suggest that the LY compounds are more potent than $1,25(OH)_2D_3$. These measurements are not consistent across the various biological endpoints, however, and the EC_{50} calculations often hinge around a single point. Thus, it is not entirely clear in these studies what the relative agonist activities are compared to $1,25(OH)_2D_3$. Not unexpectedly, the potency profiles do not appear to reflect the competitive binding activity of the LY compounds at the vitamin D receptor as compared to $1,25(OH)_2D_3$. Regardless, it is very clear that these nonsteroidal LY compounds manifest significant biological activity *in vitro*.

2. Mechanism of LY selectivity: Perhaps the most interesting aspect of the study by Ma and coworkers (1) is their exploration of the cellular activities of the LY compounds in bone and intestinal cells. These studies attempt to address the potential mechanism(s) that underlies the apparent lack of LY activity at the level of hypercalcemia *in vivo*. The working hypothesis is that the actions of the LY compounds should diverge from those seen with the hypercalcemic hormone $1,25(OH)_2D_3$ on osteoblastic and intestinal epithelial cell gene expression. The *in vitro* findings are highly intriguing. In short, the authors find that while the relative potencies of both $1,25(OH)_2D_3$ and the LY compounds are similar in osteoblastic cell lines, as assessed by a molecular two-hybrid interaction system comprised of a vitamin D receptor hybrid and a retinoid X receptor hybrid, the LY compounds are far less potent than $1,25(OH)_2D_3$ when examined similarly in the human colon adenocarcinoma cell line Caco2, a surrogate model for an intestinal epithelial cell. This finding suggests the possibility that the LY compounds are much less active than $1,25(OH)_2D_3$ in this particular cell background. The authors then demonstrate that the LY compounds are also exceedingly poor agonists of CaT1 (TRPV6) mRNA induction, a gene whose ion channel product is now believed to represent the vitamin D-regulated gatekeeper of calcium uptake into the intestinal epithelial cell (15). While full potency curves were not shown, the activity of the LY compounds on calbindin-9k induction, another protein involved in calcium uptake, was also reduced compared to $1,25(OH)_2D_3$. These data suggest that the authors have indeed uncovered two cellular models that appear to reflect weak agonist activity on the part of the LY compounds that could account for the lowered calcemic potential of the LY compounds seen *in vivo*. Interestingly, the behavior of the LY compounds in this cellular model is highly reminiscent of those observed by Peleg and coworkers using the vitamin D analog Ro-26-9228 (16). This vitamin D analog has also been shown to manifest weak calcium-uptake inducing activity (17). Further work will be necessary to clarify whether the results of these investigative teams are specific for an unusual cell line, or truly represent a manifestation of weak agonist

activity at the level of the intestine *in vivo*. If the latter, the results of Ma *et al*. (1) and Ismail *et al*. (17) may well provide an important and desperately needed inroad into the mechanism whereby vitamin D analogs or mimics fail to induce calcium uptake.

3. Molecular mechanism of LY. In a final series of experiments, Ma and coworkers (1) attempt to determine the molecular mechanism that underlies the weak agonist activity of the LY compounds. Accordingly, they explore the capacity of $1,25(OH)_2D_3$ and the LY compounds to induce an interaction between the vitamin D receptor and each of the three p160 coregulators SRC-1, TIF2 and AIB1. Interestingly, the LY compounds are much less potent in their capacity to induce these interactions when examined in the Caco2 cell line. Indeed, where AIB1 is concerned, the reduction in potency is well over two logs. Evidence that these aberrant interactions are due to changes in vitamin D receptor conformation is provided by a unique study using a vitamin D receptor mutant and the PGC-1α coregulator. These studies provide correlative support for the idea that weak induction of calcium regulating genes such as CaT1 (TRPV6) and calbindin-9k by the LY compounds is due to their inability to stimulate appropriate vitamin D receptor-coregulatory interactions. Additional investigations will be necessary, however, to establish whether these finding are unique to the Caco2 cell line, whether the p160 coregulators actually play a role in the induction of the calcium regulating genes such as CaT1 and calbindin-9k and whether these actions are directly linked to a reduction in calcium uptake across the intestine *in vivo*.

Conclusion

Ma and coworkers (1) describe the discovery of a new class of ligands that activate the vitamin D receptor and exhibit unique biological activities as a result of that activation. Additional studies will be necessary, however, to establish whether these ligands manifest a lowered calcemic potential over long periods of treatment *in vivo*, and if so, whether they demonstrate therapeutic utility for indications such as psoriasis, immune disease or cancer. Apart from the intriguing findings related to LY-mediated activation of the vitamin D receptor, it is clear that further research will be necessary before a complete molecular understanding of vitamin D receptor ligand-selectivity emerges.

Conflict of Interest: The author has declared that no conflict of interest exists.

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