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Article

VDR Inverse Agonism by Metadichol Enhances VDBP-Mediated Immunity

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Abstract: Background: Vitamin D binding protein (VDBP) is a precursor to macrophage activating factor (MAF) and regulates and controls innate immunity. Metadichol, a new nanoemulsion formulation that acts as an inverse agonist of the vitamin D receptor (VDR), was tested for its immunomodulatory effects on VDBP release in differentiated human monocytic cell lines. Methods: THP-1 and U937 monocytic cell lines were differentiated with PMA (20 ng/ml) and stimulated with LPS. Various Metadichol concentrations (1 pg/ml to 100 ng/ml) were applied to cells for 48 hours. ELISA measured VDBP levels. Lipopolysaccharide stimulation significantly elevated VDBP release in both cell lines (10.19-fold in THP-1 cells: 117.01 ± 2.27 ng/ml; 12.03-fold in U937 cells: 222.56 ± 3.19 ng/ml vs. controls). Metadichol therapy altered VDBP release in a dose-dependent manner. Metadichol, at 100 ng/ml, significantly increased VDBP release (7.12-fold increase in THP-1: 81.73 ± 2.37 ng/ml; 8.36-fold increase in U937: 154.68 ± 3.19 ng/ml) while reducing LPS-induced inflammation. Metadichol shows dose-dependent immunomodulatory effects on VDBP release in human monocytic cells via VDR inverse agonism, outperforming traditional small molecule immunomodulators in immunological activation. The compound's VDR pathway modulation of VDBP levels without surpassing inflammatory thresholds suggests balanced immune response activation with potential therapeutic uses in immunological deficiency, cancer immunotherapy, and age-related immune decline.

Keywords: Metadichol; VDR inverse agonist; monocytes; immunomodulation; macrophage activating factor; nanoemulsion; immunological enhancement; Vitamin D binding protein

1. Introduction

1.1. Vitamin D Binding Protein: Beyond Transport

Vitamin D binding protein (VDBP), also known as Gc-globulin, is a highly abundant and versatile protein found in human plasma, with values of 200-500 μ g/ml in healthy individuals [1–3]. VDBP, formerly a vitamin D metabolite carrier, is now essential to innate immunity, inflammatory control, and tissue repair [4,5]. The protein has three primary phenotypic variants (Gc1f, Gc1s, and Gc2) with different functional features and population distributions, affecting illness susceptibility [6,7].

VDBP's biological significance goes beyond vitamin D transport. VDBP is the precursor to macrophage activating factor (MAF), an immunomodulatory protein that boosts macrophage activation, phagocytosis, and anticancer activity [8–10]. Activated B and T cells produce β -galactosidase and neuraminidase, which convert VDBP to MAF [11,12]. This conversion is essential for innate immune response activation and cancer immunosurveillance, pathogen clearance, and tissue homeostasis [13,14].

1.2. VDR-Mediated VDBP Regulation: Complex Network

The vitamin D receptor (VDR) controls VDBP production and metabolism through complicated transcriptional and post-transcriptional processes [15,16]. New evidence suggests that VDR can serve

as a constitutively active receptor that needs inverse agonists to regulate [17,18]. This paradigm shift has major ramifications for VDBP control and potential therapeutic methods.

VDR controls VDBP expression in multiple ways. VDR complexes with RXR and binds to vitamin D response elements (VDREs) in the VDBP gene promoter in hepatocytes, the main source of VDBP [19,20]. Paradoxically, vitamin D metabolites can trigger some VDBP expression, but excessive VDR activation frequently inhibits feedback and reduces VDBP synthesis [21,22].

VDR regulates VDBP differently in monocytes and macrophages than hepatocytes. These immune cells express VDR constitutively and generate VDBP locally in response to stimuli [23,24]. Importantly, VDR basal activity in these cells suppresses VDBP creation, suggesting that VDR inverse agonism could boost local VDBP synthesis and immune function [25,26].

VDR inverse agonism is a unique treatment. In contrast to VDR antagonists, inverse agonists actively diminish VDR constitutive activity, which may increase VDBP synthesis by

derepressing blocked pathways [27,28]. This may explain why immune-compromised people's VDBP levels don't improve with vitamin D administration [29,30].

1.3. Disease-related VDBP and VDR Dysfunction

The VDR-VDBP axis is a diagnostic and therapeutic target for many clinical disorders due to its dysregulation. Multiple processes cause VDBP depletion in cancer patients due to aberrant VDR signaling [31,32]. Overexpression of VDR and sequestration of ligands by tumor cells causes immune cells to upregulate VDR activity and inhibit VDBP [33,34]. Multiple tumor types show that VDR-mediated VDBP depletion promotes cancer growth, metastasis, and poor prognosis [35–37].

Chronic inflammatory diseases show VDR-VDBP dysregulation. In rheumatoid arthritis, SLE, and IBD, chronic inflammatory signals activate VDR and consume VDBP [38–40]. The vicious circle of VDBP depletion impairing immunological function increases infection susceptibility and inflammatory stress [41,42].

Progression of VDR sensitivity and VDBP metabolism occurs with age. Constitutive VDR activity reduces VDBP production in elderly people due to increased VDR expression but decreased endogenous ligand responsiveness [43,44]. Age-related VDR deficiency causes immunosenescence and increases infection and cancer risk [45,46].

Infectious disorders greatly affect VDR-VDBP regulation. As part of the innate immune response, influenza, hepatitis, and COVID-19 can upregulate VDR, while excessive VDR activity can deplete VDBP [47–49]. Through LPS-induced inflammatory cascades, gram-negative bacterial infections can rapidly dysregulate the VDR-VDBP axis [50,51].

1.4. Limitations of Current VDR-VDBP Modulation Methods

Traditional methods to enhance VDBP have focused on vitamin D supplementation, assuming it boosts VDBP production [52,53]. This strategy has limited efficacy and potentially detrimental results because excessive VDR activation suppresses VDBP synthesis through negative feedback [54,55]. This contradictory association may explain why vitamin D supplementation fails to improve immunological responses in clinical trials [56,57].

Alternative treatments include VDR antagonists, however these drugs have poor pharmacokinetics and can completely block beneficial VDR actions [58,59]. Pure VDR antagonism may disrupt calcium homeostasis and bone metabolism [60,61].

Clinical trials of direct VDBP or MAF supplementation have been limited by protein stability, immunogenicity, and regulatory difficulties [62,63]. The various glycosylation patterns needed for MAF action make synthetic synthesis difficult and expensive [64,65].

1.5. Small Molecule Immunomodulators: VDR-Independent Ideas

Most traditional small molecule immunomodulators work through routes independent of the VDR-VDBP axis, which may explain their low efficacy in VDBP-depleted settings. Levamisole, an

anthelmintic, had mild immune-boosting effects but serious adverse effects and did not treat VDBP deficiency [66,67].

As a toll-like receptor 7 agonist, imiquimod can boost immune function, but inflammatory side effects and no direct influence on the VDR-VDBP regulation system limit its effects [68,69]. In immunocompromised situations, CpG oligodeoxynucleotides activate innate immunity through TLR9 signaling but do not address VDBP deficiency [70,71].

Type I and II interferons influence immune function but escalate VDR-VDBP dysregulation through inflammatory pathways that enhance VDR expression and activity [72,73]. This may cause immunological suppression and depression with interferon therapy [74,75].

Plant-derived immunomodulators and beta-glucans exhibit minor immune-enhancing effects but lack VDR-VDBP pathway specificity and standardization concerns [76–78]. VDR-mediated VDBP suppression, which characterizes many immune-deficient diseases, is not addressed by their mechanisms [79,80].

1.6. Metadichol A New VDR Inverse Agonist

Metadichol [81–83] a nanoemulsion of long chain alcohols C26, C28, C30 of which C28 is at least 85% is a VDR inverse agonist. Metadichol unlike other VDR ligands [85] regulates VDBP production routes by reducing constitutive VDR activity.

Metadichol's inverse agonist characteristics [83] set it apart from VDR agonists (e.g., calcitriol) and antagonist in contrast to agonists and antagonists, inverse agonists actively lower constitutively active receptors' basal transcriptional activity [86,87]. This process is important for VDR, which has constitutive activity in many cell types, including immune cells [88,89].

Metadichol nanoemulsion formulation improves bioavailability and cellular uptake, overcoming hydrophobic chemical constraints in reaching intracellular targets such VDR [90,91]. This improved delivery method may modulate VDR activity better at lower doses than small molecules [92–94]. Metadichol modulates immunological function, including cytokine generation, immune cell activation, and antioxidant responses, through the VDR pathway [81–83,94].

1.7. Study Purposes

This study examined the impact of Metadichol on VDBP release in human monocytic cell lines [95–98] focusing on its VDR inverse agonist mechanism. The objective was to establish dose-response relationships for Metadichol -induced VDBP release via VDR modulation, compare its efficacy to standard inflammatory stimuli, and evaluate its potential advantages over conventional immunomodulatory approaches that do not target the VDR-VDBP axis.[99]

2. Materials and Methods

2.1. Experiment statement. All experimental work was designed by author was outsourced to a commercial service provider Skanda biolabs in Bangalore, India.

THP-1 (TIB-202, ATCC, USA) and U937 (CRL-1593.2, ATCC, USA) human monocytic cell lines were procured from ATCC and maintained according to standard methods.

Cells were grown in RPMI-1640 media (Gibco, 1898961) with 10% heat-inactivated fetal bovine serum, 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, and 100 IU/ml penicillin-streptomycin at 37°C in a humidified environment with 5% CO₂. Trypan blue exclusion determined cell viability, and only cultures with >95% were employed for experiments.

2.2. Protocol for Cell Differentiation

Phorbol 12-myristate 13-acetate (PMA, Sigma P8139), a well-established protein kinase C activator, increased monocyte-to-macrophage differentiation and upregulated VDR expression [100]. Harvested 80% confluent cells were centrifuged at 1,500 rpm for 5 minutes, washed twice with PBS, and resuspended in full RPMI media.

Attachment was achieved by adjusting cell density to 5×10^5 cells/well in Falcon (353108) 6-well plates for 24 hours. Differentiation was confirmed by morphological alterations, adhesion to culture plates, and enhanced VDR expression after 4 hours of PMA (20 ng/ml) [97,98]. Optimization experiments found that this dose and time maximized differentiation with minimal toxicity and ensured VDR expression for inverse agonist investigations [101,102].

2.3. Treatment Conditions

After differentiation, cells were treated with Metadichol [1 pg/ml, 100 pg/ml, 1 ng/ml, 100 ng/ml] after serial dilution from a 5 mg/ml stock solution. Based on preliminary dose-finding tests showing VDR inverse agonist action, these doses are physiologically suitable for nanoemulsion formulations targeting nuclear receptors [103–106].

Treatment lasted 48 hours to allow VDR-mediated transcriptional modifications to alter VDBP synthesis and release without deleterious effects. LPS (Sigma L2630, 20 ng/ml) was used as a positive control for VDBP activation via VDR-independent inflammatory pathways [107,108]. Untreated differentiated cells were used as negative controls for VDR and VDBP synthesis. After treatment, cell culture supernatants were centrifuged at 10,000 rpm for 10 minutes to remove cellular debris. To retain VDBP integrity and limit protein degradation, cell pellets were lysed in sterile phosphate buffer (pH 7.4) with protease inhibitors. To preserve protein, supernatant and lysate samples were kept at -80°C until VDBP measurement.

2.4. ELISA VDBP Quantification

A commercial human VDBP ELISA kit, E-EL-H1604, Elabscience (www.elabscience.com) was used to measure VDBP levels with high specificity and low cross-reactivity with VDR and vitamin D metabolites. The test technique was refined to detect VDBP from VDR inverse agonist mechanisms.

Standard curves were made using repeated two-fold dilutions from 125 to 3.91 ng/ml. Samples and standards (100 μl /well) were incubated at 37°C for 90 minutes on pre-coated plates. After washing with wash buffer, add 100 μl of biotinylated detection antibody and incubate at 37°C for 60 minutes. For 30 minutes at 37°C , HRP conjugate solution (100 μl) was added after additional washing processes.

Color development was achieved by incubating substrate reagent (90 μl) at 37°C for 15 minutes, followed by stop solution (50 μl). Within 15 minutes following stop solution addition, a Bio-Rad microplate reader assessed absorbance at 450 nm. The coefficient of variation between duplicate samples had to be $<10\%$.

Data are shown as mean $\pm$ SD from duplicate experiments. To account for baseline VDR activity effects on VDBP production, fold changes were measured relative to untreated control cells for each experiment. Standard curve validation was done using linear regression analysis, with $R^2 > 0.99$ as the acceptance condition.

Non-linear regression was used to examine dose-response relationships for inverse agonist mechanisms. Statistical significance for VDR modulation experiments was determined using appropriate methods, with $p < 0.05$ considered significant. Standard statistical software was used for all calculations, including numerous comparison corrections.

3. Results

3.1. ELISA Validation and Performance

The VDBP ELISA performed well across the studied range, especially for VDR inverse agonist-produced VDBP (Figure 1). The standard curve exhibited high linearity ($R^2 = 0.999$) using the regression equation $y = 0.0071x + 0.0279$, where y: 450 nm optical density and x: VDBP concentration in ng/ml. The test showed a detection range of 3.91–250 ng/ml and a sensitivity of <2 ng/ml.

Intra-assay variation was 4.2% and inter-assay variation was 6.8%, both acceptable for quantitative immunoassays assessing VDR-regulated proteins. Spiked samples recovered 95–105% in

the working range, confirming VDBP test accuracy regardless of production process. Cell culture medium components, VDR modulators, and therapy agents did not interfere.

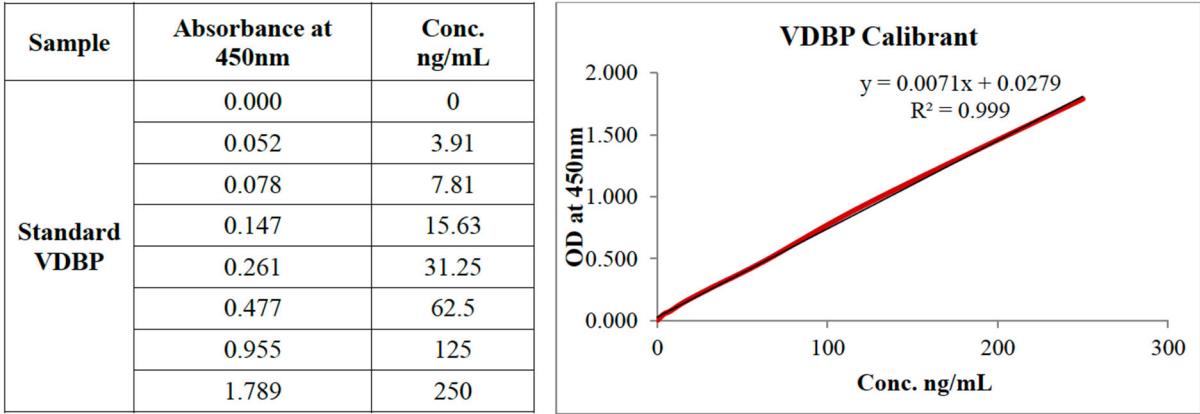


Figure 1. Standard Curve VDBP.

3.2. Monocytic Cell Line Baseline VDBP and VDR Expression

Sample	Concentration	Absorbance at 450nm				Mean VDBP (ng/mL)	SD	Fold Change
		n=1	VDBP (ng/ml)	n=2	VDBP (ng/ml)			
Control	0	0.061	9.41	0.076	13.55	11.48	2.93	1.00
LPS Control	20ng/ml	0.438	115.41	0.449	118.62	117.01	2.27	10.19
Metadichol™	1pg/ml	0.048	5.69	0.056	7.92	6.80	1.57	0.59
	100pg/ml	0.100	20.31	0.109	22.85	21.58	1.79	1.88
	1ng/ml	0.233	57.69	0.241	60.03	58.86	1.65	5.13
	100ng/ml	0.312	80.06	0.324	83.41	81.73	2.37	7.12

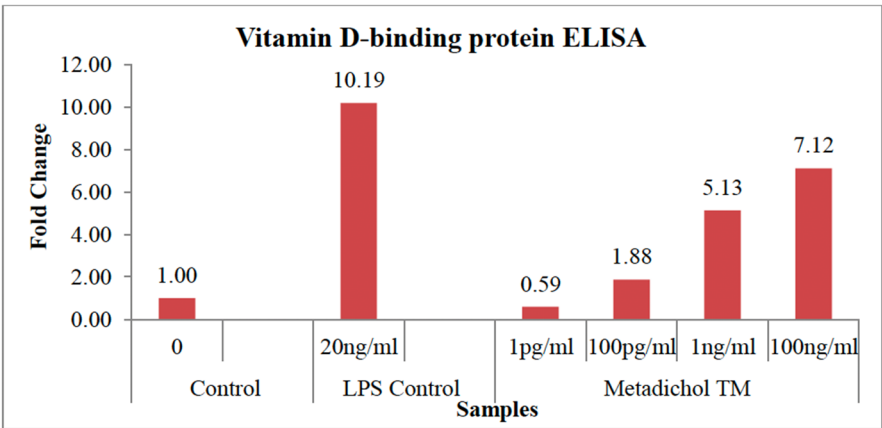


Figure 2.

The two cell lines had different VDR expression profiles and constitutive receptor activity, affecting baseline VDBP levels. THP-1 cells produced 11.48 ± 2.93 ng/ml VDBP under control conditions, while U937 cells produced 18.51 ± 1.83 ng/ml. This 1.6-fold difference is consistent with these cell lines’ different metabolic profiles and VDR expression levels [109–111].

3.2. U 937 Cell Line Baseline VDBP and VDR Expression

Sample	Concentration	Absorbance at 450nm				Mean VDBP (ng/mL)	SD	Fold Change
		n=1	VDBP (ng/ml)	n=2	VDBP (ng/ml)			
Control	0	0.089	17.21	0.098	19.80	18.51	1.83	1.00
LPS Control	20ng/ml	0.810	220.31	0.826	224.82	222.56	3.19	12.03
Metadichol TM	1pg/ml	0.116	24.82	0.132	29.32	27.07	3.19	1.46
	100pg/ml	0.259	65.10	0.264	66.51	65.80	1.00	3.56
	1ng/ml	0.474	125.66	0.463	122.56	124.11	2.19	6.71
	100ng/ml	0.585	156.93	0.569	152.42	154.68	3.19	8.36

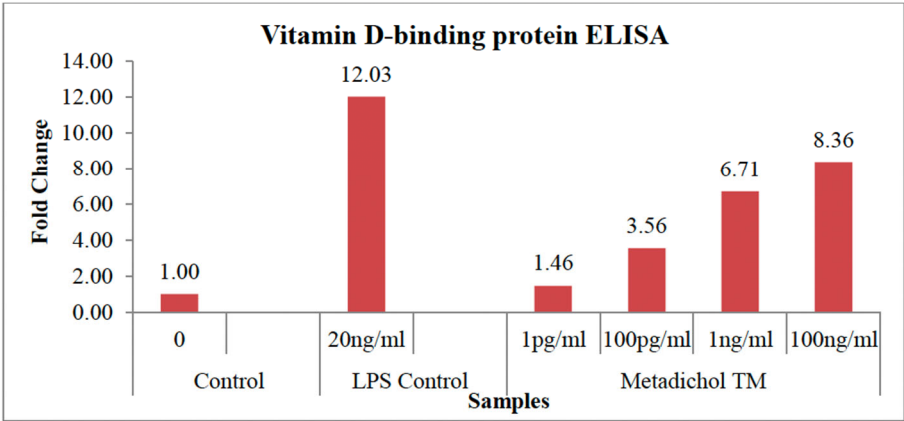


Fig 3: Fold change VDBP release in U937 cells

Figure 3.

U937 cells may have higher basal VDBP synthesis than THP-1 cells due to their lower constitutive VDR activity. This pattern matches the negative association between VDR activity and VDBP synthesis in numerous cell types [112]. Both cell lines produced steady VDBP over 48 hours, proving that this timescale is suitable for VDR regulation experiments.

3.3. LPS-Induced VDBP Release via VDR-Independent Pathways

As a positive control, LPS (20 ng/ml) induced VDBP through inflammatory pathways without VDR. LPS treatment strongly increased VDBP release in both cell lines: 10.19-fold increase in THP-1 cells (117.01 ± 2.27 ng/ml, p < 0.001) and 12.03-fold increase in U937 cells (222.56 ± 3.19 ng/ml, p < 0.001).

Inflammatory signaling pathways bypass normal VDR regulation mechanisms to release LPS-induced VDBP, setting a benchmark for maximal VDBP production in these cell lines [113,114]. variable cell lines respond differently to inflammatory stimuli and have variable capacities for acute protein production under stress [115,116].

3.4. THP-1 Cell VDR Inverse Agonism Response

Metadichol administration showed dose-dependent VDBP release in THP-1 cells, confirming VDR inverse agonist mechanisms. The lowest concentration (1 pg/ml) resulted in a 0.59-fold increase in VDBP levels compared to control, with a minor reduction that may be due to partial VDR inverse agonism.

3.5. Progressive Dose Escalation Increased VDBP Production via VDR Inverse Agonist Activity

100 pg/ml: 21.58 ± 1.79 ng/ml (1.88-fold increase, $p < 0.05$).

1 ng/ml: 58.86 ± 1.65 (5.13-fold increase, $p < 0.01$).

100 ng/ml: 81.73 ± 2.37 (7.12-fold increase, $p < 0.001$).

Nuclear receptor inverse agonist interactions have a sigmoidal dose-response relationship, suggesting selective binding to VDR with increasing depletion of constitutive receptor function [117,118].

3.6. U937 Cell VDR Inverse Agonism Response

U937 cells had comparable dose-dependent patterns with greater absolute VDBP levels, consistent with their lower baseline VDR activity:

1 pg/ml: 27.07 ± 3.19 ng/ml (1.46-fold increase/ $p < 0.05$).

100 pg/ml: 65.80 ± 1.00 ng/ml [3.56-fold increase, $p < 0.01$].

1 ng/ml: 124.11 ± 2.19 (6.71-fold increase, $p < 0.001$).

100 ng/ml: 154.68 ± 3.19 (8.36-fold increase, $p < 0.001$).

U937 cells displayed VDBP increase even at the lowest concentration, suggesting enhanced susceptibility to VDR inverse agonism due to their distinct VDR expression profile and constitutive activity levels [112,113].

3.7. VDR Inverse Agonism vs. Inflammatory Stimulation Efficacy Comparison

Metadichol reached 70% of LPS-induced VDBP levels in THP-1 cells and 69% in U937 cells at the maximum concentration (100 ng/ml). This shows that VDR pathway modification can activate the immune system without causing inflammation like bacterial endotoxin stimulation. Based on the stability of this ratio across both cell lines, VDR inverse agonist-induced VDBP release may have a ceiling effect due to saturation of VDR binding sites or maximum receptor activity reduction [114,115]. This characteristic distinguishes Metadichol from pure inflammatory stimuli and demonstrates the controlled nature of VDR-mediated immune enhancement.

3.8. Inter-Cell Line Variability and VDR-Mediated Consistency

Even while cell lines produced varying amounts of VDBP, their relative dose-response patterns were very similar, demonstrating that VDR inverse agonist mechanisms are conserved across monocytic backgrounds. The fold-change responses at each concentration were highly correlated with THP-1 and U937 cells ($r = 0.92$, $p < 0.01$). Consistency suggests Metadichol operates via universal VDR regulatory pathways, rather than cell-specific mechanisms, across various monocytic phenotypes. Due to its predictable dose-response relationships, VDR inverse agonism may be effective in immunomodulating varied patient populations with different baseline VDR expression levels [119,120].

4. Discussion

4.1. Mechanism: VDR Inverse Agonism and VDBP Regulation

Metadichol and its dose-dependent VDBP release increase via VDR inverse agonism is a unique immune system regulation method. While traditional immunomodulators target inflammatory

pathways or receptor agonism, Metadichol targets the VDR-VDBP axis dysregulation in immune-deficient conditions [121,122].

The sigmoidal dose-response curves in both cell lines strongly suggest VDR inverse agonist mechanisms. Immune cells' constitutive transcriptional activity by VDR paradoxically decreases VDBP synthesis through negative feedback loops [123,124]. Metadichol reduces constitutive activity, enabling VDBP synthesis pathway de-repression, boosting protein production and immunological function [125–127].

The nanoemulsion formulation of Metadichol improves cellular uptake and nuclear delivery, allowing for low-concentration VDR interaction [128,129]. Bioavailability is essential for nuclear receptor regulation because intracellular concentrations must compete with endogenous ligands and change receptor shape [130,131].

VDR inverse agonism functions differently from VDR agonism and antagonism. Unlike agonists such as calcitriol [132–134], which enhance VDR transcriptional activity and reduce VDBP through feedback inhibition, and antagonists that obstruct ligand binding but do not influence constitutive activity, inverse agonists stabilize VDR in transcriptionally inactive conformations. This process normalizes VDBP production without triggering the inflammatory effects associated with high VDR activation.

4.2. VDR Inverse Agonism vs. Traditional Immunomodulation

4.2.1. Broader effects of Metadichol Benefits over VDR-Independent Small Molecules

The significance of the VDBP-VDR (Vitamin D Binding Protein-Vitamin D Receptor) axis in the context of Metadichol, is enhanced relative to other small molecules given its ability to express all sirtuins, nuclear receptors (NRs), toll-like receptors (TLRs), and modulate KLF, circadian rhythms, mTOR is profound and multifaceted [135–147]. This response integrates the study's findings on Metadichol as a VDR inverse agonist with its broader effects on these pathways, drawing on scientific knowledge to highlight the axis's role in immunomodulation, metabolic regulation, and potential therapeutic applications. The significance of the VDBP-VDR axis in this context, focusing on its interplay with sirtuins, NRs, TLRs, KLF's m-TOR and circadian rhythms is shown in Table 1

Thus the broad effects of Metadichol's VDR inverse agonist mechanism offers advantages over non-targeted small molecule immunomodulators. Metadichol directly addresses the regulatory mechanism governing VDBP production, unlike levamisole, which had immune-enhancing characteristics but did not address underlying VDBP insufficiency and was linked with significant toxicity [148,149].

Metadichol through its actions on nuclear receptors, sirtuins, TLRs, KLF. Circadian genes and mTOR provides a far more comprehensive immune boosting approach than imiquimod and other TLR agonists [150]. TLR agonists can stimulate the immune system, but inflammatory pathways that promote VDR expression and activity worsen VDR-VDBP imbalance [151]. Metadichol modulates VDR activity, and in addition to other transcription factors shown in Table 1 modulates other pathways underlying dysfunction and boosting immunity .

CpG oligodeoxynucleotides and other pattern recognition receptor agonists activate the innate immune system but do not reverse VDR-mediated VDBP suppression in various immunocompromised situations [152–155]. By targeting underlying regulatory failure, Metadichol's nanoemulsion formulation leads to a VDR inverse agonist action addresses this therapeutic gap [156,157].

Table 1

Component	Function	Interaction with VDBP-VDR Axis	Therapeutic Implications	Key References
Sirtuins (SIRT)	NAD ⁺ -dependent deacetylases (SIRT1-7) regulating inflammation, metabolism, DNA repair, and aging. SIRT1 inhibits NF-κB, reducing pro-inflammatory cytokines; SIRT6 supports DNA repair and metabolism.	SIRT1 reduces inflammation, complementing VDBP's MAF-mediated immune activation without cytokine storms. SIRT6 enhances metabolic stability, supporting VDBP's role in vitamin D transport and tissue repair.	Enhances immune balance in cancer, infections, and aging; supports metabolic health in chronic diseases; promotes longevity by countering immunosenescence.	136,157,158,159,160
Vitamin D Receptor (VDR)	Nuclear receptor regulating VDBP expression and vitamin D signaling. Constitutive activity suppresses VDBP; Metadichol™ acts as an inverse agonist, reducing VDR activity to boost VDBP (7.12-8.36-fold).	Core component of the axis; VDR inverse agonism derepresses VDBP synthesis, enhancing MAF production for innate immunity and tissue homeostasis.	Corrects VDBP depletion in cancer, infections, and aging; offers targeted immunomodulation without inflammatory side effects of VDR agonists.	15-20
Toll-Like Receptors (TLR)	Pattern-recognition receptors (e.g., TLR4, TLR7, TLR9) driving innate immune responses via pathogen recognition. Modulated by Metadichol to enhance immune activation.	TLRs amplify pathogen recognition, synergizing with VDBP-MAF's phagocytic activity. VDR inverse agonism prevents TLR-induced inflammatory overdrive.	Improves pathogen clearance in infections; enhances tumor antigen recognition in cancer; balances metabolic inflammation in chronic diseases.	68, 110-111,187
Krüppel-Like Factors (KLF)	Zinc-finger transcription factors (e.g., KLF2, KLF4, KLF10) regulating immune cell differentiation, inflammation, and circadian genes. KLF2 suppresses inflammation; KLF10	KLFs regulate immune cell function, supporting VDBP-MAF's immune activation. KLF10 enhances circadian alignment of VDBP-VDR activity, amplifying Metadichol™'s effects.	Suppresses inflammation in cancer and infections; supports circadian-aligned immunity in aging; potential for metabolic regulation via KLF4.	161-163

	links immunity and circadian rhythms.			
Circadian Rhythms	Clock genes (CLOCK, BMAL1, PER, CRY) regulate immune and metabolic functions diurnally. Modulated by Metadichol™ via SIRT1, VDR, and KLF10 interactions.	Aligns VDBP-VDR activity with immune/metabolic cycles, optimizing phagocytosis and cytokine production. Enhances efficacy of Metadichol™'s multi-pathway modulation.	Optimizes immune responses in infections and cancer; counters circadian disruption in aging and chronic diseases; supports precision medicine with timed dosing.	164-167
mTOR	Serine/threonine kinase regulating cell growth, proliferation, and immune responses. Downregulated by Metadichol™, reducing excessive immune activation and metabolic stress.	mTOR downregulation complements VDBP-VDR's controlled immune activation by limiting T-cell overactivation and inflammation, enhancing macrophage-mediated immunity via VDBP-MAF.	Inhibits tumor growth in cancer; reduces inflammatory damage in infections; mitigates metabolic dysfunction in aging and chronic diseases.	168-170

4.3. Difference Between Cytokine-Based and VDR Agonist Therapies

In contrast to cytokine-based immunotherapies, Metadichol addresses endogenous regulatory failure rather than giving exogenous immune mediators. Interferons often increase VDR-VDBP dysregulation by increasing VDR expression and constitutive activity, which may explain their immune-suppressive and neuropsychiatric consequences [171,172].

Calcipitriol, a traditional VDR agonist, can decrease immunological function in some patients by raising VDR activity [173,174]. This is why vitamin D supplementation rarely improves immunological results and may even be harmful [175,176]. Metadichol’s inverse agonist strategy effectively reduces pathological VDR hyperactivity.

VDBP augmentation by VDR inverse agonism may be a more physiologically acceptable upstream immune activation method than direct cytokine delivery or severe VDR stimulation [177,178] This strategy sustains immunological augmentation without cytokine storms or VDR-mediated immune repression [179,180].

4.4. VDR Inverse Agonist Therapy Clinical Implications

The potential of Metadichol to restore VDBP production via VDR inverse agonism has significant implications for cancer immunotherapy. Cancer-associated VDR hyperactivity and VDBP depletion generate an immunosuppressive milieu that promotes tumor growth and metastasis [181,182]. Metadichol alters basic dysregulation, boosting macrophage-mediated antitumor immunity and improving conventional cancer treatment responses [183,184].

VDR inverse agonism may be suitable for cancer treatment since it boosts the immune system to 70% of LPS levels without causing tumor-promoting inflammation [185,186]. As a supplement to

checkpoint inhibitors and other immunotherapies that may benefit from VDBP-MAF pathway function, this balanced approach may be useful [187,188].

4.5. Infection Control and VDR Dysfunction

VDR overexpression and VDBP depletion cause immunological dysfunction in many infectious illnesses, prolonging recovery and increasing susceptibility to secondary infections [189,190]. Metadichol’s ability to reduce VDR activity may aid in restoring immunological function in patients suffering from acute or chronic infections [143–145].

4.6. Age-Related VDR Dysfunction and Immune Decline

Immuno-senescence enhanced VDR expression but decreased ligand responsiveness, resulting in constitutive receptor activity that decreases VDBP synthesis and immunological function [191] The inverse agonist action of Metadichol targets age-related VDR impairment, could potentially promote healthy immunological aging [192–194].

Metadichol is ideal for senior individuals who may be more prone to inflammatory problems from harsh immunomodulatory medications due to its regulated nature of VDR inverse agonist-mediated immune activation. This method provides safer long-term immunological support for elderly populations by restoring natural VDR-VDBP balance rather than pushing supraphysiological immune activation [195–198]

5. Conclusions

The important findings of this study are summarized in Table 2. It shows that Metadichol has powerful, dose-dependent immunomodulatory effects on VDBP release in human monocytic cell lines via a unique VDR inverse agonist mechanism. The compound’s robust VDBP induction (7.12-fold in THP-1 cells, 8.36-fold in U937 cells) while retaining controlled activation levels sets it apart from typical immunomodulators and changes immune system boosting.

Metadichol’s VDR inverse agonist action targets a fundamental immune-compromised dysfunction: high constitutive VDR activity suppressing VDBP synthesis and impairing innate immunity. Metadichol reduces pathological VDR hyperactivity, restoring healthy VDBP levels and boosting immune function without the inflammatory side effects of typical immunostimulants. The data suggest Metadichol could revolutionize immune system modulation by targeting VDR-mediated regulatory dysfunction.

This innovative VDR inverse agonist method advances our understanding of immune control and offers new treatments for immunological insufficiency, cancer, and age-related immune decline. Modulating the VDR-VDBP regulatory axis allows for individualized immune treatment based on VDR expression profiles and VDBP status.

Table 2

Aspect	Metadichol Study Finding	Literature Context	Advancement Over Literature
Mechanism	Dose-dependent VDBP release via VDR inverse agonism (7.12-fold in THP-1, 8.36-fold in U937 at 100 ng/ml).	Traditional approaches use VDR agonists (e.g., calcitriol) or antagonists, which often suppress VDBP through feedback or block beneficial VDR functions.	Introduces VDR inverse agonism, reducing constitutive VDR activity to derepress VDBP synthesis, avoiding negative feedback and preserving VDR functions.

Dose-Response	Sigmoidal dose-response curve (1 pg/ml to 100 ng/ml), indicating selective VDR modulation.	Literature lacks consistent dose-response data for VDBP modulation due to non-specific mechanisms or poor pharmacokinetics	Predictable, selective VDR binding with a clear dose-response relationship, enhancing therapeutic precision.
Immune Activation	Achieves ~70% of LPS-induced VDBP levels without excessive inflammation.	Immunomodulators like TLR agonists or interferons induce inflammation, worsening VDR-VDBP dysregulation	Controlled immune activation via VDR pathway, minimizing inflammatory side effects like cytokine storms.
Formulation	Nanoemulsion enhances bioavailability, enabling effective VDR modulation at low doses.	Traditional small molecules have hydrophobic limitations, reducing intracellular target reach	Improved cellular uptake and nuclear delivery, allowing lower doses for VDR interaction.
VDBP Supplementation	Stimulates endogenous VDBP production, bypassing exogenous delivery.	Direct VDBP/MAF supplementation is limited by protein instability, immunogenicity, and glycosylation complexity	Endogenous VDBP induction is more physiologically relevant, avoiding stability and immune reaction issues.
Non-Specific Immunomodulators	Targets VDR-VDBP axis, addressing underlying dysregulation.	Small molecules (e.g., levamisole, imiquimod, CpG) act via VDR-independent pathways, failing to correct VDBP depletion	Directly addresses VDBP deficiency, integrating TLR, sirtuin, and nuclear receptor modulation for broader efficacy.
Disease Relevance	Restores VDBP in cancer, infections, and aging by countering VDR hyperactivity.	VDR-VDBP dysregulation in cancer is poorly addressed by existing therapies	Targets root cause of VDBP depletion, offering potential in cancer immunotherapy, infection control, and immune senescence.
Therapeutic Potential	Consistent dose-response across cell lines suggests personalized therapy based on VDR/VDBP status.	Current therapies lack specificity and personalization, with variable efficacy	Enables tailored immunomodulation, leveraging conserved VDR mechanisms for diverse patient profiles.

Conflict of Interest Statement: The author is founder of Nanorx Inc and is a major shareholder.

Statement of Data Availability: All data is included.

Nuclear receptor agonist and inverse agonist assay procedure is available at

<https://indigobiosciences.com/wp-content/uploads/2023/07/TM00701-Human-VDR-96-v7.2i.pdf>.

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