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A New Steroidal 5,7-Diene Derivative, 3β-Hydroxyandrosta-5, 7-Diene-17β-Carboxylic Acid, Shows Potent Anti-proliferative Activity

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Abstract

The new steroidal 5,7-diene, 3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid (17-COOH-7DA), was synthesized from 21-acetoxypregnenolone, with the oxidative cleavage of the side chain being dependent on the presence of oxygen. In human epidermal (HaCaT) keratinocytes, 17-COOH-7DA inhibited proliferation in a dose-dependent manner, starting at a dose as low as 10^{-11} M. This inhibition was accompanied by decreased expression of epidermal growth factor receptor, bcl2 and cyclin E2 mRNAs and by increased expression of involucrin mRNA. Inhibition of proliferation was associated with slowing of the cell cycle in G1/G0 phases but not with cell death. 17-COOH-7DA was significantly more potent than pregnenolone, 17-COOH-pregnenolone, 17-$COOCH_3$-7DA and calcitriol. 17-COOH-7DA also inhibited proliferation of normal human epidermal melanocytes and human and hamster melanoma lines, however, with lower potency than for keratinocytes. In normal human dermal fibroblasts 17-COOH-7DA stimulated proliferation in serum-free media but inhibited it in the presence of 5% serum. 17-COOH-7DA inhibited cell colony formation of human and hamster melanoma cells, and induced monocyte-like differentiation of human HL60 leukemia cells. Thus, the new steroidal 5,7-diene, 17-COOH-7DA, can serve as an inhibitor of proliferation of normal keratinocytes and normal and malignant melanocytes, as a condition-dependent regulator of fibroblast proliferation and a stimulator of leukemia cell differentiation.

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Keywords

3β-Hydroxyandrosta-5,7-diene-17β-carboxylic acid; 5,7-dienes; skin; keratinocytes; melanoma; melanocytes; fibroblasts

Introduction

Pregna-5,7-dienes and their hydroxylated derivatives can be formed *in vivo* when there is a deficiency in 7-dehydrocholesterol (7DHC) Δ-reductase, as seen in the Smith-Lemli-Opitz syndrome (SLOS) [1-4]. 7DHC Δ-reductase is the enzyme responsible for the conversion of 7DHC to cholesterol. The lack of this key enzyme leads to the accumulation of 5,7-dienes and 5,7,9(11)-trienes which is believed to result in multiple metabolic defects. In addition, recent data have shown that steroidal 5,7-dienes are generated through the P450scc-mediated cleavage of the side chain of 7DHC [4,5]. Production of pregna-5,7-dienes and their hydroxylated derivatives from 7DHC has also been described in normal adrenal glands incubated ex-vivo [6].

In the skin 7DHC is converted to vitamin D3 following the absorption of photons of ultraviolet light B (wavelength 290 – 320 nm) [7-9]. Vitamin D3 undergoes 25-hydroxylation in the liver then 1α-hydroxylation in the kidney producing 1α,25-dihydroxyvitamin D3 (calcitriol; 1,25 $(OH)_2D_3$)), the active regulator of calcium metabolism. 1,25$(OH)_2D_3$ can also be produced locally in the skin [7,8]. 1,25$(OH)_2D_3$ and its precursors have immune and neuroendocrine activities, and tumorostatic and anticarcinogenic properties, affecting proliferation, differentiation and apoptosis in cells of different lineages, and protecting DNA against oxidative damage [7,10-13].

Benign or malignant hyperproliferative skin disorders such as psoriasis, solar keratosis, basal and squamous cell carcinoma as well as melanoma represent a significant clinical problem. Melanoma especially, has the fastest growing incidence of any cancer in the United States. While early stage melanoma can be cured by surgery, advanced melanoma is notoriously resistant to all existing treatments. Dacarbazine (DTIC) is the only FDA approved drug to treat metastatic melanoma in terms of chemotherapy but the complete remission rate is less than 5% in patients. [14,15] Tremendous efforts have been made in recent years to search for more effective agents for the treatment of advanced melanoma [16]. However, none of them has clearly demonstrated better efficacy than DTIC. With the fast rising melanoma occurrences in the United States and the world, searching for more effective agents is becoming a pressing task for drug makers. Furthermore, development of new drugs that could be used in treatment of psoriasis, solar keratosis or squamous or basal cell carcinomas represents a challenge in clinical and experimental dermatology.

Here we report the structure, synthesis and biological testing of 3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid (17-COOH-7DA), a new steroidal 5,7-diene resulting from our search for new therapeutic agents for melanoma and hyperproliferative skin disorders. This is the first report of the synthesis and biological evaluation of this compound.

Experimental

Chemical synthesis

The synthesis of compounds **5, 6, 8** are shown in Scheme 1 and 2.

General methods

All reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), Alfa Aesar (Ward Hill, MA) or Steraloids Inc. (Newport, RI), and were used without further purification. The solvents for moisture sensitive reactions were freshly distilled and the reactions were carried out under an argon atmosphere. Routine thin layer chromatography (TLC) was performed on aluminum backed Uniplates (Analtech, Newark, DE). Melting points were measured with Fisher-Johns melting point apparatus (uncorrected). Mass spectra were recorded using a Bruker Esquire-LC/MS spectrometer equipped with an electrospray ionization (ESI) source as described previously [17]. The purity of the final compounds was determined using a Supelco Ascentis™ 5μM RP-Amide column (250 × 4.6 mm) at ambient temperature on a Waters 2695 HPLC system equipped with a Photodiode Array Detector. Individual HPLC conditions were optimized and were labeled along with the purity determination (supplementary materials).

NMR Analysis

All NMR measurements were performed using an inverse triple-resonance probe on a Varian Unity Inova 500-MHz spectrometer (Varian, Inc., Palo Alto, CA, USA). Samples were dissolved in DMSO-d_6. Temperature was regulated at 22°C and was controlled to an accuracy of ±0.1°C. Chemical shifts were referenced to residual solvent peaks for DMSO-d_6 (2.50 ppm for proton and 40.0 ppm for carbon). Standard 2D NMR experiments (^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{1}H COSY or ^{1}H-^{13}C HMBC, supplementary materials) were acquired to fully elucidate the structures of the new compounds. All data were processed using Varian's VNMR 6.1C software, with zero-filling in the direct dimension and linear prediction in the indirect dimension. Full structural assignments were performed described before [4,17, 18].

Pregn-5-en-20-one-3β,21-diyl diacetate (3β,21-dihydroxypregn-5-en-20-one diacetate) (2)

The acetylation of compound **1** was carried out following a known procedure [19]. A mixture of steroid **1** (3.74 g, 10 mmol) in a small amount of acetic anhydride (20 ml) and catalytic amount of pTSA·H_2O (38 mg, 0.2 mol) was placed in an open glass tube. This mixture was irradiated in the microwave-assisted synthesizer for 10 min. After completion of the reaction, the mixture was brought to room temperature, mixed with ice-cold water and stirred until solid product precipitated out. The solid was collected by filtration then washed with saturated sodium bicarbonate solution (30 ml) and water (40 ml). The dried material was used for next step without further purification Yield: 95%. ^{1}H NMR (500MHz, $CDCl_3$)**:** δ 5.38-5.41 (m, 1H), 4.75 (d, ***J*** = 18.0 Hz, 1H), 4.60-4.66 (m, 1H), 4.56 (d, ***J*** = 18.0 Hz, 1H),2.54 (t, ***J*** = 9.8 Hz, 1H), 2.33-2.36 (m, 2H), 2.22-2.26 (m, 1H), 2.18 (s, 3H), 2.06 (s, 3H), 2.01-2.08 (m, 2H), 1.87-1.90 (m, 2H), 1.40-1.76 (m, 10H), 1.28-1.34 (m, 1H), 1.14-1.22 (m, 1H), 1.04 (s, 3H), 0.70 (s, 3H). ESI-MS: calculated for $C_{25}H_{3636}O_5$, 416.3, found 439.3 $[M+Na]^+$

3β, 21-Dihydroxy-pregna-5,7-dien-20-one diacetate (3)

Compound **3** was synthesized according to a literature-described procedure [20]. To a solution of compound **2** (2.08 g, 5.0 mmol) in benzene-hexane (120 ml, 1:1 in volume) was added dibromantin (0.86 g, 3.0 mmol) and 2,2−azobisisobutyronitrile (33 mg, 0.2 mmol). The mixture was refluxed under argon for 30 min in a preheated oil bath (100°C) and then placed in an ice bath to cool. The insoluble material was removed by suction filtration, followed by evaporation of the filtrate to yield a yellow-brown solid. To a solution of this yellow-brown material in anhydrous tetrahydrofuran (40 ml) was added tetrabutylammonium bromide (0.4 g, 1.25 mmol), the resulting solution was stirred for 75 min under argon at room temperature. To this reaction mixture was added tetrabutylammonium fluoride (10 ml of 1.0 M solution in tetrahydrofuran, 10 mmol) and the resulting dark brown solution was stirred for an additional

50 min to yield a brown solid after removing solvent. A solution of this solid in ethyl acetate (200 ml) was washed with water 3 times (3 × 100 ml) and dried over anhydrous Na_2SO_4. Solvent was removed to give crude compound **3**. The crude compound **3** was subjected to flash chromatography (column eluted with hexane–ethyl acetate 20:1, 10:1, 5:1, 1:1 in order). Compound **3** is a white solid. Yield: 40-50%. ^{1}H NMR (500MHz, $CDCl_3$): δ 5.60 (dd, *J* = 9.6 Hz, 2.8 Hz, 1H), 5.44-5.47 (m, 1H), 4.78 (d, *J* = 16.0 Hz, 1H), 4.70-4.76 (m, 1H), 4.58 (d, *J* = 16.0 Hz, 1H), 2.64 (t, *J* =9.6 Hz, 1H), 2.52-2.56 (m, 1H), 2.39 (t, *J* = 14.8 Hz, 1H), 2.25-2.32 (m, 1H), 2.20 (s, 3H), 2.12-2.15 (m, 1H), 2.08 (s, 3H), 2.04-2.10 (m, 2H), 1.50-1.96 (m, 8H), 1.50 (dt, *J* = 14.8 Hz, 8.0 Hz, 1H),1.40 (dt, *J* = 14.0 Hz, 5.0 Hz, 1H), 0.96 (s, 3H), 0.65 (s, 3H). ESI-MS: calculated for $C_{25}H_{34}O_5$, 414.2, found 437.3 $[M+Na]^+$, m.p. 139-141 °C, consistent with the value (141-142) reported in the literature. [21]

3β-Hydroxyandrosta-5, 7-diene-17 β-carboxylic acid (5)

Compound **5** was synthesized as shown in scheme 1. To a solution of compound **3** (414 mg, 1 mmol) in THF:MeOH (60 ml, 1:2 in volume) was added potassium carbonate (414 mg, 3 mmol) and the solution stirred for 2 days in the presence of air. HCl (1% aqueous solution) was added to the reaction mixture to make it slightly acidic. The precipitated solid was filtered and washed by methanol to give a white solid. Yield: 49%. ^{1}H NMR (500MHz, DMSO-d_6): δ 12.00 (s, 1H), 5.49 (dd, ***J*** = 3.5 Hz, 1.6 Hz, 1H), 5.35-5.37 (m, 1H), 4.66 (s, 1H), 3.32-3.42 (m, 1H), 2.38 (t, ***J*** = 9.0 Hz, 1H), 2.09-2.15 (m, 1H), 1.92-2.06 (m, 4H), 1.72-1.81 (m, 5H), 1.59-1.61 (m, 2H), 1.43-1.47 (m, 1H), 1.30-1.35 (m, 2H), 1.21-1.25 (m, 1H), 0.85 (s, 3H), 0.56 (s, 3H). Table 2 summarized detailed chemical shifts for all atoms of this compound. ESI-MS: calculated for $C_{20}H_{28}O_3$**,** 316.2, found 315.0 $[M - H]^-$; HPLC: isocratic, water:methanol=20:80, flow rate 1.0 mL/min, *t*R= 8.58 min, purity 96.8%, m.p. 237-239 °C.

Methyl 3β-hydroxyandrosta-5, 7-diene-17β-carboxylate (6)

Compound **6** was synthesized following a known procedure [22]. To a solution of compound 5 (63.2 mg, 0.2 mmol) in THF (5 ml) was added DBU (46 mg, 0.3 mmol) and methyl iodide (156 mg, 0.22 mmol). The mixture was stirred at room temperature for 3 hours. Water was added to the mixture and the precipitate collected by filtration and washed with ethyl acetate to provide a white solid. Yield: 95%. ^{1}H NMR (500MHz, DMSO-d_6): δ 5.49 (dd, ***J*** = 4.0 Hz, 2.0 Hz, 1H), 5.36-5.37 (m, 1H), 4.66 (s, 1H), 3.36-3.40 (m, 1H), 3.60 (s, 3H), 2.30-2.33 (m, 1H), 1.91-2.18 (m, 5H), 1.71-1.85 (m, 4H), 1.59-1.62 (m, 2H), 1.45-1.49 (m, 1H), 1.19-1.38 (m, 4H), 0.85 (s, 3H), 0.52 (s, 3H). Table 2 summarized detailed chemical shifts for all atoms of this compound. ESI-MS: calculated for $C_{21}H_{30}O_3$, 330.2, found 353.3 $[M +Na]^+$. HPLC: isocratic, water:methanol=10:90, flow rate 1.0 mL/min, *t*R= 6.11 min, purity 98.8%, m.p. 210-212 °C.

3β-Hydroxyandrost-5-ene-17β-carboxylic acid (8)

Compound **8** was synthesized following the procedure shown in scheme **2** by using the same method as described for the synthesis of compound 5. Yield: 55%. ^{1}H NMR (500MHz, DMSO-d_6): δ 11.90 (s, 1H), 5.24 (d, ***J*** = 7.0 Hz, 1H), 4.60 (s, 1H), 3.21-3.30 (m, 1H), 2.24 (t, ***J*** = 15.0 Hz, 1H), 2.05-2.11 (m, 2H), 1.89-1.95 (m, 3H), 1.03-1.76 (m, 14H), 0.90 (s, 3H), 0.59 (s, 3H). Table 2 summarized detailed chemical shifts for all atoms of this compound. ESI-MS: calculated for $C_{20}H_{30}O_3$, 318.2, found 317.0 $[M - H]^-$. HPLC: isocratic, water:methanol=30:70, flow rate 0.6 mL/min, *t*R= 17.03 min, purity 98.7%, m.p. 271-273 °C.

Cell culture

Immortalized human epidermal keratinocytes (HaCaT) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% charcoal-treated FBS (Hyclone, Logan, UT) and 1% antibiotics (penicillin/streptomycin/amphotericin, Sigma-Aldrich, St. Louis, MO).

Human SKMEL-188 and hamster AbC1 melanoma cells were grown in Ham's F10 supplemented with 5% charcoal-treated fetal bovine serum (ctFBS) and 1% antibiotics. Human WM35 and WM1341 melanoma cells were grown in DMEM supplemented with 5% ctFBS, 1% antibiotics, and 5 μg/ml insulin. Normal melanocytes were cultured in MBM-4 medium (Lonza, Walkersville, MD) containing MGM-4 (Lonza, Walkersville, MD). Fibroblast cells were cultured in DMEM containing 5 μg/ml insulin, 1% antibiotics, and with or without ctFBS as indicated in the figure legend. All cultures were performed at 37 °C in 5% CO_2.

DNA synthesis

Incorporation of [^{3}H]-thymidine into DNA was used as a measure of cell proliferation [23]. Cells were inoculated into 24-well plates at 5,000 - 25,000 cells/well, depending on cell type. After overnight incubation at 37°C, the cultures were placed in serum free media to synchronize cells at G0/G1 phase of the cell cycle [23,24]. After 24 h 17-COOH-7DA (dissolved in DMSO and diluted in culture medium) was added with fresh media containing growth supplements (as indicated in t figures legends) and incubated for an additional 24 – 72 h. After a defined period of time (see above), [^{3}H]-thymidine (specific activity 88.0 Ci/mmol; GE Healthcare, Piscataway, NJ, USA) was added to a final concentration of 0.5 μCi/ml in medium. After 4 h of incubation at 37°C, media were discarded, cells precipitated with 10% TCA in PBS (phosphate-buffered saline) for 30 min, washed twice with 1 ml PBS and then incubated with 1 N NaOH/1% SDS (250 μl/well) for 30 min at 37°C. The extracts were collected in scintillation vials and 5 ml of scintillation cocktail were added. ^{3}H-radioactivity incorporated into DNA was measured with a beta counter (Direct Beta-Counter Matrix 9600; Packard, USA).

Colony forming assay

The assay followed standard methodology used in our laboratory as described previously [24,25]. Briefly, cells were plated in six-well plates at a density of 20 cells/9.6 cm^2 in medium containing 5% ctFBS, 1% antibiotic solution, and 17-COOH-7DA at graded concentrations or DMSO (vehicle control). Cells were cultured at 37°C for 7 - 17 days with media being changed every 3 days. Colonies were fixed with 4% paraformaldehyde in PBS overnight at 4°C, washed, stained with 2% crystal violet in PBS for 15 min, rinsed, and air-dried. The number and size of the colonies were measured using an ARTEK counter 880 (Dynex Technologies Inc., Chantilly, VA). Colony forming units were calculated by dividing the number of colonies by the number of cells plated and then multiplying by 100.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA from cells was extracted using the Absolutely RNA RT–PCR Miniprep kit (Stratagene, La Jolla, CA). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). The reaction was performed with LightCycler 480 Probes Master (Roche Applied Science, Indianapolis, IN). The primers and probes were designed with the Universal Probe Library (Roche Applied Science, Indianapolis, IN). The primer sequences are shown in Table 1. Real-time PCR was performed using TaqMan PCR Master Mix at 50°C for 2 min, 95°C for 10 min, and then performing 45 cycles (95°C for 15 s, 60°C for 1 min). The data were collected with a Roche Light Cycler 480. The amounts of mRNA were normalized by comparative Ct method, using cyclophilin B as a housekeeping gene.

Cell cycle analysis

HaCaT keratinocytes were cultured in DMEM media containing 5% ctFBS to 40% confluence followed by synchronizing for 24 h in serum-free media. Then, cells were growing in the media with 10^{-7} M 17-COOH-7DA for 72 h. The cells were washed with PBS and fixed in 70% ethanol at −20°C for 3 days. The fixed cells were washed with PBS to remove ethanol and

stained in PBS containing 0.05 mg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/ml RNase for 1.5 h at 37°C. After incubation, cell cycle was determined using flow cytometry (UTHSC Flow Cytometry and Cell Sorting Laboratory) with 10,000 cells scored.

Cell differentiation of leukemia cell line

HL-60 human promyelocytic cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Atlanta Biologics) and 1% penicillin/streptomycin/amphotericin antibiotic solution (Sigma) at 37°C. The compounds to be tested were dissolved in DMSO and added to the culture to a final concentrations of 10^{-7} M. Differentiation of HL-60 cells toward monocytes-like morphology and NBT-reduction was assessed after 5 days. Cells (2×10^{6}) were washed with PBS four times and resuspended in 200 μl of NBT solution (4 mg/ml) in water. After the addition of 200 μl of TPA solution (2 μg/ml) in PBS cells were incubated at 37°C for 60 min in 24-well plates. The cell differentiation was assessed by intracellular blue formazan deposits. The NBT positive and negative cells were scored under light microscopy examination (20x) with a minimum of 200 cells scored [26].

Statistical analysis

Data are presented as means ± SEM and have been analyzed with Student's t-test (for two groups) or one-way analysis of variance, and appropriate post hoc test (for more than two groups), using Microsoft Excel and Prism 4.00 (GraphPad Software, San Diego, CA). Statistically significant differences are denoted by roman numerals as follows: I, $P < 0.05$; II, $P < 0.01$; III, $P < 0.001$; IV, $P < 0.0001$ in student's t-test. IA, < 0.05; IIA, $P < 0.01$ in one-way ANOVA analysis.

Results

Synthesis of 3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid (17-COOH-7DA) and related compounds

The new steroidal 5,7-diene, 17-COOH-7DA, was synthesized following scheme 1 (details of the synthesis are in Materials and Methods; NMR and UV spectra as well as proof of purity by HPLC are in supplementary material). The NMR spectra of 17-COOH-7DA and its methyl ester are shown in supplementary material, while chemical shift assignments in Table 2.

The synthesis of **3** was performed similarly, as described previously for pregna-5, 7-dienes [17] (Scheme 1 and Material and Methods for details). The 17-COOH-7DA (**5**) was obtained from **3** by hydrolysis of the 21-acetoxy group accompanied by base-catalyzed oxidative cleavage of the side chain by molecular oxygen present in the reaction mixture. [27-29]. The exclusion of oxygen from the reaction mixture (reaction under argon) prevented the formation of **5** (not shown). The 17-COOH-7DA (**5**) was further converted to 3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid methyl ester (**6**; 17-COOCH3-7DA) by treating it with methyl iodide and DBU.

The above reaction (**d**) is analogous to the conversion of 21-acetoxypregnenolone (**1**) to 3β-hydroxyandrost-5-ene-17β-carboxylic acid (**8**) (Scheme 2). Its NMR spectra, UV and purity assessment by HPLC are shown in supplementary material, while full chemical shift assignments in Table 2.

17-COOH-7DA inhibits proliferation of HaCaT epidermal keratinocytes

We tested the effect of 17-COOH-7DA on proliferation of HaCaT keratinocytes by measuring [^{3}H]-thymidine incorporation into DNA (Fig. 1). 17-COOH-7DA inhibited DNA synthesis in a dose-dependent manner with significant inhibitory effect seen at a concentration as low as 10^{-11} M. 17-COOH-7DA was more potent than pregnenolone (Fig. 1), indicating a degree of

specificity for 17-COOH-7DA. The anti-proliferative effect of 17-COOH-7DA was associated with an increase of involucrin mRNA and inhibition of cyclin E1, bcl-2 and EGFR mRNA expression (Fig. 2). Morphological evaluation of the cells and measurement of the LDH activity (an assay for cytotoxicity) showed a lack of any cytotoxic effect (not shown). Flow cytometry studies have confirmed the above findings and showed that that 17-COOH-7DA slows cycling of HaCaT cells through an increase of cells at G1/G0 and a decrease in G2/M, but with no effect on subG1 (Fig. 3).

To further define the specificity of 17-COOH-7DA, 17-COOH-pregnenolone (17-COOH-A) and 17-$COOCH_3$-7DA were synthesized according to scheme 2 and their effect on DNA synthesis in HaCaT keratinocytes were compared to that of 17-COOH-7DA. Fig. 4 shows that 17-COOH-7DA is significantly more potent than 17-COOH-A and 17-$COOCH_3$-7DA, with the latter having no effect on proliferation (Fig. 4). Thus there is a strong specificity for 17-COOH-7DA in the inhibition of cell proliferation.

The effect of 17-COOH-7DA on DNA synthesis in normal and malignant skin cells

We tested the responses of normal epidermal melanocytes and melanoma cells to 17-COOH-7DA at concentrations ranging from 10^{-11} to 10^{-7} M and compared them to those of HaCaT keratinocytes (Fig. 5). Dose dependent inhibition curves for DNA replication were constructed and the EC50 values were 4.34×10^{-12}, 4.60×10^{-11}, 6.36×10^{-10}, 9.13×10^{-10}, 6.60×10^{-12}, and 4.82×10^{-12} M for HaCaT keratinocytes, neonatal melanocytes and SKMEL-188-, AbC1-, WM35- and WM1341-melanoma cells, respectively (Fig. 5). Thus potency was dependent on cell type with HaCaT keratinocytes and human melanoma cells being more sensitive to 17-COOH-7DA than normal melanocytes or hamster (AbC1) melanoma cells. The latter were the least sensitive to the growth inhibition. The magnitude of the inhibition was also cell-type dependent with WM1341 melanoma cells showing the largest effect and AbC1 cells the smallest.

For normal human dermal fibroblasts, the effect of 17-COOH-7DA on DNA synthesis was dependent on the presence or absence of serum (Fig. 6). When fibroblasts were cultured in serum-free media or in the presence of 5% or 1% of charcoal-treated FBS, 17-COOH-7DA increased, inhibited or had no effect on DNA synthesis, respectively (Fig. 6).

17-COOH-7DA effects on melanoma and leukemia cells

To further define the effect of 17-COOH-7DA on cell colony formation, SKMEL-188-, WM35- and AbC1-melanoma cells were plated at a density of 20 cells/9.6 cm^2 in medium containing 5% charcoal-treated FBS and incubated at 37°C for 7 (SKMEL-188 and AbC1) or 17 days (WM35), in the presence of graded concentrations of the drug (Fig. 7). 17-COOH-7DA inhibited colony formation with an effect that was dependent on the cell line. The strongest inhibitory effect on SKMEL-188 and AbC1 cells was seen for the largest colonies (>1 mm), while significant inhibition of colony formation by 17-COOH-7DA (10^{-11} and 10^{-9} M) was seen for small colonies (>0.2 mm) of WM35 melanoma cells. The parent steroid for the chemical synthesis of the 17-COOH-7DA, pregnenolone was used as a control and again showed no significant effect (Fig. 7).

Next we investigated the effect of 17-COOH-7DA on the human HL-60 cell line. Differentiation toward monocytes-like morphology and NBT-reduction was assessed after 5 days of treatment. Fig. 8 shows that 17-COOH-7DA stimulated monocytes-like differentiation of HL-60 cells. In contrast, 17-$COOCH_3$-7DA and 17-COOH-A slightly but significantly decreased cell differentiation induced by the DMSO vehicle.

17-COOH-7DA is more potent inhibitor of cell proliferation than 1,25(OH)$_2$D$_3$, the hormonally active form of vitamin D3

Since active form of vitamin D3 (1,25(OH)$_2$D$_3$) has well documented antiproliferative activity against normal and malignant skin cells [30-32] and is used topically in the therapy of psoriasis [33], we compared the anti-proliferative effects of 17-COOH-7DA and 1,25(OH)$_2$D$_3$. 17-COOH-7DA caused significantly more inhibition of proliferation of HaCaT epidermal keratinocytes, and SKMEL-188 and AbC1 melanoma cells, than 1,25(OH)$_2$D$_3$ (Fig. 9).

Discussion

We report for the first time on the steroidal 5,7-diene, 3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid (17-COOH-7DA). We have devised an efficient and reproducible method for its synthesis that is dependent on the presence of oxygen in the reaction mixture. We have found that 17-COOH-7DA is biologically active in normal (keratinocytes, melanocytes, and fibroblasts) and malignant (melanoma and leukemia) cells, is non-toxic, and has higher potency than its methyl derivative (17-COOCH$_3$-7DA), its 5-diene steroidal counterpart (17-COOH-preg), as well as pregnenolone and 1,25(OH)$_2$D$_3$.

In vivo steroidal 5,7-dienes are detected in patients with Smith-Lemli-Opitz syndrome, a pathological condition characterized by deficiency of the 7-Δ-reductase, [1-4], or in horses under physiological conditions during synthesis of equilin (delta-7-estrone) [34]. Furthermore, recent findings documenting that P450scc can transform 7DHC to 7DHP with a possible processing to other steroidal 5, 7-dienes, depending on the activity and availability of steroigogenic enzymes [4,6], raises an intriguing question whether 17-COOH-7DA can be produced in vivo. Interestingly, hydrolysis of the 21-acetoxy group of the 21-acetoxy-7DHP is accompanied by a spontaneous oxidative cleavage of the side chain when molecular oxygen is present leading to generation of 17-COOH-7DA. In contrast, 21(OH)7DHP is produced when oxygen is excluded (see above), and this steroid has indeed been detected in clinical samples from patients with SLOS [2]. Although we are currently unable to answer the question on in vivo production of 17-COOH-7DA, the information above encourages future experimental and clinical studies aimed at the detection of 17-COOH-7DA in biological fluids in SLOS patients or fetal horse tissues.

Of particular interest and potential clinical importance is the biological activity of 17-COOH-7DA. Specifically, we have found that it inhibits proliferation of normal (keratinocytes and melanocytes) and malignant (melanoma) cells with an effect that is seen at a concentration as low as10^{-11} M for HaCaT keratinocytes, Inhibition of proliferation was found to be associated with an increased fraction of cells arrested at G1/G0 phase of cell cycle but with no induction of apoptosis or necrosis. Studies on melanoma cells also showed that 17-COOH-7DA inhibits colony formation, indicating an anti-tumor potential. Importantly, 17-COOH-7DA was more potent than pregnenolone, 17-COOH-A or 17-COOCH$_3$-7DA indicating not only structural selectivity for a free carboxyl group attached to C17 but also the presence of the double bond at the 7-position of the 17-COOH-7DA for full biological activity. This specificity was also seen in leukemia cells where 17-COOH-7DA stimulated monocyte-like differentiation, while 17-COOH-A or 17-COOCH$_3$-7DA inhibited it. Also, the cell type and culture conditions were found to affect the activity of 17-COOH-7DA on dermal fibroblasts. When cultured in serum-free medium, 17-COOH-7DA stimulated their proliferation whereas with 1% serum there was no effect and with 5% inhibition occurred. This shows that the bioactivity of this novel compound can be context dependent. These findings are consistent with recently reported anti-proliferative activities of selected Δ^7unsaturated steroidal compounds in selected skin cell lines [6,18]. Also, profound behavioral abnormalities of SLOS are thought to result from the production of Δ^7unsaturated neurosteroid-like compounds [35].

Therefore, careful testing of the biological activity of steroidal 5,7-dienes may provide clinically and perhaps therapeutically important information.

The active form of vitamin D3 ($1,25(OH)_2D_3$) has well documented anti-proliferative activity against normal and malignant skin cells [30-32]. It is also used topically in therapy of a benign hyperproliferative skin disorder, psoriasis [33]. Therefore, our finding that 17-COOH-7DA is significantly more potent than $1,25(OH)_2D_3$ in inhibiting proliferation of human epidermal keratinocytes and human and hamster melanoma cell lines could have potential therapeutic implications. For example, it could be a good candidate for a topically applied agent for treatment of psoriasis or other hyperproliferative skin disorders. Furthermore, metastatic melanoma is a deadly disease for which there is no effective therapy. Therefore, any relatively non-toxic compound showing anti-melanoma activity warrants future animal testing, and we believe that 17-COOH-7DA is an example of such a compound. Eventually, it could serve as a candidate for a topically applied adjuvant drug for lentigo maligna or mucosal melanoma that, because of size or location, cannot be entirely resected.

In summary, we report for first time the structure and synthesis of the new steroidal 5,7-diene, 17-COOH-7DA. We show that it has specific and potent anti-proliferative or antitumor activities against human keratinocytes, human melanocytes and malignant melanomas (human and hamster), leukemia cells, and acts as a condition-dependent regulator (stimulator or inhibitor) of fibroblast proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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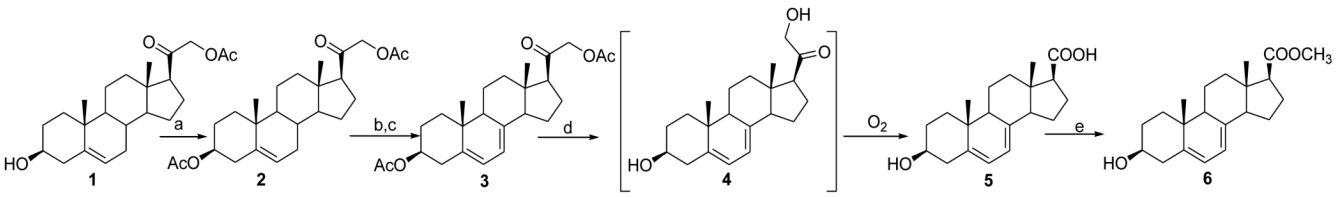
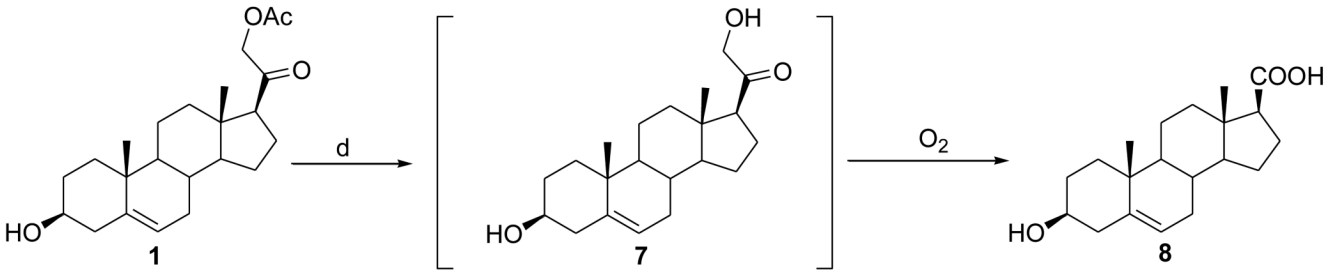


Scheme 1.

Synthesis of 17-COOH-7DA and its methyl ester. Reagents and conditions: (a) Ac_2O, microwave, *p*-toluenesulfonic acid monohydrate; (b) Dibromantin, 2, 2′-azobisisobutyronitrile, benzene/hexane (1:1), 100°C, reflux; (c) Bu_4NBr, Bu_4NF, THF, room temperature; (d) K_2CO_3, O_2, MeOH-THF, room temperature, overnight; (e). DBU, MeI, THF, room temperature.



Scheme 2.

Synthesis of 3β-hydroxyandrost-5-ene-17β-carboxylic acid. Reagents and conditions: (d) K_2CO_3, O_2, MeOH-THF, room temperature, overnight.

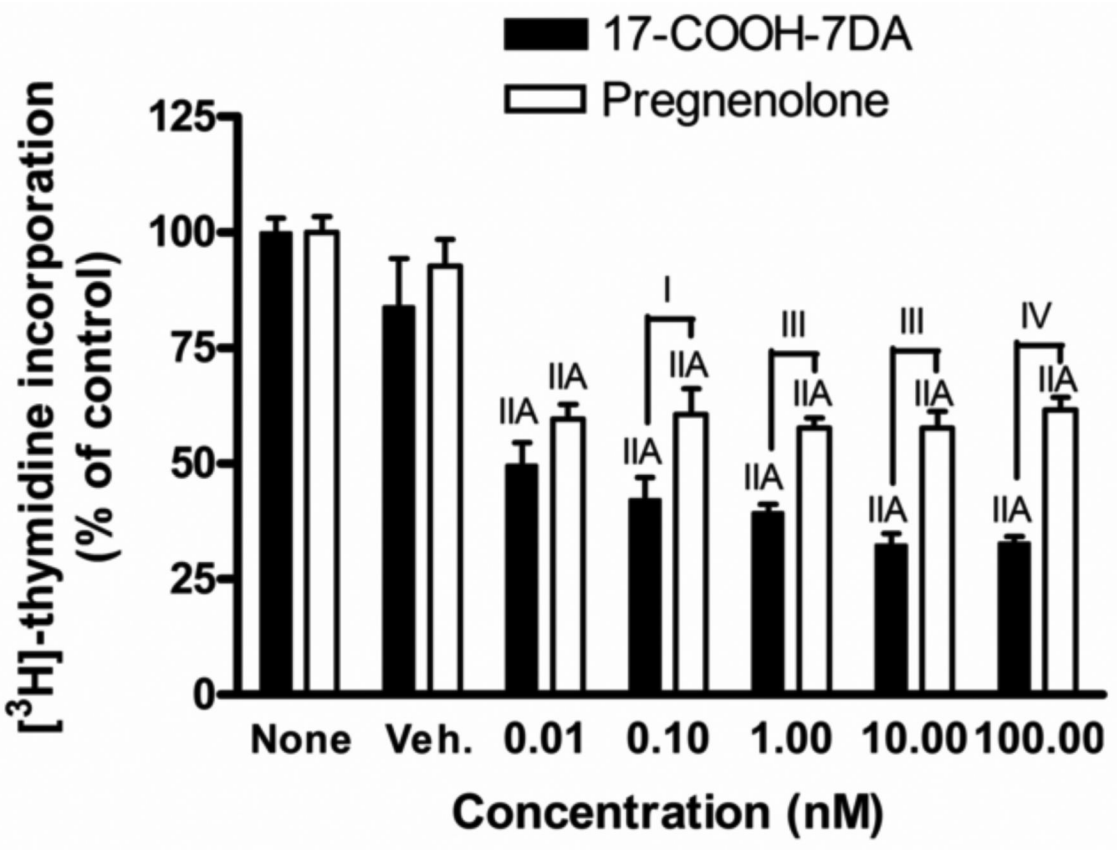


Fig. 1. 17-COOH-7DA inhibits DNA synthesis in HaCaT keratinocytes and has greater effect than pregnenolone

HaCaT cells were incubated with drugs for 72 h in DMEM containing 5% charcoal treated FBS. [^{3}H]-thymidine was added for last 4 h of incubation. DNA synthesis was measured by counting the radioactivity incorporated into TCA precipitable material. Data are presented as means ± SEM (n = 4). The dose dependent inhibition was analyzed by one-way ANOVA with IA, $P < 0.05$ and IIA, $P < 0.01$. The differences between 17-COOH-7DA and pregnenolone was analyzed with student's t-test where II, $P < 0.01$ and III, $P < 0.001$.

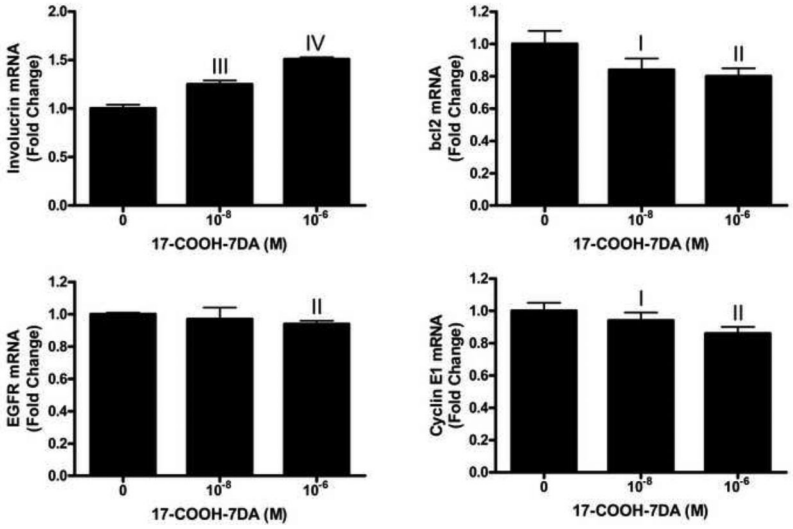


Fig. 2. 17-COOH-7DA stimulates involucrin and represses cyclin E1, bcl-2 and EGFR mRNA expression in HaCaT keratinocytes

HaCaT keratinocytes were treated with 17-COOH-7DA for 6 (involucrin) or 24 h (cyclin E1, bcl-2 and EGFR), total RNA was extracted and subjected to real time RT-PCR analysis with cyclophilin B used as a housekeeping gene. Data are presented as means ± SEM (n = 3). III, $P < 0.001$; IV, $P < 0.0001$ in student's t-test.

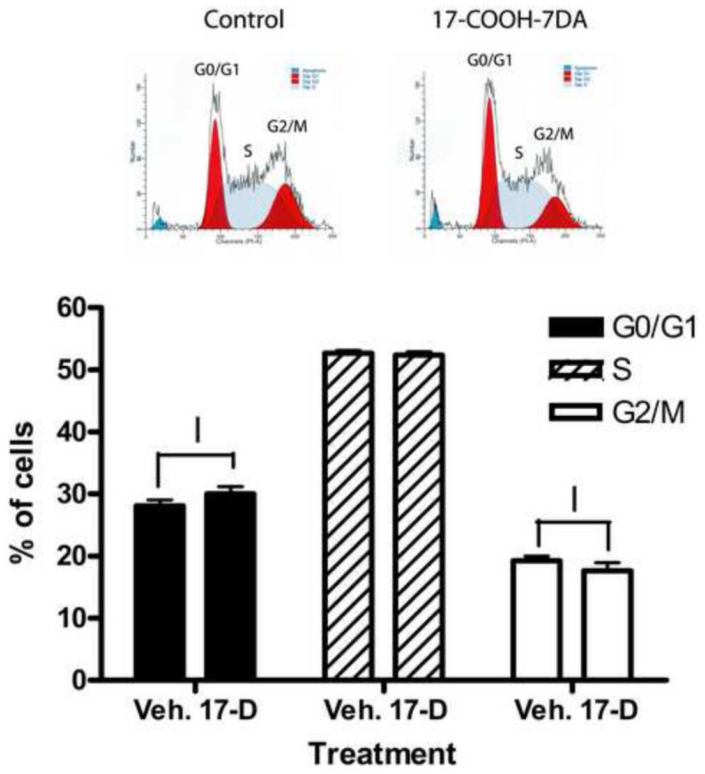


Fig. 3. Cell cycle analysis with flow cytometry

HaCaT keratinocytes were cultured in DMEM plus 5% ctFBS and 10^{-7} M 17-COOH-7DA for 72 h. The cells were fixed with 70% ethanol and stained with propidium iodide as described in materials and methods. Cell cycle was determined using flow cytometry with 10,000 cells scored. Veh., DMSO control; 17-D, 17-COOH-7DA. I, $P < 0.05$ in student t-test.

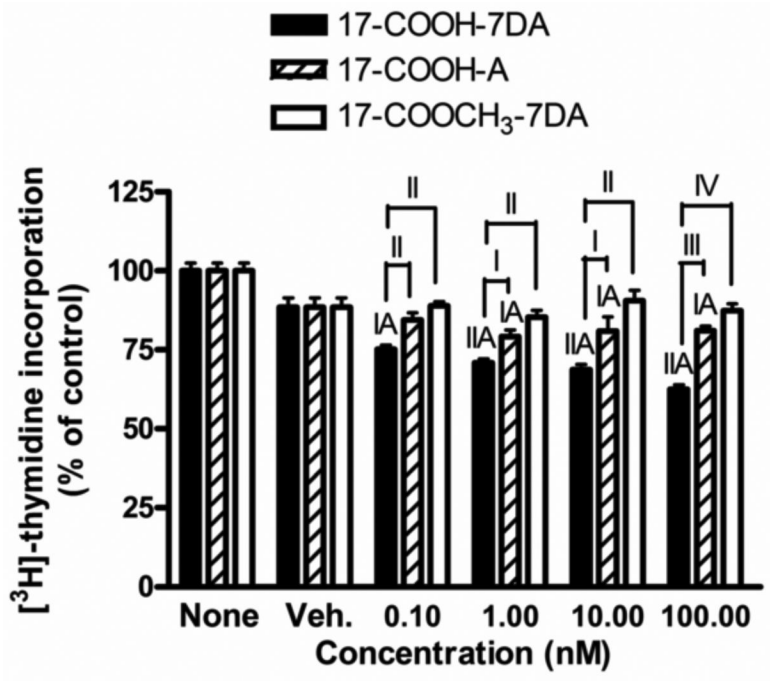


Fig. 4. Comparison of the effects of 17-COOH-7DA, 17-COOH-pregnenolone (17-COOH-A) and 17-$COOCH_3$-7DA on DNA synthesis in HaCaT keratinocytes

Data are presented as means ± SEM (n = 4). The dose-dependent inhibition was analyzed by one-way ANOVA with IA, $P < 0.05$ and IIA, $P < 0.01$. The differences between 17-COOH-7DA and 17-COOH-A or 17-$COOCH_3$-7DA were analyzed with student's t-test where II, $P < 0.01$ and III, $P < 0.001$.

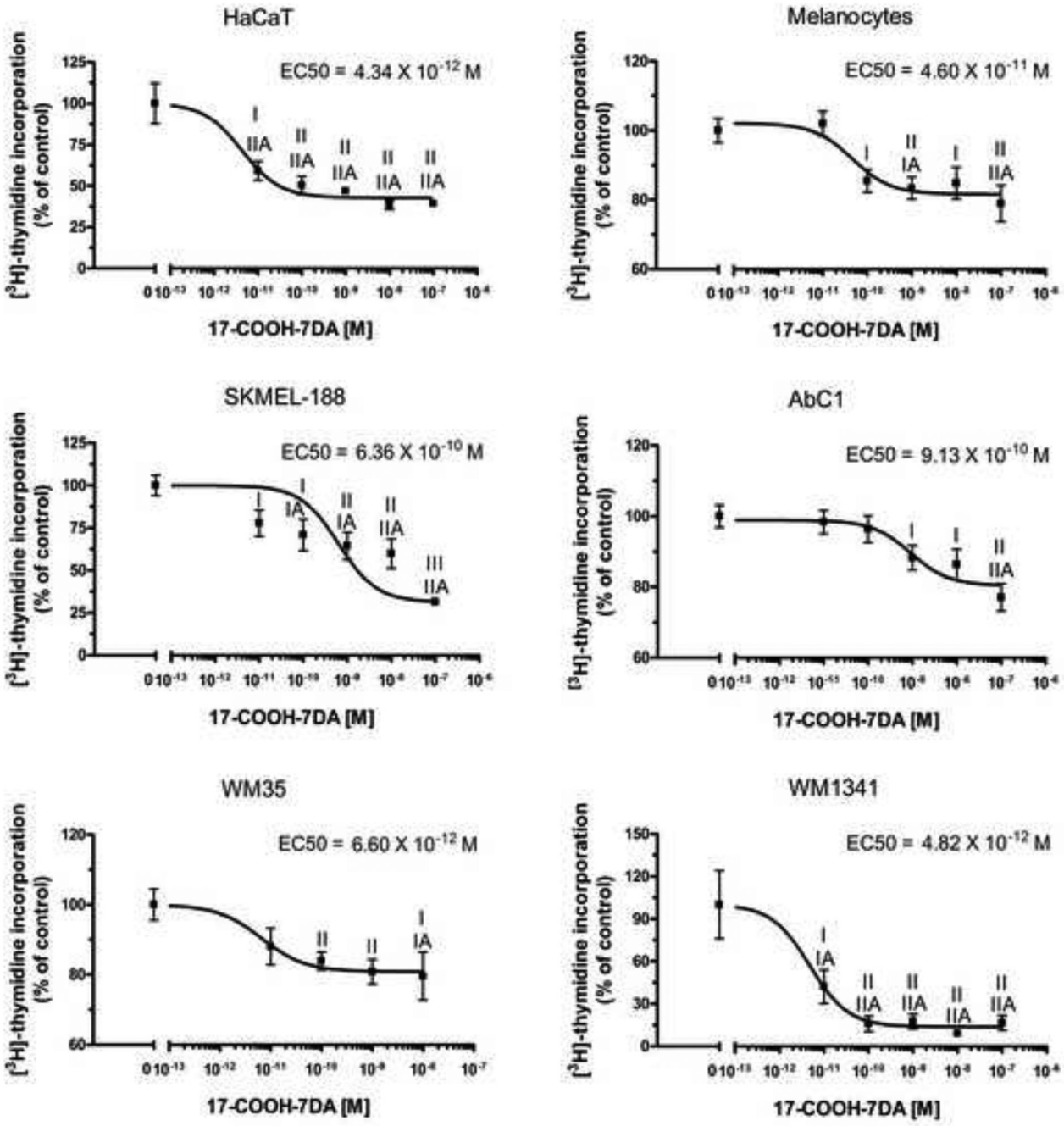


Fig. 5. 17-COOH-7DA inhibits DNA synthesis in human keratinocytes (HaCaT), normal melanocytes, melanoma cells (SKMEL-188, WM35, WM1341) and hamster AbC1 melanoma cells

Data are presented as means ± SEM (n = 4). I, $P < 0.05$; II, $P < 0.01$; III, $P < 0.001$ in student's t-test. IA, $P < 0.05$; IIA, $P < 0.01$ in one-way ANOVA analysis.

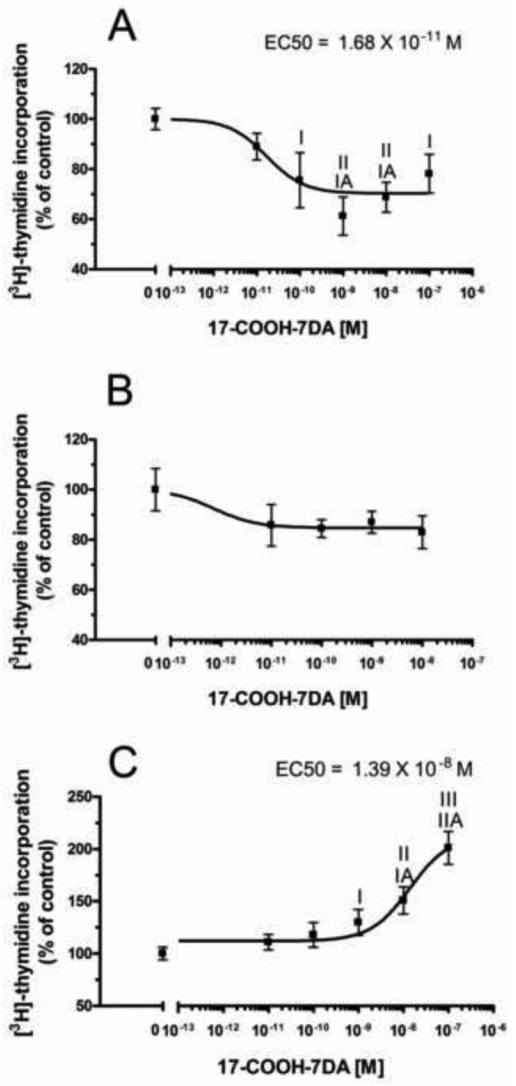


Fig. 6. The effect of 17-COOH-7DA on DNA synthesis in normal human dermal fibroblasts is dependent on the presence or absence of serum

Normal human dermal fibroblasts were cultured in DMEM containing 5 μg/ml insulin and 17-COOH-7DA in the absence (serum free media) or presence of 5% or 1% of charcoal treated FBS. After 68 h, [^{3}H]-thymidine was added and after 4 h the incorporation of [^{3}H]-thymidine into DNA was measured using a beta-counter. **A**, 5% FBS; **B,** 1% FBS; **C,** serum free media plus 0.1% BSA. Data are presented as means ± SEM (n = 4). II, $P < 0.01$; III, $P < 0.001$ in student's t-test. IA, $P < 0.05$; IIA, $P < 0.01$ in one-way ANOVA analysis.

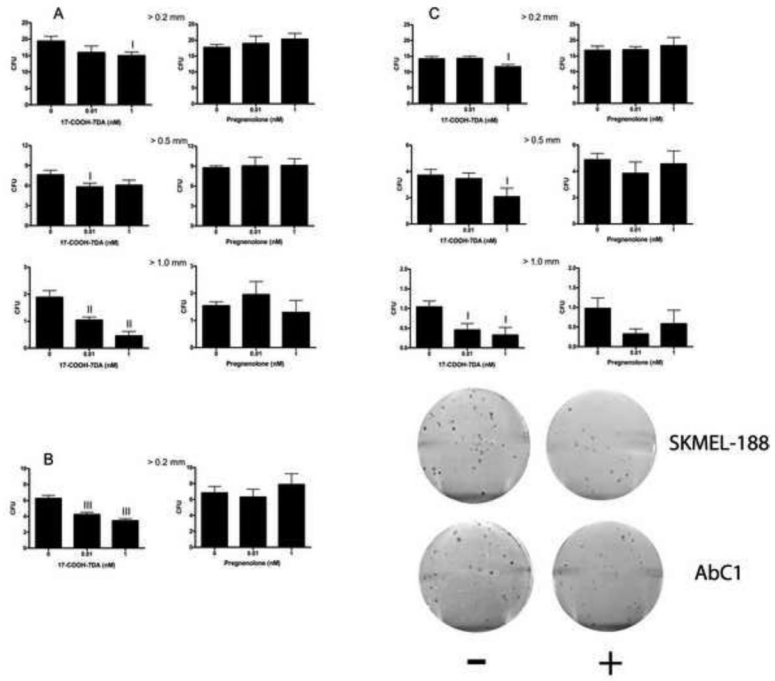


Fig. 7. 17-COOH-7DA inhibits colony formation of SKMEL-188 (A), WM35 (B) and AbC1 (C) melanoma cells

The plates were incubated with drugs for 7 (SKMEL-188 and AbC1) or 17 days (WM35) and colonies were stained with crystal violet. The number of formed colony units (CFU) were counted for all colonies (>0.2 mm), medium size colonies (>0.5 mm) or large colonies (>1 mm). Data are presented as means ± SEM for colony forming assay (n = 4). I, $P < 0.05$; II, $P < 0.01$; III, $P < 0.001$ in student's t-test. Lower right corner shows representative plates of SKMEL-188 and AbC1 treated with vehicle control (−) or 10^{-9} M 17-COOH-7DA (+).

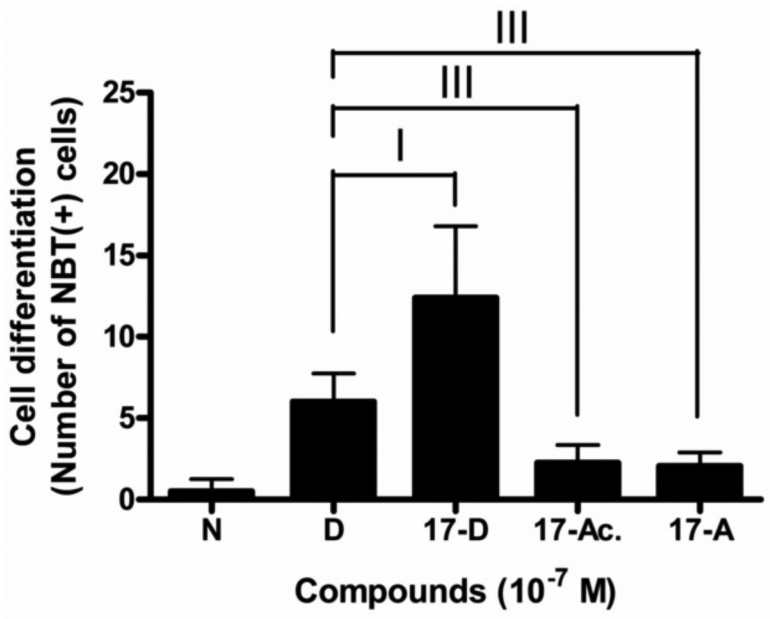


Fig. 8. Effects of 17-COOH-7DA, 17-COOH-pregnenolone (17-COOH-A) and 17-$COOCH_3$-7DA on monocyte-like differentiation of HL-60 leukemia cells

After 5 days of treatment, the cells were resuspended in NBT/TPA solution and incubated at 37°C for 60 min. The differentiated cells were identified by their intracellular blue formazan deposits. The NBT-positive and –negative cells were scored under light contrast microscopy (20x) with a minimum of 200 cells scored. N, untreated control; D, DMSO treated control; 17-D, 17-COOH-7DA; 17-Ac., 17-$COOCH_3$-7DA; 17-A, 17-COOH-A. Data are presented as means ± SEM (n = 3). I, $P < 0.05$; III, $P < 0.001$ in student's t-test.

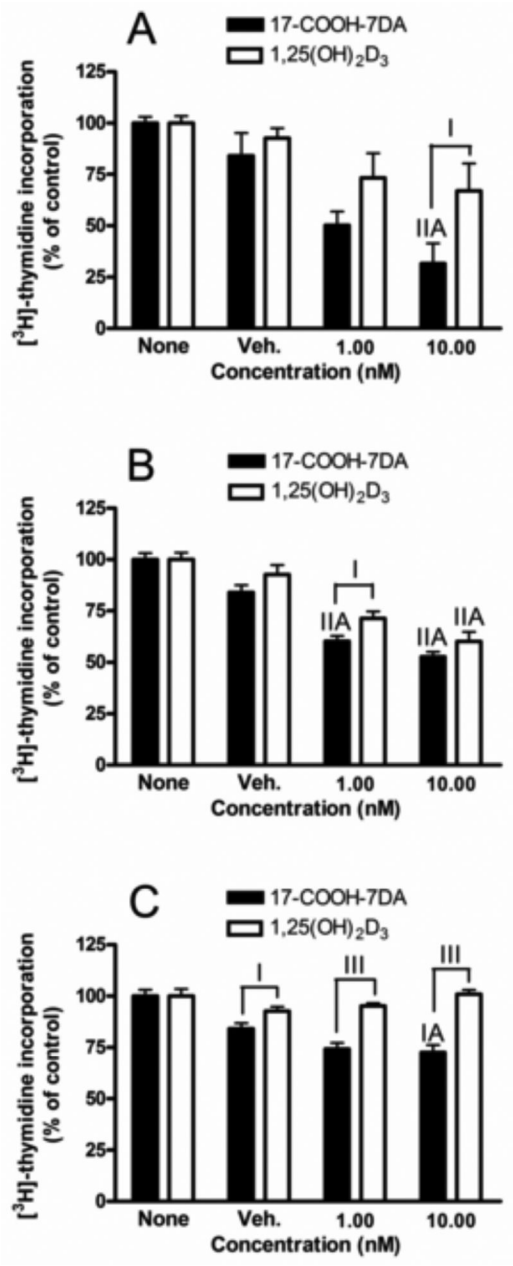


Fig. 9. 17-COOH-7DA inhibits DNA synthesis in HaCaT keratinocytes, SKMEL-188 and AbC1 and has a larger effect than $1,25(OH)_2D_3$

The cells were incubated with drugs for 72 h in DMEM (HaCaT) and Ham's F-10 (SKMEL-188 and AbC1) containing 5% charcoal-treated FBS. [^{3}H]-thymidine was added for last 4 h of incubation. DNA synthesis was measured by counting the radioactivity incorporated into TCA precipitable material. **A**, HaCaT; **B**, SKMEL-188; **C**, AbC1. Data are presented as means ± SEM (n = 4). The dose dependent inhibition was analyzed by one-way ANOVA with IA, $P < 0.05$ and IIA, $P < 0.01$. The differences between 17-COOH-7DA and $1,25(OH)_2D_3$ was analyzed with student's t-test where I, $P < 0.05$ and III, $P < 0.001$

Table 1

Primer sequences for Real-time PCR

Gene	Primer Sequences
Cyclophilin B	5′-TGTGGTGTTTGGCAAAGTTC-3′ 5′-GTTTATCCCGGCTGTCTGTC-3′
Involucrin	5′-TGCCTCAGCCTTACTGTGAGT-3′ 5′-TCATTTGCTCCTGATGGGTA-3′
Cyclin E1	5′-GGCCAAAATCGACAGGAC-3′ 5′-GGGTCTGCACAGACTGCAT-3′
Bcl-2	5′-AGTACCTGAACCGGCACCT-3′ 5′-GGCCGTACAGTTCCACAAA-3′
EGFR	5′-ACACAGAATCTATACCCACCAGAGT-3′ 5′-ATCAACTCCCAAACGGTCAC-3′

Table 2

NMR chemical shift assignments for 17-COOH-DA, 17-$COOCH_3$-7DA and 17-COOH-A by analysis of their 2D NMR spectra (solvent: DMSO-d_6)

Atom	17-COOH-7DA(5)		17-COOCH3-7DA(6)		17-COOH-A(8)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1α	1.22	38.35	1.24	38.47	0.98	37.47
1β	1.80	38.35	1.81	38.47	1.77	37.47
2α	1.74	32.27	1.73	32.27	1.68	31.96
2β	1.33	32.27	1.34	32.27	1.34	31.96
3	3.37	68.97	3.38	69.17	3.26	70.60
3-OH	4.66	NA	4.66	NA	4.60	NA
4α	2.32	41.18	2.32	41.27	2.14	42.67
4β	2.12	41.18	2.14	41.27	2.09	42.67
5	NA	141.41	NA	141.77	NA	142.18
6	5.49	119.05	5.51	119.03	5.27	120.73
7α	5.36	117.13	5.39	117.26	1.53	31.87
7β	NA	NA	NA	NA	1.93	31.87
8	NA	139.96	NA	139.72	1.41	32.12
9	1.93	46.12	1.94	46.14	0.91	50.24
10	NA	37.16	NA	37.10	NA	37.64
11α	1.61	20.90	1.62	20.97	1.37	20.97
11β	1.61	20.90	1.62	20.97	1.54	20.97
12α	1.33	37.74	1.36	37.39	1.23	38.16
12β	2.05	37.74	2.01	37.39	1.98	38.16
13	NA	44.18	NA	44.50	NA	43.60
14	1.97	53.55	2.00	53.37	1.09	56.08
15α	1.74	23.39	1.77	23.34	1.62	24.63
15β	1.45	23.39	1.48	23.34	1.20	24.63
16α	1.78	23.60	1.84	23.63	1.69	23.72
16β	2.00	23.60	2.08	23.63	1.95	23.72
17	2.39	54.54	2.49	54.35	2.26	55.11
18	0.56	13.44	0.53	13.54	0.64	13.54

Atom	17-COOH-7DA(5)		17-COOCH3-7DA(6)		17-COOH-A(8)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
19	0.86	16.53	0.86	16.51	0.95	19.67
20-COOH	11.96	175.15	NA	174.06	11.90	175.18
21-CH_3	NA	NA	3.61*	51.87*	NA	NA

NA – Not applicable (ternary carbons) ; 7α and 7β is only applicable for 17-COOH-A

*chemical shifts are for the methyl group in 17-COOCH3-7DA