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Synthesis and photochemical transformation of 3 β ,21-dihydroxypregna-5,7-dien-20-one to novel secosteroids that show anti-melanoma activity

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Abstract

We have synthesized 3 β ,21-dihydroxypregna-5,7-dien-20-one (21(OH) 7DHP) and used UVB radiation to induce its photoconversion to analogues of vitamin D (pD), lumisterol (pL) and tachysterol (pT). The number and character of the products and the dynamics of the process were dependent on the UVB dose. The main products: pD and pT compounds were characterized by UV absorption, MS and NMR spectroscopy after RP-HPLC chromatography. In addition, formation of multiple oxidized derivatives of the primary products was detected and one of these derivatives was characterized as oxidized 21-hydroxyisotachysterol compound (21(OH)oxy-piT). These newly synthesized compounds inhibited growth of human melanoma cells in a dose dependent manner, with greater or equal potency to calcitriol. 3 β ,21-Dihydroxy-9 β ,10 α -pregna-5,7-dien-20-one (21(OH)pL) and 21(OH)oxy-piT had higher potency against pigmented melanoma cells, while the EC₅₀ for compounds 21(OH)7DHP and (5Z,7E)-3 β ,21-dihydroxy-9,10-secopregna-5,7,10(19)-trien-20-one (21(OH)pD) were similar in both pigmented and non-pigmented cells. Moreover, 21(OH)7DHP and its derivatives inhibited proliferation of human epidermal HaCaT keratinocytes, albeit at a lower activity compared to melanoma cells. Importantly, 21(OH)7DHP derivatives strongly inhibited the colony formation of human melanoma cells with 21(OH)pD being the most potent. The potential mechanism of action of newly synthesized compounds was similar to that mediated by 1,25(OH)₂D₃ and involved ligand-induced translocation of vitamin D receptor into the nucleus. In summary, we have characterized for the first time products of UVB-induced conversion of 21(OH)7DHP and documented that these compounds have selective, inhibitory effects on melanoma cells.

Keywords

secosteroids; UV radiation; lumisterol; vitamin D; oxidation; SLOS; skin; melanoma

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1. Introduction

Human skin is the main organ for photo-induced synthesis of vitamin D₃ (cholecalciferol, 3 β ,5Z,7E-9,10-secocholesta-5,7,10(19)-trien-3-ol) from 7-dehydrocholesterol (7DHC, cholesta-5,7-dien-3 β -ol) [1–3]. This reaction is initiated by photolysis of the unsaturated B ring of 7DHC, resulting in the formation of a pre-D₃ intermediate and subsequent isomerization to vitamin D₃, tachysterol (6E-9,10-secocholesta-5(10),6,8-trien-3 β -ol, T₃) and lumisterol₃(9 β ,10 α -cholesta-5,7-diene-3 β -ol, L₃). Depending on strength and duration of ultraviolet B (UVB) exposure, tachysterol might undergo further isomerization to isotachysterol, and subsequent oxidation of A, B or C rings [3–6]. The other factors influencing the relative ratio and structure of the products of UV photolysis of steroidal 5,7-dienes are temperature, wavelength, and the presence of biological membranes or cellular compartments of the skin [4,6]. Production of 5,6-transvitamin D₃, suprasterols I and II in the skin in response to high doses of UVB has also been observed [4]. Irradiation of 5,7-dienes also results in formation of 5,7,9(11)-trienes, with the probable involvement of singlet oxygen and photosensitizers [7–11]. Detection of non-classical products of UV mediated photolysis of 5,7-dienes might represent a naturally occurring mechanism of regulation of vitamin D₃ synthesis and response to UV.

A deficiency of 7DHC Δ -reductase can lead to formation of pregna-5,7-dienes *in vivo* [12–16]. Similar to 7DHC, such compounds [9,10,13,17–19], are sensitive to UVB-induced photoconversion to vitamin D, lumisterol and tachysterol analogues [17–19]. Recently, it was shown that cytochrome P450scc is capable of the oxidation and cleavage of the side chain of 7-DHC to produce 7-dehydropregnenolone (7DHP) [17,20]. 7DHP can serve as a substrate for further metabolism by classical enzymes of the steroidogenic pathway [17,20], which raises the possibility of generation of a new class of vitamin D₃ derivatives after UVB irradiation [18,19]. Moreover, cytochrome P450scc was shown to hydroxylate vitamins D₃ and D₂ and ergosterol without the subsequent side chain cleavage [21–25], broadening the spectrum of possible secosteroidal products of CYP11A1 activity.

Vitamin D₃ is known as a master regulator of body calcium level [2,26–28]. Furthermore, vitamin D₃ affects proliferation, differentiation and apoptosis of normal and malignant cells [2,26]. However, its use as an anti-proliferative agent is largely limited due to toxicity (hypercalcemia) at high doses [1,2]. It has been shown that one way to significantly reduce this effect is by shortening or removal of the side chain of 7DHC [29,30]. Since UVB can photolyze pregna-5,7-dienes and their hydroxylated derivatives into vitamin D analogs with short side chains, it is logical to examine their potential anticancer properties [31]. Indeed, some of these compounds have already demonstrated antiproliferative activity against skin cells [19,20] and leukemia [32].

The aim of this study was to generate new vitamin D derivatives with low calcemic effects and good antiproliferative properties. Here we report on the UVB photolysis of 3 β ,21-dihydroxypregna-5,7-dien-20-one [33] synthesized from 3-acetylated 5-diene precursor [34,35]. The photolytic reaction led to synthesis of novel 9,10-secosteroids with vitamin D and lumisterol backbone structures, and oxidized derivatives. The biological activity of these derivatives was determined against human melanoma cells since this cancer is poorly responsive to current treatment regimens, especially once metastasis has occurred.

2. Experimental

Chemical synthesis

The synthesis of compounds **4** is shown in Scheme 1.

2.1. 3 β ,21-Dihydroxypregn-5-en-20-one diacetate (2)—Compound **1** (3 β ,21-Dihydroxypregn-5-en-20-one acetate) was acetylated following a known procedure [36]. A mixture of compound **1** (7.48 g, 20 mmol) and pTSA·H₂O (76 mg, 0.4 mol) in acetic anhydride (40 ml) was placed in an open glass tube. This mixture was irradiated in the microwave-assisted synthesizer for 10 min. Ice-cold water was added and stirred until solid product precipitated out. The solid was collected by filtration followed by washing with saturated sodium bicarbonate solution and water. The dried material was used for next step without further purification. Yield: 95%. ¹H NMR (500MHz, CDCl₃): δ 5.38–5.41 (m, 1 H), 4.75 (d, J = 18.0 Hz, 1 H), 4.60–4.66 (m, 1 H), 4.56 (d, J = 18.0 Hz, 1 H), 2.54 (t, J = 9.8 Hz, 1 H), 2.33–2.36 (m, 2 H), 2.22–2.26 (m, 1 H), 2.18 (s, 3 H), 2.06 (s, 3 H), 2.01–2.08 (m, 2 H), 1.87–1.90 (m, 2 H), 1.40–1.76 (m, 10 H), 1.28–1.34 (m, 1 H), 1.14–1.22 (m, 1 H), 1.04 (s, 3 H), 0.70 (s, 3 H). ESI-MS: calculated for C₂₅H₃₆O₅, 416.3, found 439.3 [M+Na]⁺.

2.2. 3 β ,21-Dihydroxypregna-5,7-dien-20-one diacetate (3)—Compound **3** was synthesized according to a known procedure [35]. To a solution of compound **2** (4.16 g, 10.0 mmol) in benzene–hexane (250 ml, 1:1 in volume) was added dibromantoin (1.72 g, 6.0 mmol) and 2,2'-azobisisobutyronitrile (68 mg, 0.4 mmol). The mixture was refluxed under argon for 25 min in a preheated oil bath (100°C) and then placed in an ice bath to cool. The insoluble material was filtered off. The filtrate was concentrated to yield a yellow-brown solid. To a solution of this yellow-brown material in tetrahydrofuran (60 ml) was added tetrabutylammonium bromide (0.8 g, 2.5 mmol) and stirred for 75 min at room temperature. To this reaction mixture was added tetrabutylammonium fluoride (20 ml of 1.0 M solution in tetrahydrofuran, 20 mmol) and the resulting solution was stirred for 50 min. Water was added and the mixture was extracted by ethyl acetate. The organic layer was dried over anhydrous Na₂SO₄ and concentrated to yield crude compound **3**. The crude compound **3** was subjected to flash chromatography (eluted with hexane–ethyl acetate 2:1) to produce a white solid. Yield: 41%. ¹H NMR (500MHz, CDCl₃): δ 5.60 (dd, J = 9.6 Hz, 2.8 Hz, 1 H), 5.44–5.47 (m, 1 H), 4.78 (d, J = 16.0 Hz, 1 H), 4.70–4.76 (m, 1 H), 4.58 (d, J = 16.0 Hz, 1 H), 2.64 (t, J = 9.6 Hz, 1 H), 2.52–2.56 (m, 1 H), 2.39 (t, J = 14.8 Hz, 1 H), 2.25–2.32 (m, 1 H), 2.20 (s, 3 H), 2.12–2.15 (m, 1 H), 2.08 (s, 3 H), 2.04–2.10 (m, 2 H), 1.50–1.96 (m, 8 H), 1.50 (dt, J = 14.8 Hz, 8.0 Hz, 1 H), 1.40 (dt, J = 14.0 Hz, 5.0 Hz, 1 H), 0.96 (s, 3 H), 0.65 (s, 3 H). ESI-MS: calculated for C₂₅H₃₄O₅, 414.2, found 437.3 [M+Na]⁺.

2.3. 3 β , 21-Dihydroxypregna-5,7-dien-20-one (4, 21(OH)7DHP)—Compound **4** was synthesized according to a reported procedure [37]. To a solution of compound **3** (414 mg, 1 mmol) in THF:MeOH (60 ml, 1:2 in volume) was added potassium carbonate (276 mg, 2 mmol) and stirred for 10 hours at room temperature. Argon bubbling into the reaction mixture was applied to exclude oxygen since the presence of oxygen initiates oxidative cleavage of 21-CH₂ leading to the formation of 17-carboxylic acid [38]. Water was added and the mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous Na₂SO₄ and concentrated. The residue was purified by flash chromatography (eluent: hexane–ethyl acetate 1:1) to produce a white solid. Yield: 66%. ¹H NMR (500 MHz, CDCl₃): δ 5.51 (dd, J = 8.0 Hz, 2.8 Hz, 1 H), 5.36–5.38 (m, 1 H), 4.10–4.20 (m, 2 H), 3.57–3.60 (m, 1 H), 3.17–3.20 (m, 1 H), 2.5 (t, J = 8.0 Hz, 1 H), 2.40–2.44 (m, 1 H), 2.18–2.24 (m, 2 H), 1.91–1.98 (m, 3 H), 1.76–1.84 (m, 4 H), 1.52–1.66 (m, 2 H), 1.36–1.44 (m, 2 H), 1.18–1.26 (m, 1 H), 0.86 (s, 3 H), 0.54 (s, 3 H). ESI-MS: calculated for C₂₁H₃₀O₃, 330.3, found 353.3 [M+Na]⁺.

2.4. General procedure for UVB irradiation of 21(OH)7DHP—The procedure of UVB irradiation was performed essentially as described previously [18] with some minor modifications. Briefly, a methanol solution of **4** at 1 mg/ml concentration was subjected to UVB irradiation for 5–15 min. in a 5 mm quartz NMR tube using a Biorad UV

Transilluminator 2000 (Biorad, Hercules, CA). Spectral characteristics of the UVB (280–320 nm) source were published previously [39] and its strength ($3.8 \pm 0.2 \text{ mW cm}^{-2}$) was routinely measured with a digital UVB Meter Model 6.0 (Solartech Inc., Harrison Twp, MI). Irradiation was followed by rapid separation of the products by RP-HPLC chromatography, as described previously.[18] The major products were identified on the basis of their retention time and characteristic UV absorption. Fractions containing 21(OH)pre-pD (λ_{max} at 260 nm) were re-purified after effective conversion to 21(OH)pD (at least 90% of D, λ_{max} at 265 nm). Initial identification was confirmed by MS, and NMR spectroscopic measurements, as described below.

21(OH)pD (5Z,7E)-3 β ,21-Dihydroxy-9,10-secopregna-5,7,10(19)-trien-20-one: ^1H NMR (500 MHz, CD_3OD): δ 6.24 (d, $J = 11.0 \text{ Hz}$, 1 H), 6.09 (d, $J = 11.0 \text{ Hz}$, 1 H), 5.06–5.08 (m, 1 H), 4.76–4.78 (m, 1 H), 4.17–4.26 (m, 2 H), 2.77–2.81 (m, 1 H), 2.90–2.93 (m, 1 H), 2.78 (t, $J = 9.0 \text{ Hz}$, 1 H), 2.56 (dd, $J = 13.0 \text{ Hz}$, 4.0 Hz, 1 H), 2.41–2.45 (m, 1 H), 1.32–2.24 (m, 14 H), 0.52 (s, 3 H). ESI-MS: calculated for $\text{C}_{21}\text{H}_{30}\text{O}_3$, 330.3, found 353.3 $[\text{M}+\text{Na}]^+$.

21(OH)pL 3 β ,21-Dihydroxy-9 β ,10 α -pregna-5,7-dien-20-one: ^1H NMR (500 MHz, CD_3OD): δ 5.61 (dd, $J = 5.0 \text{ Hz}$, 2.5 Hz, 1 H), 5.49–5.51 (m, 1 H), 4.20–4.29 (m, 2 H), 4.05–4.07 (m, 1 H), 2.96 (t, $J = 8.5 \text{ Hz}$, 1 H), 2.74–2.76 (m, 1 H), 2.46 (d, $J = 16.0 \text{ Hz}$, 1 H), 2.34–2.36 (m, 1 H), 2.20–2.35 (m, 4 H), 1.25–1.91 (m, 9 H), 0.80 (s, 3 H), 0.59 (s, 3 H). ESI-MS: calculated for $\text{C}_{21}\text{H}_{30}\text{O}_3$, 330.3, found 353.3 $[\text{M}+\text{Na}]^+$.

21(OH)oxy-piT (6E)- 3 β ,21-Dihydroxy-9,10-secopregna-5(10),6,8-triene-20-one derivative (oxidized compound): ^1H NMR (500 MHz, CD_3OD): δ 6.59(d, $J = 11.5 \text{ Hz}$, 1 H), 6.11(d, $J = 9.5 \text{ Hz}$, 1 H), 4.19–4.28 (m, 2 H), 3.99–4.00(m, 1 H), 3.11 (s, 3 H), 2.79–2.85 (m, 2 H), 2.67 (dd, $J = 13.5 \text{ Hz}$, 4.0 Hz, 1 H), 2.35 (d, $J = 13.5 \text{ Hz}$, 1 H), 1.33–2.25 (m, 13 H), 1.36(s, 3 H), 0.55 (s, 3 H). ESI-MS: calculated for $\text{C}_{21}\text{H}_{30}\text{O}_5$, 362.3, found 401.3 $[\text{M}+\text{K}]^+$.

2.5. MTT assay—The SKMEL-188 cells are amelanotic when grown in the Ham's F10 and start to produce melanin pigment after the switch to Ham's F10:DMEM (50:50, v/v) [40,41]. SKMEL-188 cells were seeded at a density of 5,000 cells per well into 96-well plates in Hams' F10 or Hams' F10:DMEM (50:50, v/v) medium (GIBCO, Invitrogen Corp., Carlsbad, CA) supplemented with charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and an antibiotic-antimycotic solution (Sigma, St. Louis, MO). After 24 hr, stock media was exchanged with media containing serial dilutions of **21(OH)7DHP** and its derivatives. After incubation for 48 hours, 20 μL of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; 5 mg/ml in PBS; Sigma, St. Louis, MI) was added and the plates were incubated at 37°C for 4 hours in the presence of 5% CO_2 . Media was discarded and 100 μL of acidified isopropanol (contained 0.1 M hydrochloric acid) was added prior to spectroscopic measurement of optical density at 570 nm, with a 96-well plate reader.

2.6. [^3H]-thymidine incorporation—The cells were grown in Ham's F-10 media containing 5% charcoal treated FBS. After synchronization at G0/G1 phase of the cell cycle by incubation in serum free media, the chemicals were added with fresh media with 5% charcoal treated FBS and incubated for 72 hours. After a defined period of time, [^3H]-thymidine (specific activity 88.0 Ci/mmol; Amersham Biosciences, Piscataway, NY, USA) was added to a final concentration of 0.5 $\mu\text{Ci/ml}$ in medium, and after 4 hours of incubation cells were precipitated with 10% TCA, processed as described previously, and subjected to liquid scintillation counting using a beta counter (Direct Beta-Counter Matrix 9600; Packard) [38]

2.7. Colony forming assay—The inhibition of melanoma SKMEL-188 colony formation by derivatives of **21(OH)7DHP** (**21(OH)pD** and **21(OH)pL**) was performed as described [19,20]. Briefly, Melanoma SKMEL-188 cells were plated at density of 500 cells per well in twelve well plates and grown in F10 medium (GIBCO, Invitrogen Corp., Carlsbad, CA) supplemented with charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and an antibiotic-antimycotic solution (Sigma, St. Louis, MO) at 37°C in an atmosphere of 95% air and 5% CO₂. Cells were treated with **21(OH)7DHP**, **21(OH)pD**, **21(OH)pL**, **21(OH)oxy-piT** and 1,25-(OH)₂ D₃ as a positive control at various concentrations (0.1, 10 and 1000 nM). A corresponding dilution of ethanol was used as a negative (vehicle) control. Cells were grown for 6–10 days and colonies were stained with 0.1% crystalline blue, photographed and the number and the size of colonies were measured by ImageJ software (NIH, Bethesda, MD). Colony forming efficiency (CFU) was determined by dividing the number of colonies formed by the number of cells plated, and multiplied by 100.

2.8. Generation of cell lines expressing VDR-EGFP fusion protein—In order to construct pLenti-CMV-VDR-EGFP-pgk-puro, EGFP cDNA was amplified by PCR from pLenti-CMV-VDR-p2a-EGFP-pgk-puro (submitted for publication) using primers 5'-GGACCTCTCGAGATGGTGAGCAAGGGCGAGGAG-3' (forward); 5'-GCGAATTCCTACTTGTACAGCTCGTCCATGCC-3' (reverse), then digested with XhoI and EcoRI and subcloned into pLenti-CMV-VDR-p2a-EGFP-pgk-puro in which EGFP including p2a gene was removed. The VDR in pLenti-CMV-VDR-EGFP was expressed with EGFP together as a fusion protein. Lentivirus was produced in 293FT cells by UTHSC viral vector core using a method described previously [42]. 10 MOI of lentivirus (construct pLenti-CMV-VDR-EGFP-pgk-puro) was used to transduce SKMEL-188 melanoma in the presence of 6 µg/ml polybrene in the corresponding culture media (see above). The transduction efficiency was determined using a Nikon Eclipse TE300 microscope (Japan) by green fluorescence.

2.9. VDR translocation—Using SKMEL-188 transduced by pLenti-CMV-VDR-EGFP-pgk-puro (VDR and EGFP expressed as fusion protein), VDR translocation was determined by counting cells with a fluorescent nucleus. The transduced cells were incubated with drugs for 90 min and then fixed with 4% PFA (paraformaldehyde). Fixed cells were mounted with fluorescent mounting media (Dako, Denmark) and analyzed with a fluorescent microscope. At least 10 pictures were taken from different fields and the cells containing fluorescent nuclei were counted. Data are presented as a percentage of total cell numbers.

2.10. Statistical analyses—Data were tested for distribution and homogeneity of variances, and for statistical significance with one-way analysis of variance (Anova) with Tuckey post-hoc test (for more than two groups) using Prism 4.00 (GraphPad Software, San Diego, CA). Data are presented as the mean ± SEM; for n=4–6. *P<0.05, **P<0.01 and ***P<0.005.

2.11. Other procedures—All RP-HPLC purification and analyses were performed on an Atlantis C18 column using a Waters HPLC-system equipped with a diode-array detector, an auto-sampler and a fraction collector (Waters Associates, Milford, MA) as described elsewhere [18–20].

Mass spectra were recorded using a Bruker Esquire-LC/MS Spectrometer equipped with an electrospray ionization (ESI) source, as described previously [18,19].

NMR measurements were performed on a Varian Unity Inova-500 MHz spectrometer (Varian NMR Inc., Palo Alto, CA) using either a 3 mm or a 5 mm probe, as described before [18,19].

3. Results

3.1. Synthesis of 3 β ,21-dihydroxypregna-5,7-dien-20-one (21(OH)7DHP)

The synthesis of **21(OH)7DHP** following Scheme 1 was performed as described in Materials and Methods [18,19].

The synthesis of 3 β ,21-dihydroxypregna-5,7-dien-20-one (**21(OH)7DHP**) was carried out from 21-acetylated 5-en precursor (**1**) by acetylation in position 3 β , bromination-dehydrobromination and reduction, following the known procedure [35]. Physico-chemical properties of 3 β ,21-dihydroxypregna-5,7-diene-20-one as well as byproducts (**2** and **3**) were monitored by means of MS and NMR spectroscopy and their correctness confirmed (Materials and Methods).

3.2. UVB irradiation of 3 β ,21-dihydroxypregna-5,7-dien-20-one (21(OH)7DHP) and identification of products by combination of RP-HPLC, UV, mass and NMR spectrometry

The UV conversion of **21(OH)7DHP** was performed using a UVB light source [39,43] ($3.8 \pm 0.2 \text{ mW cm}^{-2}$) with maximum emission spectrum in a range of 290–320 nm, as described previously [18,19]. In order to optimize conditions for UVB-driven photolysis of **21(OH)7DHP**, the conversion was routinely monitored by HPLC separation, and resulting products were characterized by UV spectrometry [18,20,44]. Similar to cholesta-5,7-dien-3 β -ol (7DHC) [44], four main products (Scheme 1) were formed in a time-dependent fashion, namely 21(OH)pre-pD, 21(OH)pL and 21(OH)pD (Figure 1). In addition, substantial production of compounds with a characteristic shift of UV absorption to shorter wave-lengths was also observed ($\lambda_{\text{max}} = 240 \pm 3, 249 \pm 3, 260 \pm 3$). Those compounds are presumably isotachysterols (isotachysterol and its oxidized derivatives), as described previously [6,18,20]. Due to the rapid and time-dependent rearrangement towards isotachysterol derivatives, the reaction mixture was separated immediately after irradiation. The photo-conversion and subsequent time-dependent structural rearrangements were monitored by an HPLC equipped with a diode array that enabled fast detection of products by characteristic UV spectra [18,20,44]. All products were separated by HPLC and identified based on their unique UV absorption spectra (Figure 1).

Initial characterization of main products as pre-D (λ_{max} at 260 nm; **21(OH)pre-pD**), D (λ_{max} at 265 nm; **21(OH)pD**) and L (λ_{max} at 262, 274, 283; **21(OH)pL**) analogues was confirmed by MS and NMR (see Materials and Methods section for details). Surprisingly, the formation of T-derivative of **21(OH)7DHP**, with predicted λ_{max} at 274, 281, 290 nm, was not detected. Instead, the rapid accumulation of compounds with $\lambda_{\text{max}} = 240 \pm 3, 249 \pm 3, 260 \pm 3 \text{ nm}$ ($\pm 2 \text{ nm}$) was observed (several peaks on chromatogram; not shown). Detection of iso-T products indicates further metabolism of **21(OH)pT** (Scheme 1), with the potential formation of suprasterols [4], with a λ_{max} below 250 nm. The pre-vitamin D derivative ($\lambda_{\text{max}} = 260$; **21(OH)pre-pD**) underwent subsequent slow isomerization into vitamin D compounds (**21(OH)pD**), with maximum UV absorption at $\lambda_{\text{max}} = 265 \text{ nm}$.

Further identification was performed after purification by RP-HPLC equipped with a fraction collector (see Materials and Methods for details) and selected fractions were subsequently analyzed by MS. As expected, all pD, pL and pT products had identical mass ($m/z = 353.3 [\text{M}+\text{Na}]^+$) with the parental compound (Table 1). In addition to the predicted (Scheme 1) products with molecular ion at $m/z = 353.3 [\text{M}+\text{Na}]^+$, several products initially identified as iso-tachysterols (by UV spectra with λ_{max} below 250 nm) showed a main ion at $m/z = 401.3$ (Figure 2B). This indicates either the addition of 2 (or 3) oxygen atoms with formation of peroxide or hydroperoxide derivatives. Similar process, such as autooxidation of isotachysterol ((6E-9,10-secocholesta-5(10),6,8(14)-trien-3 β -ol) has been reported previously [6]. The oxidation of compound **21(OH)7DHP** without photolysis of the B ring

resulting in production of endoperoxide and hydroperoxide, cannot be excluded, such process has been shown for 7DHC [8,10].

The **21(OH)pD** and **21(OH)pL** irradiation products of **21(OH)7DHP**, with defined UV and mass spectra, were subjected to NMR analysis. Elucidation of the structures was based on ^1H 1D and ^1H - ^1H 2D correlation spectroscopy (COSY) experiments. The **21(OH)pD**, and **21(OH)pL** compounds were assigned based on expected chemical shifts for vinylic and methyl protons with the characteristic pattern we have previously described in detail [18,19]. The main difference between the NMR spectra for **21(OH)pL** and its parental compound is a downfield shift (~ 0.17 ppm) of 19-methyl protons of the parent compound and an upfield shift of 3-H (from 3.58 ppm to 4.06 ppm), Figure 3A and 3B).

Although we were able to detect **21(OH)pT** and **21(OH)isopT** derivatives of compound **21(OH)7DHP** by means of characteristic UV spectrum, these compounds were not stable, which prevented their in-depth characterization.

3.3. Detection and characterization of novel oxidized product of UVB irradiation of **21(OH)7DHP**

In addition to well-characterized products of 5,7-diene irradiation (**21(OH)pD**, **21(OH)pL**, and **21(OH)piT** compounds), we detected products with UV absorption $\lambda_{\text{max}} = 241, 249, 262$ nm (Figure 1) and the molecular ion at $m/z = 401.3$ (Figure 2). The unexpected mass could be calculated for $\text{C}_{21}\text{H}_{30}\text{O}_5$, 362.3 as $[\text{M}+\text{K}]^+$ or for $\text{C}_{21}\text{H}_{30}\text{O}_6$, 378.3 as $[\text{M}+\text{Na}]^+$. It is worthwhile to note that UV irradiation of **21(OH)7DHP** resulted in formation of at least 3 oxidized derivatives with λ_{max} below 250 nm and found $m/z = 401.3$.

The proton NMR (Figure 3D) study revealed only two double bond protons assigned to 6-H (6.59 ppm) and 7-H (6.11 ppm). This strongly suggested an iso-tachysterol arrangement of double bonds of compound **21(OH)oxy-piT** had predictably occurred, in agreement with the UV spectrum (λ_{max} 250 nm, characteristic for conjugated double bond). In contrast to **21(OH)pD**, this compound lacks the two double bond protons from 19- CH_2 with chemical shifts around 5.07 ppm and 4.77 ppm, instead it has an additional (when compared to **21(OH)pD**) methyl group with a chemical shift at 1.33 ppm for 19- CH_3 . Surprisingly, the chemical shift for 19- CH_3 at 1.33 ppm suggests that this methyl is not in the proximity of the double bond between C10 and C5. The expected chemical shift for 19- CH_3 should be at 1.7–1.8 ppm if it is adjacent to a double bond[6]. MS data suggested the presence of 2 (or 3) additional oxygen atoms in the structure. The COSY spectrum showed clearly all expected correlations for 3-H group (4 α -H, 4 β -H, 2 α -H, 2 β -H, 1 α -H). In addition, 6-H has a long-range correlation to 4 β -H, and 7-H with proton assign as 9-H, which strongly suggested that the double bond between 6 and 7-position remains intact. The chemical shifts for 18- CH_3 and 21- CH_2 are similar to its parental compound **21(OH)7DHP**, thus rings C, D and carbonyl group (20-C=O) are intact. Insufficient amount of **21(OH)oxy-piT** prevented us from collection of ^{13}C -NMR data or ^1H - ^{13}C HMBC which could otherwise provide an unambiguous assignment for this structure.

3.4. The compound **21(OH)7DHP** and its derivatives inhibit growth of human SKMEL-188 melanoma cells

The phenotype of human SKMEL-188 melanoma cells and the ability to produce melanin pigment are dependent on concentration of melanogenic precursors in the culture media [40,45]. Since melanogenic activity can affect the behavior of melanoma cells [41,46,47] we tested the anti-melanoma properties of **21(OH)7DHP** and its derivatives under amelanotic and melanotic phenotype. We have found that **21(OH)7DHP**, **21(OH)pD**, **21(OH)pL** and **21(OH)oxy-piT** inhibited growth of both amelanotic and melanotic melanoma cells with an

effect modified by the presence or absence of melanin pigment (Figure 4). The strongest inhibition ($EC_{50} < 1$ nM) was observed for compounds **21(OH)pL** and **21(OH)oxy-piT** against melanizing SKMEL-188 cells (Table 2). The EC_{50} values for **21(OH)pL** and **21(OH)oxy-piT** were 40 to 1300-fold lower for pigmented versus non-pigmented cells (Table 2). By contrast, **21(OH)pD** and **21(OH)7DHP** showed comparable EC_{50} values for both pigmented and non-pigmented cells (2.5 nM versus 3.4 nM and 91 nM and 118 nM, respectively).

Next, we tested the effect of vitamin D analogs against non-malignant human epidermal HaCaT keratinocytes. As expected, $1,25(OH)_2D_3$ inhibited proliferation of HaCaT keratinocytes with similar EC_{50} to non-pigmented SKMEL-188 melanoma cells (180 nM versus 190 nM, respectively) (Table 2). Interestingly, **21(OH)7DHP** and its derivatives had no effect on proliferation of HaCaT cells, with exception of **21(OH)pD**. The potency (EC_{50}) of **21(OH)pD** towards keratinocytes was about 10 times higher when compared to melanoma cells (EC_{50} of 40 nM versus 3.4 nM). These results indicate selective effects of new compounds towards melanoma cells in comparison to non-malignant epidermal keratinocytes (Table 2).

The process of DNA synthesis, required for proper growth and cell division, was measured by [3H]-thymidine incorporation assay. The inhibition of DNA synthesis in melanoma SKMEL-188 cells is shown in Figure 5. **21(OH)pL** had the highest inhibitory potency, although other 21-OH derivatives (**21(OH)-7DHP** and **21(OH)pD**) also inhibited DNA synthesis in SKMEL-188 cells. Insert in Figure 5C showed that **21(OH)pL** efficiently inhibited DNA synthesis in AbC1 cell line derived from Bomirski hamster melanoma [48]. These compounds were non-toxic as the viability of the cells cultured in their presence was >95% as measured by trypan exclusion method (not shown).

3.5. Inhibition of melanoma SKMEL-188 colony formation by 3β , 21-dihydroxypregna-5,7-diene-20-one derivatives

All of the tested compounds inhibited the formation of melanoma colonies in a dose dependent manner (Figure 6). The best inhibition was observed for **21(OH)pD** in a 10–1000 nM concentration range (Figure 6B). A less potent inhibitory effect was also seen with **21(OH)pL** and **21(OH)oxy-piT** ($p < 0.05$) at 1000 nM concentration (Figure 6C and D). Ethanol (solvent control) had no effect on colony formation (Figure 6A).

3.6. Translocation of Vitamin D receptor by **21(OH)7DHP** and its derivatives

Ligand induced translocation of VDR receptor into the nucleus was studied using SKMEL-188 cells stably transduced with pLenti-CMV-VDR-EGFP lentiviral construct (see Materials and Methods). The addition of **21(OH)pD** or **21(OH)pL** resulted in efficient translocation of VDR-GFP fusion protein into the nucleus (Figure 7). The effect of **21(OH)pD** was similar to that of $1,25(OH)_2D_3$ (positive control). **21(OH)pL** also translocated VDR-GFP to the nucleus, however, the effect was significantly lower, when compared to **21(OH)pD** or $1,25(OH)_2D_3$. These results document that novel secosteroidal derivatives of **21(OH)7DHP** can bind to VDR and induce its translocation to the nucleus in a similar manner as $1,25(OH)_2D_3$.

4. Discussion

We are reporting for the first time an efficient, reproducible and detailed method for the UVB-driven photolysis of 21-OH derivatives of 3β -hydroxypregna-5,7-dien-20-one (**21(OH)7DHP**). To our knowledge, this is the first description of secosteroidal **21(OH)pD**, **21(OH)pL** and stable oxidized iso-pT derivatives of **21(OH)7DHP**. Further analysis of

21(OH)pD and **21(OH)pL** confirmed proper identification by MS and NMR spectroscopy. In contrast to previously studied hydroxylated derivatives of 7DHP [18,19], production of pT compound was not observed by means of UV spectroscopy. This was probably due to rapid transformation of pT product to its iso-pT derivative under UVB, followed by its oxidation. The autooxidation of isotachysterol was described in detail by Jin and collaborators [6]. At least three potentially oxidized derivatives of **21(OH)7DHP** were characterized by combination of UV and MS spectra with λ_{max} below 250 nm and ESI-MS found 401.3 (Figures 1–2 and Table 1). The λ_{max} below 250 nm suggested the presence of conjugated double bonds, and an $m/z = 401.3$ could be explained by the addition of two or three oxygens ($\text{C}_{21}\text{H}_{30}\text{O}_5$, 362.3, $[\text{M}+\text{K}]^+$ or $\text{C}_{21}\text{H}_{30}\text{O}_6$, 378.3, $[\text{M}+\text{Na}]^+$). Low stability and small quantity prevented further characterization of those derivatives, with exception of product **21(OH)oxy-piT**, which was additionally analyzed by NMR. Open ring B structure was confirmed by the presence of the double bond protons of 6-C and 7-C (Figure 3). These protons in the double bond region of the spectra (7–4.5 ppm) suggested an isotachysterol type of structure. Comparison of NMR spectra for **21(OH)oxy-piT** to **21(OH)pD** and starting compound revealed that correlations between protons of ring A were intact (as judged by COSY experiment) and a similar chemical shift for 18- CH_3 suggested only slight changes in arrangement of C and D rings. Two hydroxyl groups were also present thus, it is most likely that two additional oxygens are present in A ring, replacing double bond (5-C, 10-C), as proposed by Jin and coworkers (compound 9) [6].

It has to be noted that the compound with characteristic UV spectra (λ_{max} at 320 nm) for a conjugated triene system (most probably 5,7,9(11)-triene) was also detected by relatively low efficiency of conversion (below 1%); this prevented us from further identification (not shown). Thus, observed UVB-driven formation of triene derivatives for several 5,7-dienes such as 7DHC or 17,20-diOH derivatives of 7DHP [19] is a common reaction with potential implications for photosensitivity of the skin [9].

The derivatives of vitamin D have a broad spectrum of biological functions [2,26,49] including anti-carcinogenic properties in the skin (reviewed in [27,50] [28]) and melanomas (reviewed in [51]). Our focus has been on analogs of vitamin D₃ without the cholesterol-type side-chain with potentially low calcemic activity [18,19,30–32,38]. In the present studies we have shown that 3 β ,21-dihydroxypregna-5,7-dien-20-one and its secosteroidal and oxidized derivatives had an inhibitory effect on growth (Figure 4, Table 2), DNA synthesis (Figure 5) and colony formation of human melanoma cells (Figure 6). Interestingly, the presence or absence of melanin pigment influenced the sensitivity of the cells to pL- and pT photoderivatives of 21(OH)7DHP. Thus, production of melanin pigment sensitized melanoma cells towards **21(OH)pL** and **21(OH)oxy-piT**. This effect was absent in the case of **21(OH)pD**. It might be suggested, that active melanogenesis positively affects the anti-melanoma activity of the above compounds. Although the mechanism for this action would require future extensive studies, it must be noted that intermediates of melanogenesis are highly toxic towards melanoma cells [46,52]. Importantly, our initial study showed that **21(OH)7DHP**, **21(OH)pL** and **21(OH)piT** had no effect on proliferation of HaCaT keratinocytes and the inhibitory effect of 21(OH)pD was at least 10 times lower than for non-pigmented SKMEL-188 cells. This suggests at least partial selectivity of 21-OH photoderivatives towards melanoma cells. The antiproliferative action of newly synthesized **21-OH7DHP** and its photoderivatives were additionally confirmed using radiolabeled thymidine incorporation into DNA assay. All tested compounds (**21(OH)7DHP**, **21(OH)pD** and **21(OH)pL**) significantly inhibited DNA synthesis in SKMEL-188 cells with **21(OH)pL** being the most potent compound (Figure 5). Inhibition of DNA synthesis was also observed for hamster AbC1 melanoma, indicating that the observed effect is not limited to human SKMEL-188 melanoma cells, and identifying an attractive animal model of melanoma [53] for future in vivo testing.

In order to gain some information on the mechanism of action of test compounds their effect on translocation of VDR from cytoplasm to the nucleus was studied using EGFP labeled VDR and epifluorescence microscopy (Figure 7). Treatment of SKMEL-188 resulted in efficient translocation of VDR into the nucleus (Figure 7B) indicating involvement of VDR in the observed phenotypic effects. Interestingly, the most potent compound – **21(OH)pL** showed lower capacity to translocate VDR into nucleus in comparison to **21(OH)pD** or **1,25(OH)₂D₃** (Figure 7A). This suggests that **21(OH)pL**, in addition to VDR, might possibly interact with other proteins. For example, 1,25D₃-MARRS (membrane associated rapid response to steroids, ERp57) receptor protein[54] is one of the possible candidates.

The present data may help to identify more efficient drugs for adjuvant melanoma therapy, including the described analogues of vitamin D (**21(OH)pD**) and, interestingly, its precursor (**21(OH)7DHP**) and isomer (**21(OH)pL**). These compounds may have different mechanisms of action from 1,25(OH)₂D₃, the subject of future laboratory studies. Synthesis and biological activity of oxidized isotachysterol derivatives may also suggest a receptor independent mechanism of action, e.g., intoxication of the cells already stressed by cytotoxic intermediates of melanogenesis. These areas may represent an exciting and fruitful line of future investigations.

In summary, newly generated photoderivatives of 3β,21-dihydroxypregna-5,7-dien-20-one, presented here, may serve as promising candidates for the treatment of hyperproliferative pathological processes, especially malignant melanomas [55].

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Figure 1.

UVB- driven photolysis of **21(OH)7DHP**. Representative UV spectra of irradiated sample of **21(OH)7DHP**. Peaks were assigned as follows: dotted line – **21(OH)7DHP** (substrate), solid line – **21(OH)pre-pD**, dashed line – **21(OH)pD**, dash double dot line – iso-pT or oxidized iso-pT. The UV spectra of fractions collected from RP-HPLC separation of UVB-irradiation products were acquired by Nanodrop.

Figure 2.

Identification of oxidized derivatives of **21(OH)7DHP** by mass spectrometry. Sample were purified after UV treatment using RP-HPLC and analyzed with LC-MS. Representative spectrum of potentially oxidized derivative of **21(OH)7DHP** is shown on panel B in comparison to starting material (Panel A). The compound **21(OH)piT** shows $m/z = 401.3$. The parental compound ($\text{C}_{21}\text{H}_{30}\text{O}_3$, mass = 330.25) gave $m/z = 353.25$ and compound **21(OH)oxy-piT** could be calculated as $\text{C}_{21}\text{H}_{30}\text{O}_5$, 362.3 [$M+\text{K}$] $^+$ or as $\text{C}_{21}\text{H}_{30}\text{O}_6$, 378.3 [$M+\text{Na}$] $^+$.

Figure 3. Proton NMR spectra of **21(OH)7DHP** (A), **21(OH)pL** (B), **21(OH)pD** (C), and oxidized derivative – **21(OH)oxy-piT** (D) analogs as well as their predicted structures (as insert for each panel).

Figure 4.

Inhibition of growth of SKMEL-188 melanoma cells by **21(OH)7DHP** and its derivatives. SKMEL-188 were seeded into 96-well plates and incubated in Ham's F10 medium containing low tyrosine levels, 10 μ M (amelanotic phenotype) or in DMEM:Ham's F10 (50:50, v:v) media containing high tyrosine levels, 200 μ M (melanotic phenotype). The media were supplemented with serial dilutions of compound **21(OH)7DHP** and its derivative **21(OH)pD**, **21(OH)pL** and **21(OH)oxy-piT**. After 48 hours the cells were submitted to an MTT test. The experiment was repeated with similar results. Examples of amelanotic and melanotic cells are shown as inserts for each graph. 1,25(OH)₂D₃ was used as positive control. Data is presented as means $\pm$ SEM for 6 independent measurements. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ versus control.

Figure 5.

21(OH)7DHP (A), **21(OH)pD** (B) and **21(OH)pL** (C) inhibit DNA synthesis in SKMEL-188. (D) **1,25(OH)₂D₃** was used as a positive control. The cells were incubated with compounds for 72 h in Ham's F10 media containing 5% charcoal treated FBS. [³H]-thymidine was added for last 4 hours of incubation. DNA synthesis was measured by counting the radioactivity incorporated into TCA precipitable material. Data are presented as means $\pm$ SD (n = 4). The dose dependent inhibition was analyzed by one-way ANOVA with #, P < 0.05 and ##, P < 0.01. The differences between control and the chemicals were analyzed with student's t-test; *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 6.

Inhibition of colony formation of melanoma SKMEL-188 treated with photo derivatives of **21(OH)7DHP**. Ethanol -solvent control (A); **21(OH)pD**, (B), **21(OH)pL**, (C) and **21(OH)oxy-piT** (D). The inhibition of melanoma SKMEL-188 colony formation was used to assay biological activity of newly synthesized compounds at 0.1, 10 and 1000 nM concentrations. Ethanol was used as a solvent control. The average value (n=6) for vehicle-treated cells is presented as control for drug treated cells. The total number of colonies was expressed in colony forming units (CFU). Data are presented as means $\pm$ SEM (n=3 or 4); *P<0.05, **P<0.005, and ***P<0.001.

Figure 7. 21(OH)7DHP, 21(OH)pD and 21(OH)pL (10 nM) induced translocation of VDR from the cytoplasm to the nucleus. (A) Data are presented as mean $\pm$ SD ($n \geq 10$). The differences were analyzed with student's t-test. *** $P < 0.001$: differences between control and chemicals. # $P < 0.05$: differences between chemicals. (B) Representative epifluorescence microphotographs of cells expressing VDR-EGFP fusion protein.

Scheme 1.

Synthesis of 3β,21-dihydroxypregna-5,7-dien-20-one (**4**) and its UVB-driven photolysis to vitamin D, tachysterol and lumisterol analogues. Reagents and conditions: (a) Ac₂O, microwave, p-toluenesulfonic acid monohydrate; (b) Dibromantin, 2,2'-azobisisobutyronitrile, benzene/hexane (1:1), 100°C, reflux; (c) Bu₄NBr, Bu₄NF, THF, room temperature; (d) K₂CO₃, MeOH-THF, H₂O, argon, room temperature.

Table 1

UV and MS spectra data for 21(OH)7DHP and its derivatives.

Name	Parental compound	Structure type	UV max (nm)	Formula	Predicted MW	Found MW
21(OH)7DHP	21-OHpreg *	5,7-diene	260,268,280,292	C ₂₁ H ₃₀ O ₃	330.22	353.3 [M+Na] ⁺
21(OH)pre-pD	21(OH)7DHP	preD-like	260	C ₂₁ H ₃₀ O ₃	330.22	353.3 [M+Na] ⁺
21(OH)pD	21(OH)7DHP	D-like	265	C ₂₁ H ₃₀ O ₃	330.22	353.3 [M+Na] ⁺
21(OH)pL	21(OH)7DHP	L-like	260,271,283	C ₂₁ H ₃₀ O ₃	330.22	353.3 [M+Na] ⁺
21(OH)-piT	21(OH)7DHP	iT-like	238,247,257	C ₂₁ H ₃₀ O ₃	330.22	353.3 [M+Na] ⁺
21(OH)oxy-piT	21(OH)7DHP	isoT-like (oxide)	241,250,259	C ₂₁ H ₃₀ O ₅ **	362.21 **	401.3 [M+K] ⁺
21(OH)oxy-piT1	21(OH)7DHP	isoT-like (oxide)	241,250,259	C ₂₁ H ₃₀ O ₅ **	362.21 **	401.3 [M+K] ⁺
21(OH)oxy-piT2	21(OH)7DHP	isoT-like (oxide)	241,250,259	C ₂₁ H ₃₀ O ₅ **	362.21 **	401.3 [M+K] ⁺

Bold – purified and characterized (NMR),

* 3β,21-Dihydroxypregn-5-en-20-one acetate

** alternative calculation base on predicted formula C₂₁H₃₀O₆ and found 401.3 [M+Na]⁺

Table 2
Inhibition of growth of SKMEL-188 melanoma cells

EC50 values were calculated as described in Materials and Methods and Figure 4.

	SKMEL-188		HaCaT
	amelanotic	melanotic	
1,25(OH) ₂ D ₃	190 nM	12.4 nM	180 nM
21(OH)7DHP	91 nM	118 nM	>1000 nM
21(OH)pD	3.4 nM	2.5 nM	40 nM
21(OH)pL	130 nM	0.1 nM	>1000 nM
21(OH)oxy-piT	40 nM	0.8 nM	>1000 nM