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Interaction of Vitamin D-BODIPY With Fat Cells and the Link to Obesity-associated Vitamin D Deficiency

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Abstract. *Background/Aim: Obese individuals often exhibit vitamin D deficiency, potentially due to sequestration in fat cells. Little is known about how vitamin D_3 enters adipocytes and associates with the intracellular lipid droplet. Materials and Methods: Newly differentiated human and mouse (3T3-L1) adipocytes and primary mouse adipocytes were treated with vitamin D_3 covalently linked to green fluorescent BODIPY (VitD-B) or Green BODIPY (GB) as control. Cells were exposed to 10-100 nM concentrations for various lengths of time (1-48 h). Fluorescence microscopy assessed vitamin D distribution. Results: VitD-B demonstrated stable incorporation into adipocytes without enzymatic cleavage, as HPLC showed no free vitamin D_3 after 72 h. Fluorescence microscopy showed GB uptake was rapid and persisted for 48 h. VitD-B uptake was more gradual compared to GB in the human and 3T3-L1 adipocytes. Primary mouse adipocytes exhibited similar uptake patterns, with VitD-B appearing within 1 h and fluorescence intensity increasing 1.2-fold at 8 h and 5.7-fold at 24 h. GB exhibited rapid fluorescence uptake in these same cells, 29-fold higher than VitD-B at 1 h. At 24 h, some VitD-B treated cells exhibited greater fluorescence intensity around the surface of the lipid droplets, which was not observed in GB. Isolated lipid droplets exhibited rapid and immediate uptake of both VitD-B and GB, indicating a strong affinity for these lipid structures. The time-dependent accumulation of vitamin D_3 in human adipocytes mirrored VitD-B uptake. Conclusion: VitD-B is a reliable proxy for studying the dynamics of vitamin D_3 uptake in adipocytes.*

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Key Words: Adipocyte, vitamin D_3, lipid droplet, fluorescent-labelled vitamin D_3.

Adipose tissue plays a crucial role as an energy reservoir, a regulator of energy balance, and an endocrine organ. It is no longer viewed merely as an inert fat storage organ; instead, it is now recognized as a key player in whole-body energy metabolism and inflammation, with significant implications for chronic disease (1).

Vitamin D is a fat-soluble vitamin that can be obtained either from the diet or synthesized by the skin (2). Obese adults are at high-risk for vitamin D deficiency considered to be due to vitamin D being diluted or sequestered in excess adipose tissue, leading to reduced bioavailability (2-5). Currently, our understanding of the interaction between fat-soluble vitamin D_3 and body fat is limited, beyond the knowledge that it is stored in adipose tissue. It is unclear how vitamin D_3 enters adipocytes, how it associates with the intracellular lipid droplet(s), and whether vitamin D within or on the lipid droplets is bioavailable.

Few studies have directly quantified vitamin D levels in adipose tissue. Most early studies identified adipose tissue as a major vitamin D storage organ by measuring radiolabeled

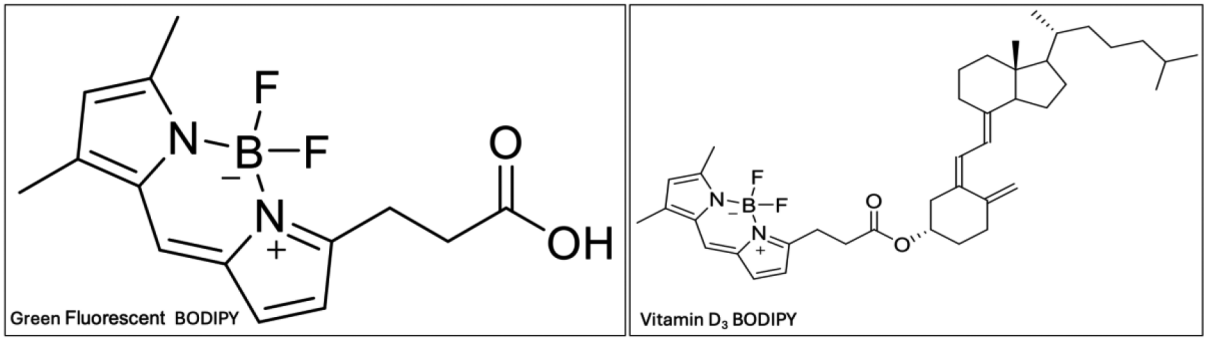

Figure 1. *The structure of Green fluorescent BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, and Vitamin D_3 BODIPY (VitD-B).*

vitamin D_3 in adipose tissue (6-8). When rats were given 5 μg of vitamin D_3 per day for 12 days, the tissue with the highest concentration of vitamin D_3 was the kidney, followed by adipose tissue, blood, and the liver. After two weeks of ingesting ^{14}C-vitamin D_3, rodents showed that 75-90% of the radioactivity in adipose tissue was in the form of vitamin D_3 (9). The remaining radioactivity was associated with esters of vitamin D_3 and metabolites. After dietary deprivation of vitamin D_3 the radioactivity disappeared rapidly in the first three weeks in most tissues (blood, liver, and kidney). In contrast, the radioactivity of the adipose tissue did not decrease nearly as fast after 80 days (9). Heaney *et al*. reported vitamin D was found to be predominantly stored in human adipose tissue (35%) as cholecalciferol (vitamin D_3) based on previous studies that measured vitamin D levels in human tissues (10).

We now report the use of a green fluorescent-labeled vitamin D_3 to investigate its incorporation into cultured human adipocytes, mouse 3T3-L1 adipocytes, primary murine adipocytes, and their lipid droplets.

Materials and Methods

Compounds. Green fluorescent BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY FL=GB in the following) was covalently linked to vitamin D_3 (CARBOGEN AMCIS B.V., Veenendaal, the Netherlands). The product's structure was confirmed by NMR and mass spectroscopy (Figure 1).

Cells. Human preadipocytes were obtained from the Boston University Adipose Biology and Nutrient Metabolism Core. Fat tissue from one-year old male C57BL/6 mice was used to isolate primary adipocytes. 3T3-L1 fibroblasts were gifted from BU-BMC Cancer Research Center.

Differentiation of 3T3-L1 and human preadipocytes. 3T3-L1 preadipocytes were cultured in Dulbecco's modified eagle medium (DMEM) containing 4.5 g/l glucose, L-glutamine and sodium pyruvate) supplemented with 10% fetal bovine serum (FBS), 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin (all from Corning, Corning, NY, USA) until 70-80% confluent, then incubated in the same medium for an additional seven days. Differentiation was induced by addition of 1 μM dexamethasone, 0.5 mM IBMX, and 1.67 μM insulin for three days, at which time the medium was replaced with growth medium containing 1.67 μM insulin, as previously described (11). Human preadipocytes were cultured in 12-well plates (5,000 to 15,000 cells/cm^2) and differentiated as previously described (12). Briefly, the preadipocytes were grown in Growth medium containing alpha-MEM with 10% FBS and antibiotics until confluent. Differentiation was induced using an adipogenic cocktail of insulin, dexamethasone, IBMX and rosiglitazone for seven days. The medium was then changed to a lipogenic maintenance medium for seven days.

Murine primary adipocytes. Primary mouse adipose tissue was provided by the Department of Biochemistry and Cell Biology at Boston University (Boston, MA, USA). Adipose tissue was harvested from C57BL/6 mice and placed in a 10 cm dish containing PBS. Subcutaneous fat pads were finely minced and washed with 10 ml of PBS. The resulting suspension was filtered through a 250 μm SpectraMesh filter and transferred into a 50 ml tube containing 25 ml of DMEM, 2 mg/ml collagenase type I, and 0.5 g BSA. The mixture was incubated at 37˚C with shaking at 150-155 RPM for 1 h. Following digestion, the solution was filtered into another 50 ml tube and 5 ml of sterile fetal bovine serum (FBS) was added to neutralize the collagenase. The top cloudy layer consists of adipocytes rich in lipids and was collected using a wide-bore pipette tip. The cells were then resuspended and incubated in DMEM containing 25 mM glucose, 10% FBS, and 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin at 37˚C in 5% CO_2.

VitD-B incubation. Cultured mouse 3T3-L1 cells, newly differentiated human adipocytes or primary mouse adipocytes were exposed to 1 μM VitD-B. GB was added to cultured cells and served as the control. Cells were imaged at 1, 4, 8, 24, and 48 h following exposure on a wide-field epifluorescence microscope and analyzed with NIH ImageJ software.

Lipid droplets Isolation. Lipid droplets were isolated using a method adapted from Brasaemle *et al*. (13). Briefly, cells were first homogenized in a HLM buffer to disrupt their membranes and release the intracellular components. The homogenate was centrifuged to remove cellular debris and nuclei. This supernatant was then centrifuged at 18 psi in an airfuge (Beckman, Brea, CA, USA), where lipid droplets, due to their lower density, accumulated at the top, forming a distinct, cloudy layer. This layer was carefully collected using a wide-bore pipette tip to minimize contamination from other fractions, washed and further purified through additional centrifugation steps to remove contaminants and ensure the isolation of lipid droplets.

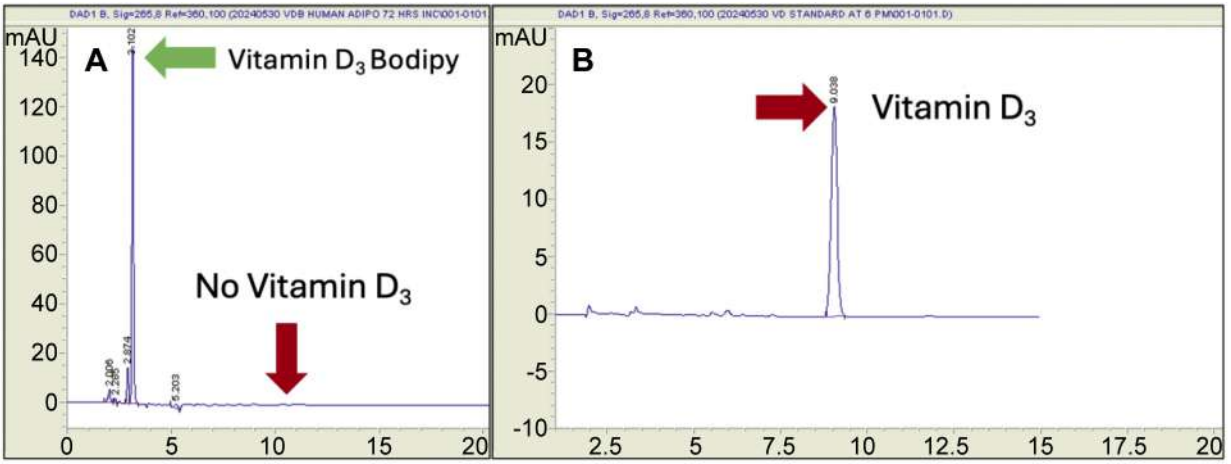

Figure 2. *HPLC chromatograms of the vitamin D_3 standard chromatographed on a straight phase column with 1% isopropanol in hexane (A) and a lipid extract from adipocytes exposed for 72 h with VitD-B chromatographed on a straight phase column with 1% isopropanol in hexane (B).*

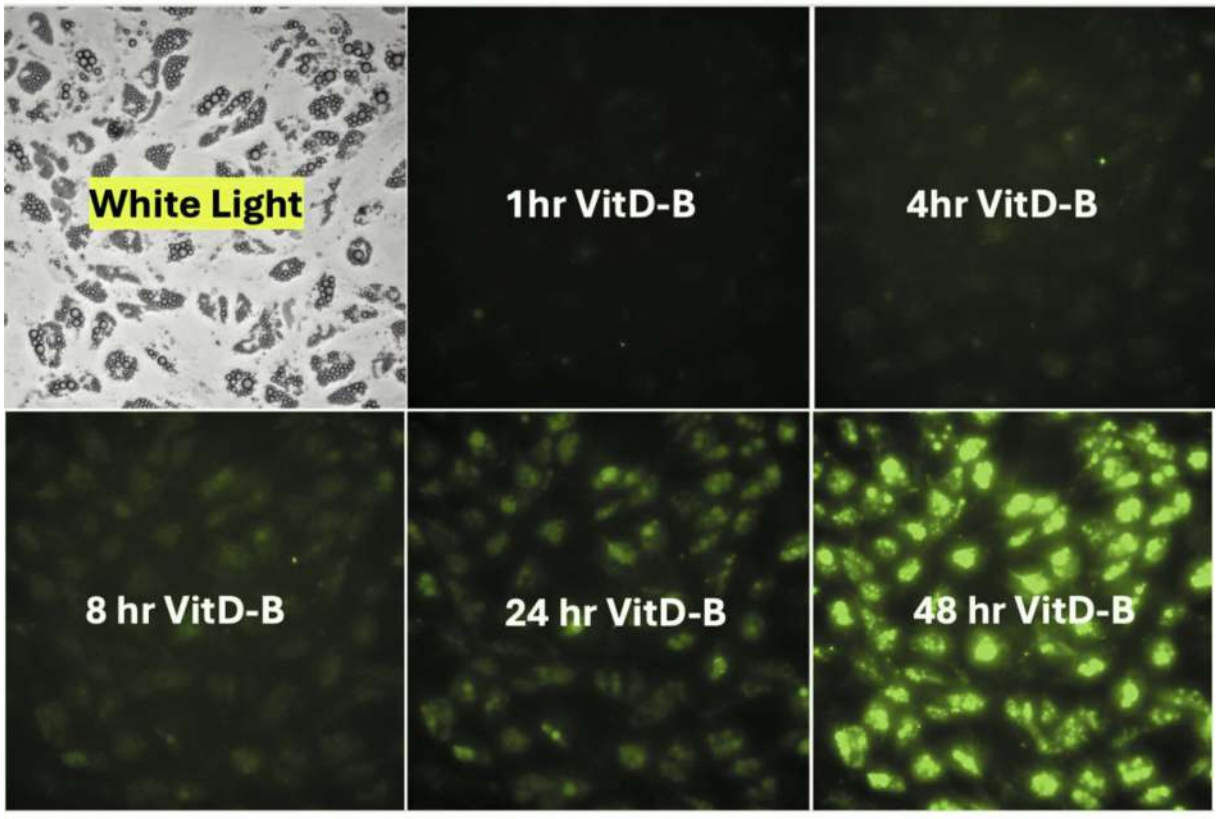

Figure 3. *100 nM VitD-B uptake into newly differentiated human adipocytes is shown in these images from the same culture dish. The images include white light and fluorescence images of the same differentiated human adipocytes incubated with VitD-B for 1, 4, 8, 24, and 48 h (30 ms, 488 excitation at 20× magnification).*

Microscopy. Cultured cells were plated into 35 mm glass bottom dishes (#1.5, 0.17 mm, MatTek, Ashland, MA, USA) and imaged using either a Nikon Deconvolution Wide-Field Epifluorescence microscope (Nikon Instruments, Tokyo, Japan) or a Leica SP5 Confocal Microscope (Leica Microsystems, Wetzlar, Germany). Images were recorded using NIS Elements software for acquisition parameters, shutters, filter positions, and focus control. Primary adipocytes and isolated lipid droplets were

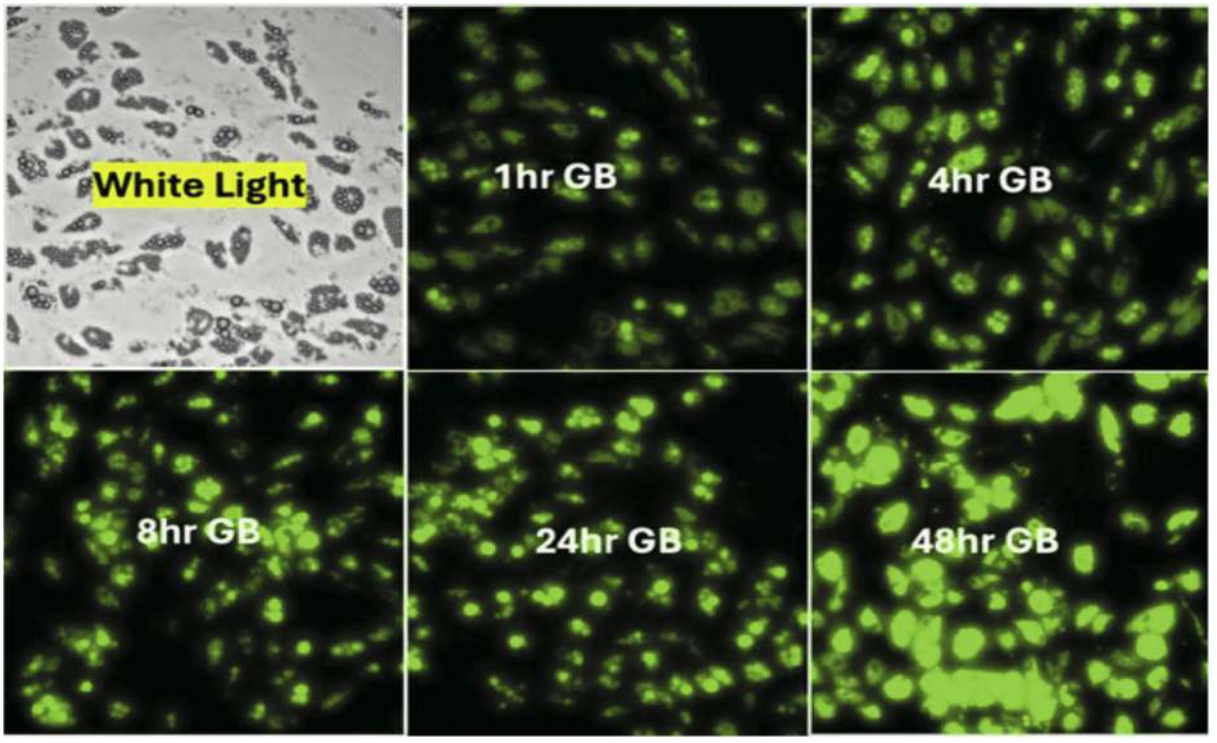

Figure 4. *Newly differentiated human adipocytes incubated with 100 nM GB. White light image and fluorescence images; 1, 4, 8, 24, and 48 h (985 μs, 488 excitation at 20× magnification).*

floated to attach to inverted glass bottom dishes coated with Cell-Tak (Corning).

High-performance liquid chromatography (HPLC). Following incubation with VitD-B or vitamin D, the cells were extracted for HPLC analysis. The cells were first harvested with 2 ml methanol. The cell lysate was then collected into tubes and dried under reduced pressure to concentrate the extracts and remove the methanol. The dried residue was reconstituted in 1 ml HPLC solvent for centrifugation. After removing cell debris, the samples were dried down again and reconstituted in 130 μl of solvent for HPLC analysis. All HPLC solvents were of analytical grade. HPLC was performed using Zorbax RX-SIL column. The solvent system used was 1% isopropanol (IPA) in hexane at a flow of 1.5 ml/min. Detection was carried out using a variable wavelengths UV detector at 265 nm with a minimum sensitivity 2.5 ng (14).

Results

Stability and uptake dynamics of vitamin D-BODIPY conjugate in human and mouse adipocytes. The present study makes use of vitamin D covalently linked to BODIPY through an ester linkage (VitD-B), which raises the possibility of cellular esterase activity separating the fluorescent tag from the vitamin D. In order to test the stability of VitD-B in adipocytes we incubated newly differentiated human adipocytes with 10 nM VitD-B for 72 h. The adipocytes were then subjected to lipid extraction and then analyzed by normal-phase HPLC using a 265 nm detector to determine the presence of free vitamin D_3. The HPLC chromatograph

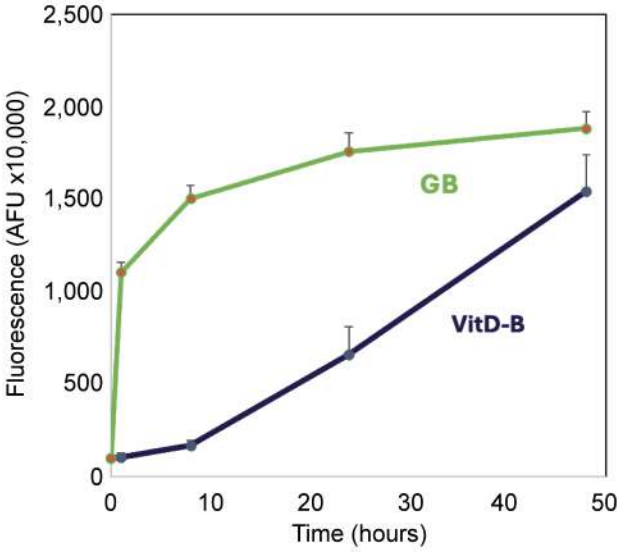

Figure 5. *Time course of 100 nM incorporation of VitD-B and GB into lipid droplets in newly differentiated adipocytes. The graphs represent an average of 30 individual measurements. (n=3, p<0.05).*

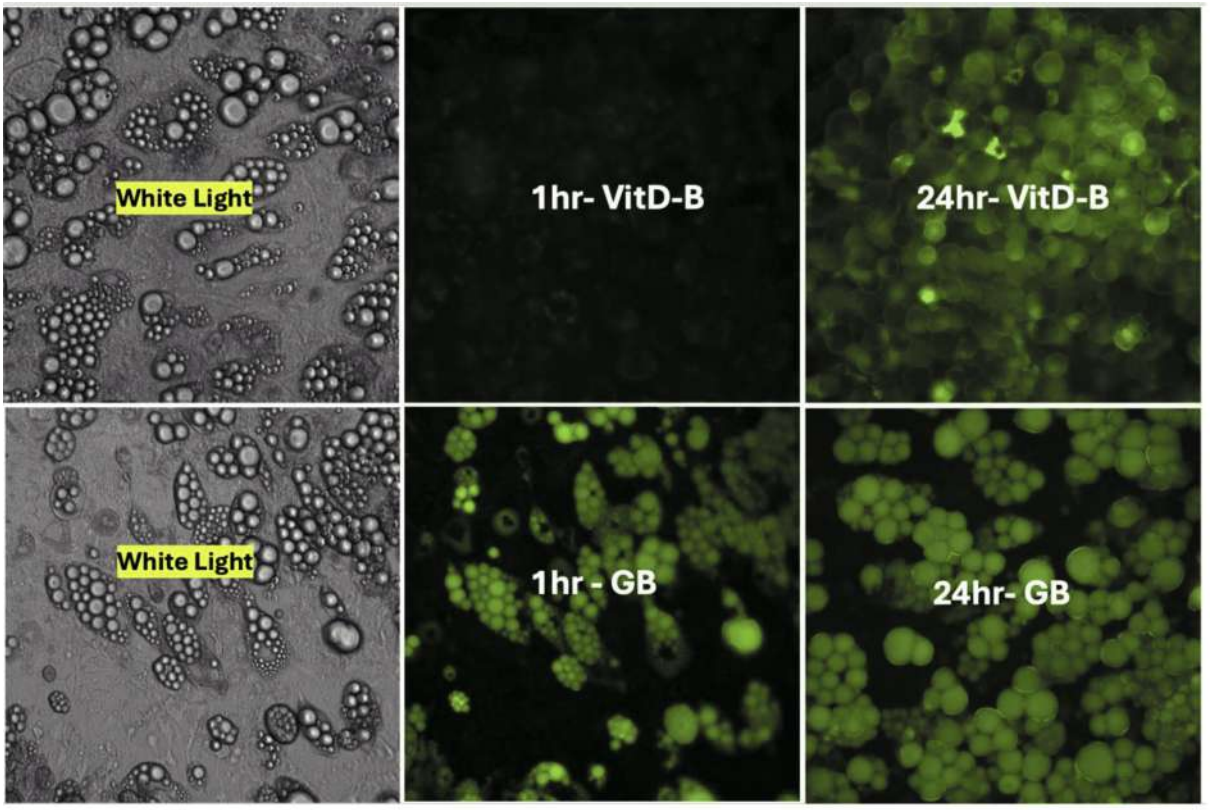

Figure 6. *Newly differentiated 3T3-L1 cells incubated with 100 nM VitD-B and GB. Fluorescence images at 1 h and 24 h. (Note: 1-h images were taken at 20× magnification, while 24-h images were captured at 40× magnification. Exposure times were normalized between conditions but not between time points, reflecting an important distinction in the imaging setup).*

showed that the nonpolar esterified VitD-B eluted with a retention time of 3.1 min (Figure 2A), with no perceptible peak where the standard vitamin D_3 eluted at 9.03 min (Figure 2B). The absence of detectable free vitamin D_3 in adipocytes incubated with VitD-B for 72 h demonstrates that VitD-B is stable and that BODIPY is not released from VitD-B by adipocyte esterases. This confirms previous findings with $1,25(OH)_2D_3$-BODIPY (15) and provides a useful tool to examine the distribution of VitD-B in the adipocyte as demonstrated below.

VitD-B uptake in newly differentiated human adipocytes and its accumulation in lipid droplets were observed over 48 h. In newly differentiated human adipocytes, VitD-B was barely visible in the lipid droplets after 1 h of incubation, with fluorescence intensity steadily increasing over the next 48 h (Figure 3). The stability of VitD-B supports that our fluorescent microscopy results are related to VitD-B, not BODIPY released from vitamin D. GB was clearly detected in lipid droplets within 1 hour and showed a comparable increase in intensity after 48 h (Figure 4). However, the time course and appearance of these two probes differ markedly. Figure 5 shows the time course of the uptake of VitD-B compared to GB in newly differentiated human adipocytes. VitD-B uptake was initially slow compared to GB but exhibited a linear 7-fold increase in uptake after 8 hrs while the uptake of GB was almost maximal at this time point. The fluorescence intensity of GB increased sharply within the first hour compared to VitD-B and then plateaued.

A similar study was conducted using mature 3T3-L1 cells with similar results. Following incubation with VitD-B, fluorescence was barely detected within lipid droplets after 1 h, with a continued increase in intensity observed over the subsequent 24 h. GB fluorescence was already close to maximal intensity in lipid droplets as early as 1 h post incubation and persisted for up to 24 h (Figure 6).

We then explored the association of VitD-B with primary mouse adipocytes isolated from subcutaneous fat tissue. Fluorescence uptake of VitD-B and GB into primary mouse adipocytes after 24 h incubation is shown in Figure 7. VitD-B was barely detected in cultured murine primary adipocytes after 1 h. Fluorescence intensity increased 1.2-fold at 8 h and 5.7-fold at 24 h compared to 1 h. For comparison, GB exhibited rapid fluorescence uptake into primary adipocytes that was increased 29-fold compared to VitD-B at 1 h (Figure 7 and

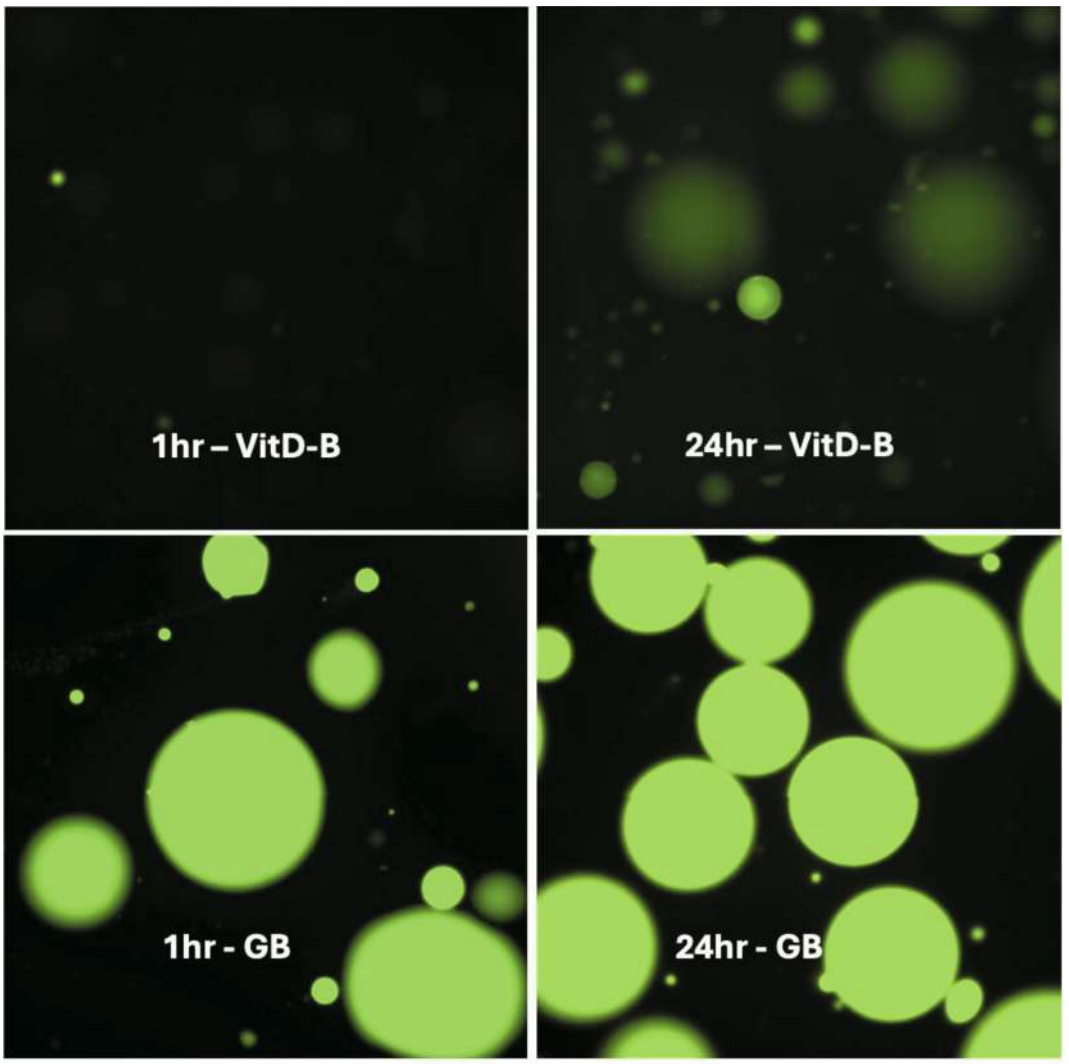

Figure 7. *Primary murine adipocytes incubated with 100 nM VitD-B and GB. Fluorescence images; 1 h and 24 h (1 h and 24 h pictures at 20× magnification under Nikon microscope).*

Figure 8). Uptake in mouse primary adipocytes was well matched to the observed uptake in differentiated preadipocytes from both human and mouse over 24 h. Figure 9 demonstrates that primary mouse adipocytes were sometimes observed with a halo around the lipid droplet after loading with VitD-B for 24 h, which was not seen surrounding the lipid droplet from mouse primary adipocytes treated with GB. This preliminary observation suggested the possibility that VitD-B was incorporated on the surface of the lipid droplet.

In order to demonstrate that VitD-B was a good surrogate for normal unlabeled vitamin D we incubated newly differentiated human adipocytes with vitamin D_3 at various durations: 2, 4, 8, 12, 24, and 48 h. The adipocytes were collected and underwent lipid extraction followed by analysis on straight phase HPLC. We observed a time-dependent increase in the concentration of vitamin D_3 in human adipocytes (Figure 10) similar to what was observed with human adipocytes incubated with VitD-B. This observation supports the use of VitD-B as a sensitive probe to mimic the uptake of vitamin D_3 into adipocytes and lipid droplets.

Rapid uptake of vitamin D-BODIPY in isolated lipid droplets. We then explored VitD-B uptake into freshly isolated lipid droplets. Intracellular lipid droplets were isolated following the method described by Brasaemle and Wolins (14). Subsequently, the isolated lipid droplets were attached to glass bottom dishes and incubated with VitD-B or GB. Unlike uptake into intact cells the fluorescence of both VitD-B and GB appeared immediately indicating rapid

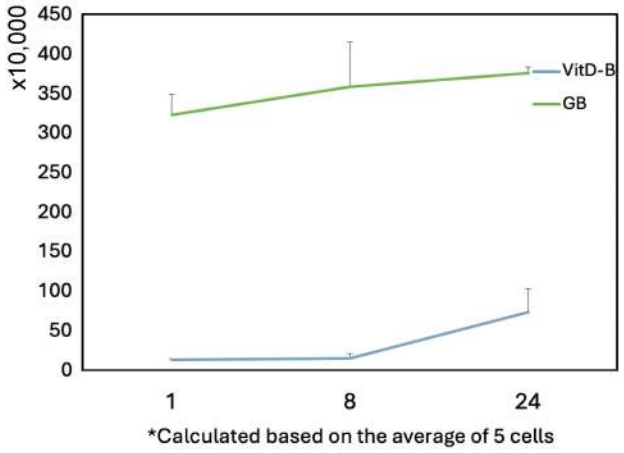

Figure 8. *Time course of 100 nM VitD-B and GB appearance in primary murine adipocytes. Fluorescence intensity was measured at 1, 8, and 24 h. The Y-axis represents fluorescence intensity (×10,000 units). The asterisk (*) indicates that the values were calculated based on the average fluorescence intensity of five individual cells per time point. Error bars represent the standard deviation of these measurements. It should be noted that these errors are based on measurements from five cells, not on experimental replicates across different experiments. Further clarification on whether these represent biological or technical replicates is needed for accuracy.*

uptake or deposition, respectively. VitD-B and GB fluorescence in isolated lipid droplets was measured after 5 min (Figure 11).

Discussion

Our study demonstrates the utility of fluorescently labeled Bodipy-vitamin D_3 (VitD-B) as a tool to investigate uptake of vitamin D_3 in adipocytes and partitioning into intracellular lipid droplets. Here we show that VitD-B is taken up into differentiated preadipocytes from both human and mice as well as isolated primary mouse adipocytes with a time course that matches that of unlabeled vitamin D_3 but much slower than GB used as a control. In contrast, VitD-B incorporation into isolated lipid droplets from adipocytes matched that of the GB control. Preliminary results that reveal increased VitD-B fluorescence intensity associated with the periphery of the intracellular lipid droplet require more scrutiny for confirmation but may provide insight into the distribution of vitamin D_3 within the lipid droplet of the adipocyte.

The time course of VitD-B accumulation in the adipocytes suggests that it is the vitamin D component and not the fluorescent label GB that regulates its cellular uptake and deposition in adipocytes. Our results suggest that human adipocytes, 3T3-L1 cells and primary murine adipocytes recognize the VitD-B differently than GB (Figure 3, Figure 4, Figure 6, and Figure 7) implying that there is a selective mechanism of uptake and processing of VitD-B that is different from GB.

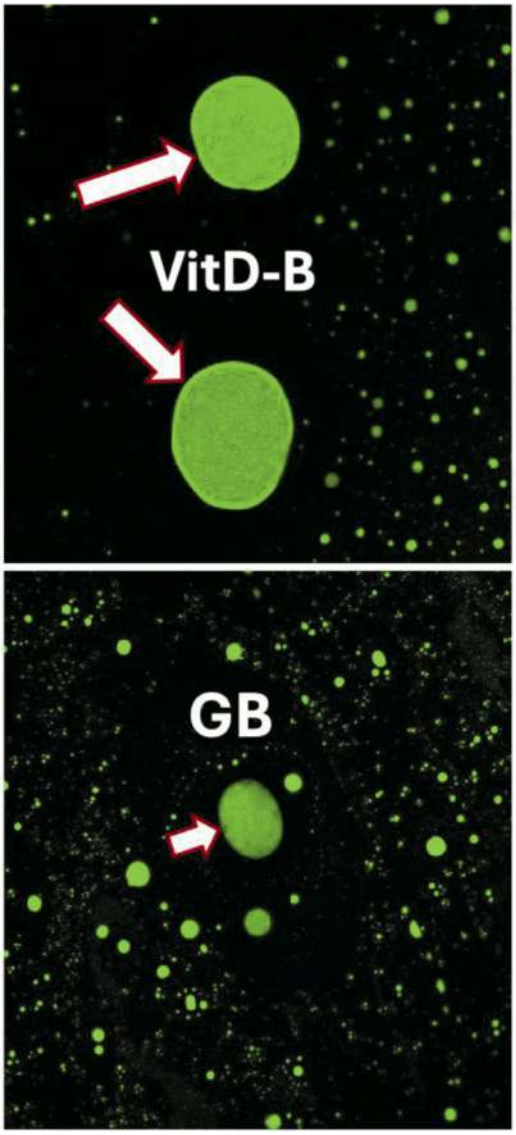

Figure 9. *Leica confocal fluorescence microscopy of primary adipocytes incubated with 100 nM VitD-B and GB for 24 h; arrows demonstrate halo around the lipid droplets.*

The immediate uptake of VitD-B and GB into isolated lipid droplets compared to the much slower uptake into intact adipocytes may be due to the uptake and incorporation into lipid being limited by the adipocyte plasma membrane (PM). After cellular uptake through the PM, the association of VitD-B with intracellular lipid may be very rapid and indistinguishable from the GB control. One reasonable alternative is that proteins regulating association of vitamin D_3 with the intracellular lipid droplet may be lost during

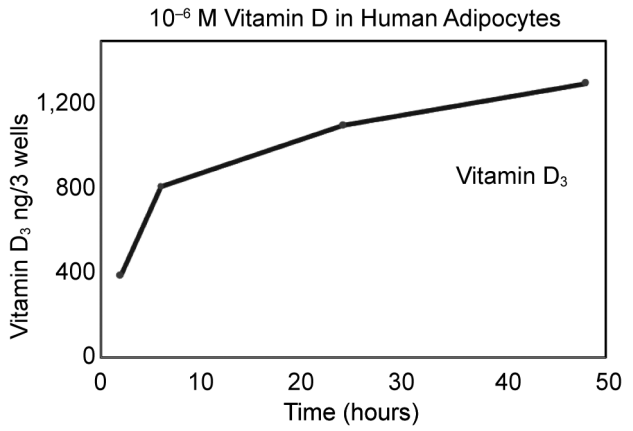

Figure 10. *Time course of vitamin D_3 appearance in newly differentiated human adipocytes incubated with 1 μM vitamin D_3, determined by HPLC. Each time point represents the mean of three technical replicates.*

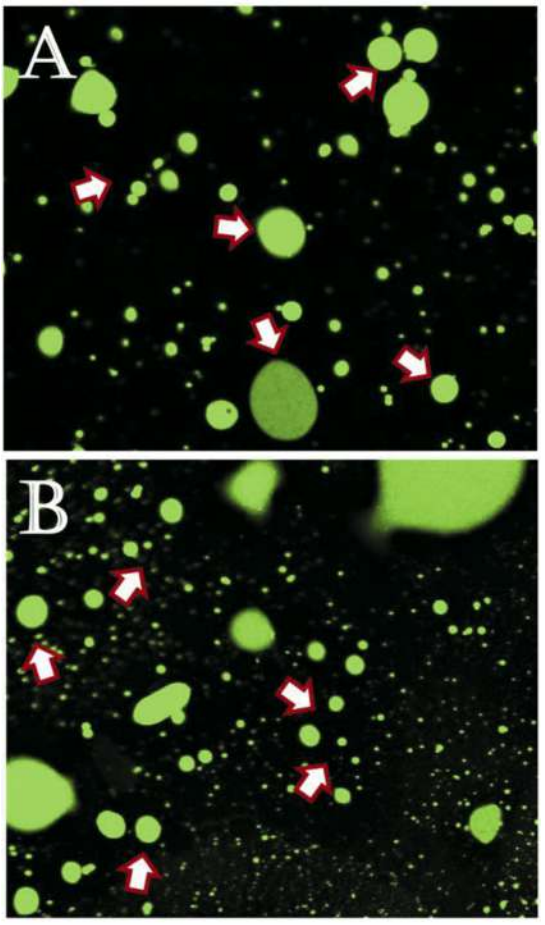

Figure 11. *Isolated lipid droplets incubated with 100 nM VitD-B (Panel A) and GB (Panel B). Arrows indicate selected lipid droplets among the population. While all the bright green structures represent lipid droplets, the arrows are used to highlight their presence and distribution within the field of view. Panel A shows the distribution of lipid droplets following incubation with VitD-B, while Panel B shows lipid droplets following incubation with GB. Differences in fluorescence intensity and droplet size can be observed between the two conditions.*

lipid droplet isolation. These proteins may be cytosolic binding proteins or constituents of the protein coat enveloping the lipid droplet. The isolation process may have thus disrupted mechanisms of transport, potentially affecting the observed differences in lipid uptake.

The preliminary observation of a halo around the lipid droplets in adipocytes treated with VitD-B suggests that VitD-B may be accumulating on the surface of the lipid droplets, similar to cholesterol (16). This halo effect was not seen in lipid droplets of adipocytes treated with GB or on isolated lipid droplets. Further experiments are necessary to confirm these findings providing additional evidence of cellular regulation of VitD-B association with intracellular lipid droplets.

Importantly, the uptake of vitamin D_3 measured by HPLC was similar to the uptake of VitD-B in adipocytes, as observed through fluorescence microscopy. This finding supports the use of VitD-B as a reliable surrogate for vitamin D_3 in studying its mechanisms of uptake into adipocytes and association with intracellular lipid droplets. These results also suggest a potential link between the accumulation of vitamin D in adipocytes and its sequestration in adipose tissue, particularly in obesity, which may contribute to vitamin D deficiency. As obesity increases adipose tissue surface as well as mass, more vitamin D may become sequestered, limiting its bioavailability and leading to deficiency. Investigating the impact of vitamin D on lipolysis and adipocyte function could therefore have significant therapeutic implications for addressing both metabolic dysfunction and vitamin D deficiency (17).

While this study provides valuable insights into the uptake and accumulation of VitD-B in adipocytes, several limitations should be acknowledged. First, the use of in vitro models, including human preadipocytes, 3T3-L1 cells, and primary murine adipocytes, may not fully replicate the complexity of adipose tissue function *in vivo*, where systemic factors, such as hormonal regulation and nutrient availability, play a significant role. Additionally, the isolation of lipid droplets may disrupt key proteins that regulate the interaction between vitamin D_3 and intracellular lipid storage, potentially affecting our observations. Although VitD-B serves as a useful surrogate for vitamin D_3, differences between the labeled compound and natural vitamin D_3 could influence the accuracy of our findings. Furthermore, the study's relatively short duration (up to 48 h) limits our understanding of longer-term dynamics, particularly in the context of obesity, where vitamin D_3 sequestration in excess adipose tissue may contribute to deficiency. Finally, while we explored the uptake of VitD-B, the functional implications of vitamin D_3 storage

and release within adipocytes were not assessed. Future studies should address these limitations by investigating longer time courses, employing obesity-specific models, and exploring the bioavailability and functional consequences of stored vitamin D_3.

Conflicts of Interest

Michael F. Holick receives grants from CARBOGEN AMCIS and Solius Inc is a consultant for Solius Inc., Ontometrics Inc. Adela Inc. KBD Inc. and Biogena and is a member of the speaker's bureau for Pulse LTD, Sanofi, and Menarini Inc. Nazlı Uçar received a grant from CARBOGEN AMCIS. Peter M. Mueller is consultant for CARBOGEN AMCIS. Ralf Loo is an employee of CARBOGEN AMCIS. Jude T. Deeney and R. Taylor Richard Pickering have no conflicts of interest.

Authors' Contributions

M.F. Holick, J.T. Deeney, P.M. Mueller, R.T. Pickering, N. Uçar participated in the design, interpretation of the experiments, and the writing of this manuscript. J.T. Deeney, R.T. Pickering, N. Uçar participated in conducting the experiments. R. Loo synthesized the vitamin D- BODIPY. M.F. Holick provided final approval. Ting-Yu Fan provided mouse adipose tissue and training in isolation.

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