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Evaluation of a novel light-emitting diode device for producing vitamin D

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EVALUATION OF A NOVEL LIGHT-EMITTING DIODE DEVICE FOR PRODUCING VITAMIN D

by

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2015
DEDICATION

I would like to dedicate this thesis to my loving family, friends, and Gurudev, who have invested so much in me to ensure I could have the opportunity to learn and grow endlessly.
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Vitamin D is a fat-soluble hormone essential for humans as it is a key player in calcium and phosphorus homeostasis for bone mineralization, and is linked to many nonskeletal health outcomes such as autoimmune diseases and cardiovascular disease as well. The primary source of vitamin D is the conversion of 7-dehydrocholesterol (7-DHC), which naturally exists in the plasma membranes of skin cells, to previtamin D₃ by the exposure to the ultraviolet-B (UV-B) portion of sunlight. Despite humans’ ability to cutaneously synthesize vitamin D, many factors limit this process, and consequently vitamin D deficiency has become a common medical issue worldwide. Deficient individuals may not respond well to traditional vitamin D replacement through dietary supplementation if suffering from fat malabsorption syndromes while unable to get sufficient vitamin D from sun exposure due to location, sunscreen use, or cultural practices, among other reasons.

It has been previously reported that exposure to artificial sources of UV-B radiation (UV lamps, tanning beds, among others) produces cutaneous vitamin D, but heat generation, poor portability, and other inconveniences to the deficient patient limit therapeutic use of these devices. In addition, broad-spectrum sources of UV radiation reduce the production of vitamin D compared to narrow-band sources because of the photoequilibrium that is established. The advent of the light-emitting diode (LED)
provided a compact, energy-efficient, high-intensity, low-heat alternative radiation source, and the recent development of the UV LED offers a viable alternative for developing a personalized vitamin D-producing device.

This thesis presents evidence that UV LEDs have the capacity to efficiently synthesize vitamin D₃ in vitro and in human skin. Ampoules of 7-DHC were irradiated in triplicate with LEDs at 280, 285, 290, 295, 298, 300, and 310 nm. The 298 nm LED was found to have the most efficient previtamin D₃ production of 7.0% in vitro at the equivalent of 0.75 minimal erythemal dose (MED, rated at 1 MED = 32 milliJoules per centimeter squared for type II skin), compared to all other assessed LEDs. Irradiation of human skin samples (IRB-exempt) with the 298 nm diode (~39 seconds of radiation) indicated that 1.5% of the original 7-DHC in type II (Caucasian) skin could be converted to vitamin D₃ in situ after exposure to 0.75 MED. These results imply that manufacturing a cuff containing 298 nm LEDs that covers 3.8% of the total surface area of skin could provide 600 IUs of vitamin D₃ if operated for just 39.0 seconds. The data provide a promising new approach to treat vitamin D-deficient patients suffering from fat malabsorption syndromes.
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The percent conversion of 7-DHC to tachysterol$_3$ over 10 minutes for skin types II, III, and IV after irradiation with the 298 nm LED
LIST OF ABBREVIATIONS

BMI ................................................................. Body Mass Index
BU ........................................................................ Boston University
BUMC ............................................................... Boston University Medical Center
DBP ...................................................................... Vitamin D Binding Protein
CYP27B1 ............................................................ 25-hydroxyvitamin D-1α-hydroxylase
DNA ................................................................. Deoxyribonucleic acid
EtAc .................................................................. Ethyl Acetate
Fig. ...................................................................... Figure
HPLC .................................................................. High-Pressure Liquid Chromatography
IOM ...................................................................... Institute of Medicine
IPA ...................................................................... Isopropyl Alcohol
IU ......................................................................... International Units
LED ...................................................................... Light-Emitting Diode
mA ........................................................................ Milli-Amperes
MED ...................................................................... Minimal Erythematic Dose
Nm ....................................................................... Nanometers
PreD3 .................................................................... Previtamin D3
PTH ...................................................................... Parathyroid Hormone
RANK .................................................................... Receptor Activator of NFκB
RANKL ............................................................... Receptor Activator of NFκB Ligand
INTRODUCTION

Evolutionary Perspective of Vitamin D

Vitamin D, the “sunshine vitamin”, is potentially the oldest hormone in existence, supported by the discovery of some of the earliest phytoplankton species on earth can make vitamin D from natural stores of ergosterol when exposed to sunlight. While the reasons for vitamin D production in nonvertebrates are not yet fully understood, it likely plays a key role in survival.\(^1\) In fact, ergosterol and its photoproducts efficiently absorb ultraviolet-B (UV-B) radiation at wavelengths between 280-315 nanometers (nm) which are capable of damaging deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins. As a result, ergosterol likely developed to act as a natural sunscreen to protect the organism from the harmful radiation.\(^2\)

Calcium is essential for healthy bone mineralization in the skeletal structure of vertebrates. As evolution proceeded, organisms left the calcium-rich oceans to explore land, where calcium was only available in plants that could extract it from the soil. To adapt to their calcium-poor surroundings and maintain skeletal health, they needed to increase the efficiency of intestinal calcium absorption from dietary plant sources. Vitamin D evolved to fulfill this role.\(^3\) When these early land-dwelling organisms were exposed to sunlight on their skin, provitamin D\(_3\) (7-dehydrocholesterol or 7-DHC) and/or ergosterol in their plasma membranes converted to vitamin D. For reasons not well understood, this photosynthetic process became responsible for modulating calcium absorption. Today, most land-dwelling species require sunlight for vitamin D production, especially humans.\(^2,3\)
**Vitamin D Sources**

While there are multiple forms of vitamin D, the predominant forms are D\textsubscript{2} and D\textsubscript{3} (derived from ergosterol and 7-DHC, respectively), referring to differences in side chain structure (Fig. 1). 7-DHC is the principal precursor for cutaneous synthesis in animals; it exists in human skin, where it is converted to vitamin D\textsubscript{3} upon exposure to sunlight. This being said, the primary source of vitamin D is exposure to sunlight.\textsuperscript{4,5}

![Fig. 1. Structures of vitamin D\textsubscript{2} and D\textsubscript{3} and their respective precursors, ergosterol and 7-DHC. Both forms of vitamin D differ only by their side chain structures. Reproduced from (21).](image)

Very few foods are natural sources of vitamin D. These include salmon, mackerel, herring, and other oily fish. Mushrooms and yeast contain ergosterol, which converts to previtamin D\textsubscript{2} upon UV-B irradiation. Vitamin D\textsubscript{3} used in food fortification can be synthesized from lanolin and added to select foods including milk, cheese, yogurt, and cereals.\textsuperscript{3-6} Supplemental forms (as solutions, injections, and dietary capsules) of
vitamin D$_2$ and D$_3$ are available as well.$^5$ Both vitamin D$_2$ and D$_3$ are equally effective in maintaining vitamin D levels.$^{2,3}$

**Photobiology of Vitamin D**

7-DHC naturally exists in the plasma membranes of epidermal keratinocytes and dermal fibroblasts of human skin.$^{4-6}$ 7-DHC will absorb UV-B photons upon exposure to sunlight, causing the cleavage of the C$_9$–C$_{10}$ bond in the B ring and thereby forming a 9,10*-seco*-steroid, S-cis,S-cis-previtamin D$_3$. $^{3,7}$ This photolytic product is thermodynamically unstable and subsequently undergoes a rearrangement of double bonds to form the stable vitamin D$_3$. $^{1-7}$ Interestingly, the cell membrane is critical for cutaneous vitamin D production. The S-cis,S-cis conformer of previtamin D$_3$ (preD$_3$) is energetically unstable and isomerizes to the S-trans,S-cis conformer *in vitro*; however, only the S-cis,S-cis conformer can thermally isomerize to vitamin D$_3$. *In situ* human skin
studies reveal that hydrophilic and van der Waals interactions in the phospholipid bilayer of the cell membrane stabilize the $S$-cis,$S$-cis conformer so it does not become $S$-trans,$S$-cis (Fig 2), indicating the importance of the cell membrane for vitamin D$_3$ production.$^3$

Evidence indicates that skin has a very large capacity to produce vitamin D; in a bathing suit (nearly 100% of the surface area of the body), exposure to enough sunlight that causes a minimal erythemal dose (MED) generates enough vitamin D equivalent to orally consuming at least 10,000 international units (IU).$^8$ Thus, exposure of 1% of the body can provide 100 to 250 IUs of vitamin D$_3$. Any alterations to the cutaneous presence of 7-DHC will influence photosynthesis of vitamin D.$^{1,4,8}$ Previous findings show that the amount of 7-DHC in skin declines with age. In fact, a 70 year-old individual will make one-fourth of the vitamin D that a 20 year-old individual would make with the same amount of sun exposure.$^{2,4,6,8}$ Cutaneous production also varies based on the ability of UV-B photons to penetrate the skin.$^{4,8}$ Melanin, the body’s natural sunscreen which absorbs UV-B, is present in higher quantities in heavily pigmented individuals. Because melanin can “compete” with 7-DHC for absorption of UV-B photons, individuals with darker skin require a greater time of sun exposure to synthesize a comparable amount of vitamin D to their lighter-skinned counterparts.$^{5,8}$ Sunscreens serve to protect skin from UV radiation and, when applied, will absorb UV-B before skin can.$^{6,9}$ A sunscreen with a sun protection factor of 8 (SPF-8) absorbs 92-95% of UV-B, therefore diminishing the capacity for vitamin D synthesis.$^{1,3}$ Given that only the UV-B portion of sunlight is responsible for producing vitamin D, the solar zenith angle is also an important determinant.$^{6,9}$ When the sun is directly above us in the
summer at noontime, 1-5% of solar UV-B radiation is able to reach the surface; as the solar zenith angle becomes more oblique with changes in latitude, season, and time of day, this amount decreases because more UV-B is absorbed by the ozone layer, diminishing the capacity for cutaneous vitamin D synthesis. This accounts for the minimization of vitamin D production during the early morning before 10 AM and in the afternoon after 3 PM, and in the winter above 37° North and below 37° South latitudes. If the ozone layer does not absorb UV-B, pollution can. Vitamin D deficiency is prevalent in areas of widespread pollution, especially in large cities.

Prolonged sun exposure cannot produce excessive vitamin D₃, because any preD₃ or D₃ that remains in skin and does not reach circulation will absorb solar UV radiation and isomerize to several biologically inactive photoproducts. Upon UV absorption, excess vitamin D₃ isomerizes to suprasterol I or II, or 5,6-transvitamin D₃, while excess preD₃ isomerizes to tachysterol₃ and lumisterol₃ (Fig. 3A). MacLaughlin et al. demonstrated that when 7-DHC is converted to preD₃ upon exposure to sunlight in a light-skinned Caucasian, the amount of preD₃ reaches a plateau after about 15 minutes and a subsequent rise in tachysterol₃ and lumisterol₃ is observed. The production of preD₃ and these photoisomers is dependent on the wavelength of UV radiation, due to differences in the UV absorption characteristics of each. The optimal wavelengths for preD₃ production are between 295 and 300 nm, with a maximum around 297 nm (Fig. 3B). In situ studies revealed that human skin exposed to narrow-band radiation at 295±5 nm can yield a maximum conversion to preD₃ of 60±5 percent of the original concentration of 7-DHC, while tachysterol and lumisterol were about 25 and 5 percent
Fig. 3. (A) Photosynthesis, photoisomerization, and photodegradation of vitamin D$_3$ from 7-DHC. (B) The action spectrum of preD$_3$ formation from 7-DHC in human skin. Note that PreD$_3$ formation reaches an apparent maximum around 297-298 nm. Adapted from (10) and (11).

Interestingly, simulated solar radiation only results in a maximum of 15 to 20 percent, while tachysterol and lumisterol were at 3 to 6 and 50 to 60 percent respectively, suggesting that the spectral character of sunlight may influence the photosynthesis of vitamin D$^{11,12}$. These studies suggest that narrow-band radiation could potentially be an ideal substitute for sun exposure, especially for individuals who may have limited sun exposure and/or intestinal malabsorption preventing supplementation.
Metabolism of Vitamin D

Vitamin D$_2$ and vitamin D$_3$ are biologically inert until activated. Once vitamin D$_2$ is ingested or vitamin D$_3$ synthesized in the skin, it is drawn into the dermal capillary bed by the vitamin D binding protein (DBP) and enters circulation. It travels to the liver where it is converted to 25-hydroxyvitamin D [25(OH)D], the major circulating form of vitamin D assayed to determine vitamin D status. 25(OH)D is biologically inert, so it travels to the kidneys where it is metabolized to the active form 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1). The renal production of active vitamin D is controlled by serum parathyroid hormone (PTH), calcium, and phosphate levels.

Upon activation, 1,25(OH)$_2$D travels in the circulation to its vitamin D receptor (VDR) in target tissues that regulate calcium and phosphate homeostasis. In the intestine, the interaction of vitamin D with its VDR enhances the expression of an epithelial calcium channel, thereby increasing intestinal absorption of calcium; it also stimulates phosphate absorption. The interaction also increases phosphorus absorption and the expression of other proteins that enhance calcium absorption, like calbindin9k. When 1,25(OH)$_2$D interacts with its VDR in skeletal osteoblasts, it increases the expression of RANKL (receptor activator of NFκB ligand), which interacts with the RANK receptor of monocytic preosteoclasts and induces the formation of mature osteoclasts that act to release calcium from bone. In the kidneys, 1,25(OH)$_2$D acts to increase calcium reabsorption. Finally, 1,25(OH)$_2$D regulates PTH production by negative feedback in the parathyroid glands. In all, the major physiologic function of
vitamin D is the regulation of calcium and phosphorus homeostasis, not only for skeletal health but for many other metabolic functions in the body. VDRs are present in numerous tissues not involved in calcium and phosphorus metabolism\textsuperscript{18,19}, and although vitamin D action is only understood for a few tissues, this broadens the scope for other possible actions of vitamin D.

**Vitamin D Deficiency and Health Consequences**

Today, vitamin D deficiency is considered a worldwide pandemic, with an estimated prevalence of upwards of 50\% deficient individuals in North America, Europe, Asia, New Zealand, and Australia.\textsuperscript{3,4,6} Ample exposure to unpolluted sunlight could fulfill humans’ vitamin D requirement. However, the advancement of civilization and the Industrial Revolution leading to extensive pollution blocking human exposure to UV-B has made vitamin D a dietary necessity, and this brought about the emergence of widespread deficiency.\textsuperscript{20} Given that vitamin D is classically associated with the development of a healthy skeleton, severe deficiency results in poor bone mineralization, causing rickets in children and osteomalacia in adults. Deficiency has also been linked to the development of secondary hyperparathyroidism, as well as a greater risk of fracture.\textsuperscript{19,21} However, VDRs are found in many tissues not involved in calcium and phosphate metabolism (e.g. the heart, brain, pancreas, and immune cells, among others),\textsuperscript{21-23} suggesting the potential health consequences of vitamin D deficiency extend much further than skeletal health. Mutations in the VDR gene have been associated with the risk of developing cardiovascular disease, cancer, type 1 diabetes mellitus, and
immune disorders. In fact, VDR ligands, mainly 1,25(OH)$_2$D, induced antiproliferative, prodifferentiative, and immunomodulatory effects in clinical and experimental settings, and these effects may not be possible without sufficient 25(OH)D.

There has been considerable debate over the precise definition of vitamin D deficiency, but the Institute of Medicine (IOM) suggests that according to existing literature, a serum concentration of 20 ng/mL is adequate for skeletal health. On the other hand, the Endocrine Society Practice Guidelines indicate that 20 ng/mL is the threshold for deficiency, between 21 and 29 ng/mL is for insufficiency, and 30 ng/mL is for sufficiency, citing the latter as the threshold to reap all potential benefits of vitamin D. The IOM, however, has not changed their recommendation on the grounds that there is insufficient evidence supporting extraskeletal benefits of vitamin D. For this thesis, a circulating 25(OH)D less than 20 ng/mL will be used as the standard indicating deficiency.

There are a host of possible causes for vitamin D deficiency. As mentioned earlier, factors that affect either 7-DHC content or the amount of UV-B radiation penetrating the skin will modulate cutaneous synthesis of vitamin D$_3$: age, increased melanin content, sunscreen, solar zenith angle, and clothing. Interestingly, there is a high prevalence of osteomalacia and rickets in Saudi Arabian women and children, respectively, likely spurred by cultural traditions of clothing the entire body. The bioavailability of vitamin D supplementation is also modulated by several factors. The use of certain medications, including bile acid sequestrants, antiseizure medications, and
glucocorticoids, can decrease bioavailability.\textsuperscript{28,29} Obesity yields low bioavailability as well; serum 25(OH)D level negatively correlates with body mass index (BMI) and body fat content, likely due to fat deposits acting as “sinks” for the fat-soluble vitamin D.\textsuperscript{30,31} Studies have also revealed that patients with intestinal/fat malabsorption syndromes, such as Crohn’s disease, Whipple’s disease, and cystic fibrosis, are also at high risk for deficiency, likely for similar reasons.\textsuperscript{32,33} Major causes and health outcomes of vitamin D deficiency are pictorially represented in Figure 4.

*Figure 4.* Major causes of vitamin D deficiency and health consequences. Reproduced from (23).
Potential Use of UV Light-Emitting Diodes for Vitamin D Deficiency

Taking into account the many determinants of efficient cutaneous synthesis ranging from location to cultural practices, exposure to sunlight may not provide adequate vitamin D for many individuals who have limitations to cutaneous synthesis. While dietary supplementation could serve a viable substitute for these situations, individuals with intestinal malabsorption syndromes or taking certain medications may not be able to effectively extract vitamin D from dietary sources. In these instances, UV phototherapy could enhance cutaneous synthesis of vitamin D, especially in the winter months when vitamin D photoproduction is low. In fact, in 1919, Huldschinsky demonstrated that rickets could be cured by exposure to sunlight or artificial sources of UV radiation. After mounting evidence in the following years, it became widely accepted that vitamin D requirements for skeletal health could be fulfilled by artificial UV exposure.\textsuperscript{34} Broad-spectrum UV-B radiation mimicking the UV-B range of sunlight (280-320 nm) is recommended in the treatment of vitamin D deficient psoriasis, but 25(OH)D levels are attenuated compared to supplementation\textsuperscript{35}, likely because the spectral character of sunlight is shown to decrease the potential for cutaneous synthesis compared to narrow-band radiation.\textsuperscript{11,35} In fact, supplemental vitamin D increased the blood level of 25(OH)D by 74% compared to the broad-spectrum radiation at 36%.\textsuperscript{35} A pilot study of an 8-week tanning lamp phototherapy for patients with malabsorption syndromes showed increases in vitamin D levels, but the results were suboptimal,\textsuperscript{36} likely for similar reasons. This again indicates that narrow-band radiation could be a better candidate for artificial radiation-based cutaneous production of vitamin D.
Light-emitting diodes (LEDs) are narrow-band artificial light sources that have gained considerable attention in recent years for their high efficiency, long lifetime, and low heat production compared to incandescent or fluorescent lighting. Visible LEDs are already used for a variety of lighting applications, but the production of a blue or UV LED was significantly more difficult until Akasaki, Amano, and Nakamura found that gallium nitride crystals were pivotal for stable UV emission in 1992, for which they received the Nobel Prize in Physics in 2014. UV LEDs have since found relevance in water purification, disinfection of medical tools, and medical diagnostics. Beyond these existing applications, UV LEDs could be used to enhance the skin’s naturally immense capacity for synthesizing vitamin D. Such an application could be especially useful for vitamin D deficient individuals with malabsorption syndromes and unable to get sufficient sun exposure.

**Goals of this Thesis**

Vitamin D deficiency is already considered a pandemic, spurred by the many factors limiting the cutaneous synthesis of vitamin D$_3$. Patients with fat malabsorption are at an especially high risk for deficiency because the effectiveness of vitamin D replacement by way of dietary supplementation is significantly attenuated. Treatments using artificial UV-B exposure enhance cutaneous synthesis of vitamin D$_3$ for these patients, but these sources of radiation suffer from energy inefficiency, poor portability, and other issues. UV LEDs do not have many of these limitations and can therefore serve as efficient devices that enhance the synthesis of vitamin D$_3$ in skin. The primary
goal of this thesis was to evaluate and characterize the effect of UV LEDs that emit different wavelengths on the production of previtamin D$_3$ \textit{in vitro} and \textit{in situ}. Based on the \textit{in vitro} data, the best LED candidate was used to determine the potential for vitamin D synthesis \textit{in situ}. The evaluation of these diodes will serve as a benchmark for future manufacturing of LED-based devices such as a portable, removable cuff that can be used for the treatment and prevention of vitamin D deficiency in patients with fat malabsorption syndromes.
MATERIALS AND METHODS

Materials

Crystalline 7-DHC (Fluka Biochemika, HPLC-grade) was dissolved in ethanol (Fisher Scientific, HPLC-grade) and diluted to a concentration of 50 µg/mL. Clear glass ampoules (Wheaton, 2 mL, pre-scored) were filled with 250 µL of this solution, sealed with Parafilm, and stored in a -80°C freezer for use throughout the project.

Human skin samples were obtained at random from surgical procedures (IRB-exempt approval) at Boston Medical Center’s Department of Plastic Surgery. Samples within the same skin type were from the same patient, but information on patient characteristics was not known. Samples were organized by traditional skin pigment classification (Type II – Caucasian, Type III – Indian, Type IV – African American). Samples were stored in a -80°C freezer immediately after they were obtained.

Diodes with peak wavelengths of 280, 290, 295, 300, and 310 nm (all ± 5 nm) were purchased from Sensor Electronic Technology, Inc (SETi). An additional LED with a peak wavelength of 285 nm (± 5 nm) was obtained from the Boston University Photonics Lab, and another with a peak at 298 nm (± 5 nm) was obtained from DOWA Electronic Materials Company, Ltd. All LEDs had a maximal current of 20 milli-Amperes (mA). All LEDs were powered by a current-limiting power source (Boston University Photonics Lab) to ensure the current did not exceed 20 mA, regardless of the voltage applied.
High-pressure liquid chromatography (HPLC) is a method that separates compounds on the basis of polarity. Because of this, the photoproducts of 7-DHC and their photoisomers have different retention times on the HPLC columns, allowing for effective separation. In this study, separation was achieved using an HPLC system (Agilent, 1100 Series) equipped with a variable wavelength detector. A UV response at 265 nm was used to quantify preD₃ and vitamin D₃, while a response at 280 nm was used to quantify 7-DHC, tachysterol₃, and lumisterol₃.

HPLC-grade solvents such as isopropyl alcohol (IPA), ethyl acetate (EtAc), and hexane obtained from commercial sources (Acros, American Bioanalytical, and Fisher Scientific, respectively) were used to prepare the mobile phases used in the HPLC analysis.

**In vitro Irradiation of 7-DHC Ampoules and Separation of Photoproducts**

7-DHC ampoules were positioned such that the bottom of the ampoule was consistently kept 5.0 mm above the LED light source (Figure 5). The MED output of each LED was measured with a SolarMeter® Digital UV Radiometer (Solartech, Inc.) measuring MEDs/hr at a distance of 5.0 mm. The specifications of this digital meter rated 1 MED/hr as equivalent to 21 milliJoules per cm² (mJ/cm²). Ampoules were irradiated for 1, 3, 5, 10, and 25 minutes (repeated measures with three ampoules of the same 7-DHC solution per time point), and LEDs were operated at a constant maximal current of 20 mA.
After irradiation, the contents of the 7-DHC ampoules were transferred to test tubes. The samples were dried under nitrogen gas, re-dissolved in 140 µL of 0.8% IPA in hexane, and transferred to auto-sampler vials. The samples then underwent single normal-phase HPLC analysis using a silica stationary phase column (Agilent ZORBAX Rx-SIL, 5 µm pore size, 4.6 x 250 mm), and a mobile phase of 0.8% IPA in hexane with a flow rate of 1.5 mL/min achieved separation of photoproducts. The UV detector set at 265 nm was used to detect the presence of previtamin D₃, 7-DHC, tachysterol₃, and lumisterol₃. Integration of the peaks provided areas under the curve (AUC) for these photoproducts whenever present. The amount of each photoproduct was determined by comparing AUCs to standard curves. From this data, the percent conversion of 7-DHC to preD₃ and other photoproducts was determined.
In situ Irradiation of Human Skin Samples and Separation of Photoproducts

Based on the results from the in vitro phase, skin samples were irradiated with the UV LED with the greatest efficiency for producing preD$_3$. As shown in Table 1, this was determined to be the 298 nm LED. Type II, III, and IV skin samples (obtained from BMC Dept. of Plastic Surgery, IRB-exempt) were positioned upside-down 5.0 mm above the diode (over a hole in a platform with an area of approximately 0.45 cm$^2$) ensuring the outer epidermal layer was exposed to the radiation (Figure 6). Samples for each type were from the same patient. Samples were irradiated for 5 and 10 minutes. Unfortunately, repeated measures could not be performed for skin due to lack of sample availability.

After irradiation, a 0.385 cm$^2$ circular area of the exposed area of the epidermis of each sample was biopsied using a circular brass punch with a diameter of 0.70 cm. The biopsied piece was immersed in a water bath at 60°C to cleave the epidermal layer at its interface with the dermal layer. The epidermis was mechanically scraped off with a sterile scalpel and placed in a test tube containing 8.0% EtAc in hexane. The contents of the test tube were subjected to pulsed sonication for 10 seconds to ensure that the
EtAc/hexane solution extracted the 7-DHC and its photoproducts. The test tubes were then capped and placed in a 60°C water bath and incubated for a minimum of 16 hours to convert any preD₃ to vitamin D₃. This step was added because vitamin D₃ migrated later, thus there was less interference from other lipids that were present in the skin.

After incubation, the EtAc containing the 7-DHC and its photoproducts was removed from the skin pellet of each sample. These solutions were dried under nitrogen gas, re-dissolved in 0.3% IPA in hexane, and transferred to autosampler vials. The samples were then separated using single normal-phase HPLC analysis, with a cyanopropylidimethylsilane stationary phase column (Agilent ZORBAX CN, 5 µm pore size, 4.6 x 250 mm), and a 0.3% IPA in hexane mobile phase at a flow rate of 1.0 mL/min. UV detection at 265 nm confirmed the presence of vitamin D₃, 7-DHC, tachysterol₃, and lumisterol₃. Integration of the peaks provided AUCs for these photoproducts whenever present. The concentration of each photoproduct was determined by comparing AUCs to standard curves. From this data, the percent conversion of 7-DHC to vitamin D₃ in situ was determined.

Statistical Analyses

For the in vitro phase of this study, a series of two-tailed student’s t tests were used to establish statistical significance between the LED with the highest production of preD₃ (and other photoproducts) and all other LEDs at each time point of irradiation. Two-tailed student’s t tests also compared the percent conversion of 7-DHC to preD₃ at 0.75 MED for the 298 nm LED to the percent conversions of each of the other assessed wavelengths. A p-value less than 0.05 was considered to be statistically significant.
Two-tailed student’s t tests were conducted using VassarStats (a free statistics calculator available online at http://vassarstats.net).

Due to limited sample availability, repeated measures with skin samples could not be conducted, and only preliminary skin data is reported. As a result, statistical analyses could not be performed for the *in situ* phase of the study.
RESULTS

For the first phase of the study, the efficiency of UV-B LEDs at various wavelengths for producing preD$_3$ \textit{in vitro} was assessed. As samples passed through the HPLC column, a UV detector set to 265 nm confirmed the presence of preD$_3$, lumisterol$_3$, tachysterol$_3$ and 7-DHC. A representative chromatogram, obtained after irradiating an ampoule at 295 nm for 10 minutes and containing the peaks of all four compounds, is shown in figure 7. The UV absorption spectra for preD$_3$, tachysterol$_3$, lumisterol$_3$, and 7-DHC are shown in figure 8.

![Chromatogram diagram](image)

Fig. 7. A representative chromatogram detected at 265 nm of (A) preD$_3$, (B) lumisterol$_3$, (C) tachysterol$_3$, and (D) 7-DHC, obtained in this study after irradiating an ampoule at 295 nm for 10 minutes.
**Fig. 8.** The UV absorption spectra obtained for each photoproduct. (A) is the spectrum for preD$_3$, (B) is for tachysterol$_3$, (C) is for lumisterol$_3$, and (D) is for 7-DHC.

The primary aim of the *in vitro* phase of this study was to characterize the photoconversion of 7-DHC to its various photoproducts at various wavelengths as a function of time. All of the LEDs were capable of producing preD$_3$. As originally predicted based on the action spectrum of preD$_3$ formation (Figure 3B), the 298 nm LED produced the maximum amount of preD$_3$ after 25 minutes (52.5±0.5%). Interestingly, however, the time-response curve of each wavelength had markedly different characteristics. The 280, 285, and 290 nm LEDs quickly reached a maximum percent conversion of 7-DHC to preD$_3$ between 35-45% in approximately 5 minutes, after which
no additional production was seen with greater irradiation time (Figure 9). The 295, 298, and 300 nm LEDs exhibited more gradual increases in percent conversion of 7-DHC to preD₃ over time. The 295 and 298 nm LEDs resulted in ~40% after approximately 8 and 15 minutes, respectively (Fig. 9). The 300 nm LED resulted in less than 30% conversion to preD₃ after 25 minutes, while the 310 nm LED barely reached 1% (Fig. 9).

![Graph showing percent conversion to previtamin D₃ over time for various wavelengths.](image)

**Fig. 9.** The production of preD₃ over time (shown as mean ± standard error of the mean (SEM) based on triplicate measurements) for each wavelength is depicted. The 280 nm LED resulted in a significantly higher conversion than all other LEDs at 3 minutes, while the 290 nm LED resulted in significantly higher conversion at 10 minutes, and the 298 nm LED resulted in significantly higher conversion, after 25 minutes (two-tailed student’s t tests used to compare LED with the highest conversion at a particular time point against all other LEDs at the same time point, p < 0.01 for all points indicated by an (*) asterisk).

Photoproducts of preD₃ were also observed in vitro, and their amounts at various times changed based on the wavelength of irradiation. The varied production of these photoproducts provides insight into the differences in the photoequilibrium of each assessed wavelength. Tachysterol₃ was the major photoproduct observed when preD₃ production began reaching photoequilibrium, peaking at 55.8±0.5% for the 280 nm
diode, and its production appeared to reduce with increasing wavelength (Fig. 10A). Lumisterol$_3$ was minimally produced for all the LEDs, but its production mimicked the wavelength-dependent variations in preD$_3$ production, significantly higher at 298 nm (3.2±0.1%) than the other wavelengths (Fig. 10B). As shown in figure 11, the comparison of the production of preD$_3$ and its photoproducts between all LEDs revealed that tachysterol$_3$ production gradually increased and continued to increase when preD$_3$ production reached a plateau. This is especially apparent for the 280, 285, and 290 nm LEDs. PreD$_3$ quickly reached maximal level within 5 minutes, after which preD$_3$ was converted to tachysterol$_3$.

**Fig. 10.** The percent conversion of 7-DHC to (A) tachysterol$_3$ and (B) lumisterol$_3$ *in vitro* over time (each point is shown as mean ± SEM derived from triplicate measurements) with LEDs of different wavelengths. The LED with the greatest production of tachysterol$_3$ or lumisterol$_3$ at a particular time point is indicated by an asterisk (*) (two-tailed student’s t tests comparing LED with the highest production at a particular time point against all other LEDs at the same time point, p < 0.01 for all).
Fig. 11. The percent of original 7-DHC converted to PreD₃, lumisterol₃, and tachysterol₃ (shown as mean ± SEM derived from triplicate measurements). Each panel (A-G) is for a different LED (wavelengths specified).
The minimal erythemal dose (MED) output for each of the LEDs must be considered when selecting the best LED candidate for producing vitamin D. For type II skin (Caucasian), one MED is defined as the amount of UV-B radiation that causes a uniform slight pinkness to the skin 24 hours after exposure. According to the FDA, the recommended amount of UV exposure for tanning should not exceed 0.75 MED per day, thus a promising LED candidate should be able to synthesize the greatest amount of preD₃ in vitro in the least amount of time it takes to reach 0.75 MED. To determine this, the MEDs per hour output of each LED were measured using a SolarMeter® Digital UV Radiometer (Solartech, Inc.). Because the specifications of this digital radiometer rated 1 MED as 21 mJ/cm² (type I skin), the MEDs/hr reading was adjusted to be consistent with the 32 mJ/cm² per 1 MED rating for type II skin. MEDs/hr was inversely proportional to increasing wavelength (Figure 12), indicating that lower wavelengths required less exposure time to cause erythema.

![Fig. 12. MEDs/hr measured with a digital UV radiometer as a function of wavelength. MEDs/hr was adjusted from a rating of 21 mJ/cm² per MED to 32 mJ/cm² per MED.](image-url)
The MEDs/hr reading and the in vitro results described in Figure 9 were used to approximate the time required of each LED to reach an exposure of 0.75 MED (24 mJ/cm² for type II skin). The percent conversion of 7-DHC to preD₃ at 0.75 MED for each sample was estimated based on the initial linear trend in percent conversion to preD₃ for each individual sample, and the mean and standard error of the mean (SEM) for each LED were calculated. These results are tabulated in Table 1. Exposure to the 298 nm LED resulted in the highest average percent conversion of 7-DHC to preD₃ at 0.75 MED compared to any of the other LEDs that were evaluated. Two-tailed student’s t tests were used to compare the estimated average percent conversion at 0.75 MED of the 298 nm LED against each of the other LEDs. Using a significance level of 0.05, the 298 nm LED displayed a significantly higher percent conversion of 7.0 ± 0.3% at 0.75 MED compared to all other diodes. A graphical interpretation of this data, showing the variation of average preD₃ production at 0.75 MED for each wavelength, is shown in Figure 13.
<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>MEDs/hour</th>
<th>Time (secs) to 0.75 MED</th>
<th>% conversion to preD&lt;sub&gt;3&lt;/sub&gt; at 0.75 MED</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>280</td>
<td>128.0</td>
<td>21.0</td>
<td>5.6 ± 0.3</td>
<td>&lt; 0.01</td>
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<tr>
<td>285</td>
<td>125.0</td>
<td>21.6</td>
<td>5.5 ± 0.7</td>
<td>&lt; 0.01</td>
</tr>
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<td>106.2</td>
<td>25.8</td>
<td>6.0 ± 0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>295</td>
<td>70.7</td>
<td>38.4</td>
<td>3.7 ± 0.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>298</td>
<td>69.9</td>
<td>39.0</td>
<td>7.0 ± 0.3</td>
<td>Reference</td>
</tr>
<tr>
<td>300</td>
<td>49.2</td>
<td>55.2</td>
<td>2.1 ± 0.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>310</td>
<td>4.9</td>
<td>553.8</td>
<td>0.5 ± 0.1</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Table 1. The estimated percent conversion of 7-DHC to preD<sub>3</sub> for each assessed wavelength in vitro after exposure to 0.75 MED of radiation. MEDs/hr has been adjusted from the instrumental rating of 21 mJ/cm<sup>2</sup> per MED to 32 mJ/cm<sup>2</sup> per MED for Type II skin. Percent conversion is shown as mean ± SEM based on triplicate measurements for each wavelength. Two-tailed student’s t tests were used to determine the statistical significance of the LED with the highest production of preD<sub>3</sub> (here, the 298 nm LED) compared to each of the other LEDs (p-values indicated).

Fig. 13. Average percent conversion to preD<sub>3</sub> (from 7-DHC) in response to 0.75 MED (rated to 1 MED = 32 mJ/cm<sup>2</sup> for type II skin). Bars are shown as means ± SEM, constructed based on the initial linear trends of percent conversion to preD<sub>3</sub> from the in vitro results, as well as MEDs/hr data for each LED. The asterisk (*) indicates the LED with the highest percent conversion to preD<sub>3</sub> at 0.75 MED (two-tailed student’s t test, p< 0.01).
Since the 298 nm LED was the most efficient in producing preD$_3$ *in vitro* after exposure to 0.75 MED, it was used for the *in situ* phase with skin samples. A representative chromatogram of type II skin irradiated with the 298 nm LED for 10 minutes, along with the UV absorption spectrum for vitamin D$_3$, is shown in Figure 14.

![Chromatogram and UV absorption spectrum](image)

**Fig. 14.** (A) A representative chromatogram of type II skin irradiated with the 298 nm LED for 10 minutes. Peaks for vitamin D$_3$, tachysterol$_3$, and 7-DHC are labeled. (B) The UV absorption spectrum of vitamin D$_3$.

0.385 cm$^2$ biopsies of Type II (Caucasian), III (Indian), and IV (African-American) skin samples irradiated with the 298 nm LED for 10 minutes resulted in 15.5%, 10.0%, and 4.0% of 7-DHC being converted to preD$_3$ (detected as vitamin D$_3$), respectively (Figure 15). This result indicates that greater irradiation time is required to make the same amount of preD$_3$ in darker skin compared to lighter skin. The percent
conversion of 7-DHC to preD₃ after exposure to 0.75 MED for type II skin was estimated based on the initial trend in preD₃ production from 0 to 5 minutes. These calculations indicate that after ~39.0 seconds, 1.5% of the original 7-DHC could be converted to preD₃ in type II skin. A minimal amount of tachysterol₃ was observed after 5 minutes for type II and III skin and 10 minutes for type IV skin (Fig. 16). No other photoproducts were observed. Due to limited availability of skin samples, the in situ phase of this study could not be done in triplicate, thus no statistical analyses were performed for these preliminary data.

**Fig. 15.** The percent conversion of 7-DHC to preD₃ (detected as vitamin D₃) for skin types II, III, and IV after irradiation with the 298 nm LED. Two samples for each skin type (both from the same patient) were available for use, one of which was irradiated for 5 minutes, and the other for 10 minutes. 0.385 cm² biopsies of the irradiated area were subjected to HPLC analysis. Due to the limited availability of skin samples, measurements could not be done in triplicate, thus bars shown are single measurements (no mean or SEM could be calculated).
Fig. 16. The percent conversion of 7-DHC to tachysterol\textsubscript{3} for skin types II, III, and IV after irradiation with the 298 nm LED. Two samples for each skin type (both from the same patient) were available for use, one of which was irradiated for 5 minutes, and the other for 10 minutes. 0.385 cm\textsuperscript{2} biopsies of the irradiated area were subjected to HPLC analysis. Due to the limited availability of skin samples, measurements could not be done in triplicate, thus bars shown are single measurements (no mean or SEM could be calculated).
DISCUSSION

Vitamin D is responsible for a wide range of metabolic effects, although primarily associated with calcium and phosphorus homeostasis and healthy bone mineralization. Vitamin D deficiency is one of the most common medical issues today, classically associated with skeletal deformities including rickets in children and osteomalacia in adults. Deficiency is also related to cardiovascular disease, type 1 diabetes mellitus, and cancer, among other issues unrelated to skeletal health. Humans’ primary source of vitamin D is naturally occurring, caused by the photoconversion of cutaneous stores of 7-DHC to preD₃ after exposure to UV-B photons in sunlight. Despite skin’s immense capacity for synthesizing vitamin D₃, there are a number of key determinants for the efficiency and effectiveness of photosynthesis: wavelength of radiation, age, season, latitude, time of day, pollution, sunscreen use, cultural clothing practices, and skin color, to name a few. Correcting deficiency by restoring healthy vitamin D status is complicated by these factors influencing cutaneous synthesis, as well as intestinal malabsorption of dietary vitamin D caused by Crohn’s disease, Whipple’s disease, cystic fibrosis, and gastric bypass surgery. Individuals residing in areas with limited UV-B exposure who are also unable to absorb dietary vitamin D are at high risk for deficiency, and replacement by supplementation or sensible sun exposure may not suffice. Huldschinsky found that exposure to a mercury arc lamp cured rickets, eventually leading to UV phototherapy as a suitable option for enhancing cutaneous synthesis of vitamin D. However, current phototherapeutic UV radiation sources suffer from a number of setbacks, including low efficiency of conversion of 7-DHC to preD₃, high
energy consumption, heat generation, and being at an exact distance from the UV radiation source, all of which can pose problems to the vitamin D-deficient patient.

LEDs are a class of semiconductors that serve as compact, high-efficiency, low-energy consuming, long-lasting alternative light and radiation sources. That being said, LEDs are increasingly used in place of traditional lighting (e.g. incandescent and fluorescent bulbs, among others) for a wide variety of applications. In fact, the advent of the UV LED gave rise to uses in water purification, disinfection, and medical diagnostics. However, current phototherapeutic methods for vitamin D replacement do not yet use LED technology. This study indicates that narrow-band UV-B LEDs can efficiently convert 7-DHC to preD₃ in vitro and in situ, thereby offering a possible alternative radiation source for efficient vitamin D₃ synthesis. Based on the results, UV LEDs could be much more effective for phototherapy not only because of their compact, high-intensity, low-energy nature, but also because their narrow-band radiation has been shown in this study to optimize preD₃ production. This confirms a previous observation that narrow-band radiation was much more effective in producing preD₃ compared to broad-spectrum radiation. The results obtained show that LEDs of 280, 285, 290, 295, 298, 300, and 310 nm were all effective in converting 7-DHC to preD₃. However, considering that lower wavelengths in the UV-B range (280-290 nm) are more damaging to DNA and RNA in skin, higher wavelengths still capable of photosynthesizing preD₃ are preferred. Another health concern for UV phototherapy is minimizing the number of photons that can cause a minimal erythemal dose. The Food and Drug Administration currently recommends no more than 0.75 MED per day for tanning. According to the
data, MEDs per hour decreases with increasing wavelength, indicating the LEDs at higher wavelengths could be better candidates for cutaneous synthesis of vitamin D with less potential for causing erythema and skin damage thereby improving the benefit-to-risk ratio. Based on the results, the 298 nm LED yielded the greatest photoconversion of 7-DHC to preD₃ compared to other wavelengths after exposure to 0.75 MED (Table 1), converting an average of 7.0% of the original 7-DHC content to preD₃ in vitro. This is consistent with findings by MacLaughlin et al, who reported that UV-B radiation around 297-298 nm resulted in optimal preD₃ production, as shown in the action spectrum for preD₃ formation in Figure 3B. Along the same lines, it was interesting to note that the 295 nm LED consistently converted significantly less 7-DHC to preD₃ (3.7 ± 0.1%) after 0.75 MED than the 280, 285, or 290 nm LEDs (5.6 ± 0.3%, 5.5 ± 0.7%, and 6.0 ± 0.3% respectively). This result is not in accordance with the action spectrum, possibly due to variations in the spectral output of this particular LED. Further studies, including the characterization of the spectral output of the LEDs, will need to be conducted to better understand the reason for this inconsistency.

Given the in vitro results, the 298 nm LED was used to determine its effectiveness in producing vitamin D₃. Type II, III, and IV skin irradiated for 10 minutes showed 15.5, 10.0, and 4.0% conversion of 7-DHC to preD₃ (detected as vitamin D₃). This result clearly shows that individuals with more pigmented skin will take longer to cutaneously produce the same amount of vitamin D₃ compared to those with less pigment, consistent with the idea that melanin competes with 7-DHC to absorb UV-B. Because the MEDs/hour readings were adjusted for type II skin, the results of the type II skin
irradiations were used to determine that exposure to 0.75 MED (or 24 mJ/cm²) of radiation could convert approximately 1.5% of the original 7-DHC content to preD₃. It has been previously reported that exposure of an adult body in a bathing suit (nearly 100% of the body’s surface area) to one MED of simulated sunlight can produce 10,000-20,000 IUs of vitamin D₃,⁸ thus 0.75 MED can theoretically produce up to 15,000 IUs. The average surface area of skin on an adult is 15,000 cm², thus 0.75 MED of sunlight should be able to produce up to 1.0 IU, or 25.0 ng, of vitamin D₃ per cm² of exposed skin. A young adult has approximately 1750 ng of 7-DHC per cm² of skin,⁹ and factoring in that 0.75 MED of simulated sunlight can produce 25.0 ng of vitamin D₃ per cm², this comes to approximately 1.4% conversion. The time of sunlight exposure required to cause 0.75 MEDs can be greater than 20 minutes depending on skin type, while the 298 nm LED requires only 39.0 seconds. Taking all of this together, the results indicate that a 298 nm LED can produce vitamin D₃ in skin with a much greater efficiency than sunlight, converting 1.5% of 7-DHC to preD₃. The IOM currently recommends 600 IUs per day of dietary vitamin D to maintain sufficiency in adults. If multiple 298 nm LEDs could be put together to cover 3.8% of the average surface area of skin (570.0 cm²), exposure to the collective radiation for 39.0 seconds could produce approximately 600 IUs (or 15,000 ng) of vitamin D₃ in a young adult, fulfilling their vitamin D requirement for that day.

The data obtained in this study is especially important for individuals with malabsorption syndromes. Given the vast number of aforementioned factors influencing natural cutaneous synthesis of vitamin D by sunlight, vitamin D supplementation is now
practically a necessity. Patients with fat malabsorption syndromes caused by Crohn’s disease, Whipple’s disease, cystic fibrosis, and gastric bypass surgery, among other causes, will not efficiently absorb the fat-soluble vitamin D and are thus at higher risk for developing deficiency. For such individuals, a method of enhancing cutaneous photosynthesis of preD$_3$ is necessary to sustain vitamin D status. Traditional methods of UV phototherapy suffer from a number of setbacks including energy inefficiency, and thus UV LEDs could overcome many of those issues.

In conclusion, these preliminary results evaluated the effectiveness of UV LEDs for producing vitamin D$_3$. A cuff or blanket containing UV LEDs could be manufactured for use by vitamin D deficient patients with malabsorption syndromes to induce the cutaneous synthesis of vitamin D$_3$. Based on these findings, if the cuff covers 3.8% of the body and is operated for approximately 39 seconds per day, a patient could fulfill their daily vitamin D requirement (as defined by the IOM). Because lack of skin sample availability for the in situ phase in this study, repeated measures will be necessary in the future to increase confidence in the in situ results obtained. If patient data can be collected for future skin samples, this could offer even more confidence in the results by adjusting for various factors (age, BMI, etc.). Before a cuff or blanket can be manufactured, more data needs to be collected on the precise intensity of UV-B radiation required for optimal synthesis while minimizing the risk of harmful effects. Characterizing the spectral output of LEDs will be the next step to achieving this. Nonetheless, this study strongly indicates that UV LEDs have immense potential as a new treatment method for vitamin D deficiency.
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UMBC Department of Chemical & Biochemical Engineering, Baltimore, MD

Undergraduate Researcher (Spring 2012-Spring 2013)

• Worked under the guidance of Dr. Jennie Leach to investigate the neuronal response to hypoxic microenvironments. Observed Neural Stem Progenitor Cells (NSPCs) and monitored the cell differentiation and behavior in these microenvironments with varying oxygen concentrations.

Duke University Center for Human Genetics

Undergraduate Researcher (Summer 2011)

• Worked with Dr. Terri Young, Dr. Tammy Yanovitch, and Khanh-Nhat Tran Viet to screen the DNA of a cohort of patients with Primary Congenital Glaucoma (PCG) in order to find new, potentially causal mutations in the CYP1B1 gene.


UMBC Department of Chemistry and Biochemistry, Baltimore, MD

Undergraduate Researcher (Summer 2010-Spring 2011)

• Worked under the guidance of Dr. Marcin Ptaszek on the development of new fluorescent tools for in vivo use – primary focus on maximizing the fluorescence quantum yield of various fluorophore probes for uses such as tumor bioimaging.

Animal Clinical Investigations, LLC, Washington D.C.

Intern (Summer 2009)

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National Institutes of Health/National Cancer Institute, Bethesda, MD

Intern/Special Volunteer (Summer 2008)

• Gained exposure to the field of Molecular Biology research in a lab setting
• Completed an independent project involving the study of the effects of various chemotherapeutic drugs (Doxorubicin, Docetaxel, Etoposide) on different cancer cell lines (K562, Jurkat, U937, HeLa)
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http://www.ajo.com/article/S0002-9394%2812%2900642-3/abstract