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ORIGINAL ARTICLE

Study of the cytotoxic/toxic potential of the novel anticancer selenodiazoloquinolone on fibroblast cells and 3D skin model

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ABSTRACT

The new synthetically prepared quinolone derivative 7-ethyl 9-ethyl-6-oxo-6,9-dihydro[1,2,5]selenadiazolo [3,4-*h*]quinoline-7-carboxylate (E2*h*) showed in our previous study cytotoxic effects towards tumor cells and immunomodulatory activities on RAW 264.7 cell line murine macrophages. E2*h* may have a potential use as a novel chemotherapeutic agent with immunomodulatory properties and the ability to induce apoptotic death of cancer cells. The aim of the present study was to examine the antiproliferative/cytotoxic activities of E2*h* on human non-cancer fibroblast BHNF-1 cells and reconstructed human epidermis EpiDerm™. Further the effects of E2*h* on tissue structure and morphology were examined. Cytotoxic/toxic studies showed that selenadiazoloquinolone is not toxic on normal human fibroblast cells BHNF-1 and dimensional skin constructs EpiDerm™. Evaluation of morphological changes in EpiDerm™ showed no change in the construction and morphology of skin tissue treated by E2h compared to control.

KEY WORDS: quinolones; cytotoxicity; fibroblast BHNF-1 cells; EpiDerm™ viability; tissue morphology

Introduction

4-Quinolones (4-oxoquinolines) represent a group of heterocyclic compounds known for years. Many of them exhibited increased activity against bacteria and tumor cells *in vitro* and *in vivo*. Some of them are currently used worldwide in medical practice (*e.g.* ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) (Blasi *et al.*, 2006; Leibovitz, 2006; Hotinski *et al.*, 2015). Several quinolone derivatives, as *e.g.* vosaroxin, dovitinib, tipifarnib, are currently explored in clinical practice as potential drugs for different hematologic malignancies or acute myeloid leukemia therapy.

Quinolone derivatives are multitarget agents with a variety of biological activities (Sharma *et al.*, 2009; Chung *et al.*, 2010; Darque *et al.*, 2009; Santos *et al.*, 2009; Sokolova *et al.*, 2009; Meyer *et al.*, 2007; Vinayaka *et al.*, 2014; Huang *et al.*, 1998; Oriel, 1998). Quinolones were shown to possess also antitumor and immunomodulatory activities (Tawfik *et al.*, 1990; Xia *et al.*, 2001; Riesbeck, 2002; Dalhoff & Shalit, 2003; Dalhoff, 2005; Drygin *et al.*, 2009; You *et al.*, 2009; Chou *et al.*, 2009; Azema *et al.*, 2009; George & Pilli, 2013).

Quinolone derivatives are known as gyrase and topoisomerase I, II, and IV inhibitors, tubulin assembly inhibitors, farnesyltransferase inhibitors, P-glycoprotein inhibitors, protein kinase CK2 inhibitors, and photosystem II and cytochrome b6/f-complex inhibitors (Yamashita *et al.*, 1992; Khodursky *et al.*, 1995; Drlica *et al.*, 1997; Reil *et al.*, 2001, Golub *et al.*,2006, Medeiros *et al.*, 2007; Adams *et al.*, 2007; Asoh *et al.*, 2009).

Quinolone derivatives have cytotoxic/antiproliferative (Xiao *et al.*, 2008; Foroumadi *et al.*, 2009) and antimitotic activities (Chang *et al.*, 2009). They are inductors of DNA damage, cell cycle arrest and apoptotic and autophagic cell death (Chang *et al.*, 2009; Hadjeri *et al.*, 2004; Sheng *et al.*, 2008).

Based on the reported effects of quinolone derivatives, a new series of substituted selenadiazoloquinolones was prepared by Bella *et al.* (2010) and derivatives were screened for photochemical, antimicrobial and

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cytotoxic/phototoxic activities *in vitro*. Some of these derivatives demonstrated biological activity.

The non-photoactivated and photoactivated 7-acetyl-6,9-dihydro-6-oxo-[1,2,5]selenadiazolo[3,4-h] quinoline (derivative E2*h*) exhibited the highest cytotoxicity/phototoxicity on selected cell lines (Jantová *et al.*, 2010; Jantová *et al.*, 2016a; Barbieriková *et al.*, 2011a; 2011b; 2013).

Detailed studies showed that E2*h* induced significant antiproliferative/cytotoxic activity on human lung carcinoma epithelial cells A549 and promyelocytic leukemia cells HL-60 and was found to be a potent inducer of apoptosis on leukemia cells. EPR studies showed that E2*h* behaved as a photosensitizer activating molecular oxygen upon photoexcitation and possessed sufficient photochemical stability under the given experimental conditions (Barbieriková *et al.*, 2011b; Barbieriková *et al.*, 2013). Our immunological studies showed immunomodulatory activities of selenadiazoloquinolone on RAW 264.7 cell line murine macrophages (Jantová *et al.*, 2016b).

The obtained results indicate that selenadiazoloquinolone derivative E2*h* is a potential anticancer drug with immunomodulatory activities on topical administration. The aim of this work was to study the E2*h* cytotoxic/toxic potential on human non-cancer fibroblast BHNF-1 cells and human derived non-transformed epidermal keratinocyte model EpiDerm™.

Materials and methods

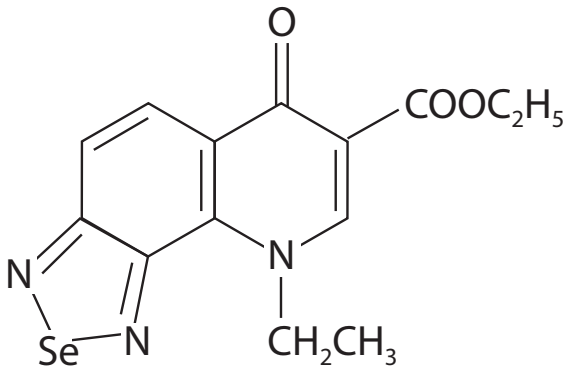
Material

DMSO was purchased from Merck (Slovakia). Trypan blue, thiazoyl blue tetrazolium bromide, phosphate buffered saline, penicillin and streptomycin were obtained from Sigma-Aldrich (Slovakia).

Dulbecco's modified eagle medium (DMEM) was from Invitrogen (Germany), fetal bovine serum was from PAA Laboratories GmbH (Austria).

Quinolone derivative

The synthesized 7-ethyl 9-ethyl-6-oxo-6,9-dihydro[1,2,5] selenadiazolo[3,4-*h*] quinoline-7-carboxylate (E2*h*, Scheme) was prepared by Bella *et al.* (2010). The stock solution of E2*h* was dissolved in 100% dimethylsulfoxide (DMSO), further diluted in the cell culture medium. Final concentration of DMSO added to cells did never exceed 0.1%.



Scheme. Chemical structure of 7-ethyl 9-ethyl-6-oxo-6,9-dihydro[1,2,5]selenadiazolo[3,4-*h*] quinoline-7-carboxylate (E2*h*).

Cell culture

Non-cancer human neonatal diploid cell line BHNF-1 (obtained from the collection of the Institute of Medical Biology, Genetics and Clinical Genetics, Comenius University in Bratislava, Slovakia) was used for cytotoxicity tests. These cells were grown at 37 °C in DMEM medium in humidified 5%-CO_2 and 95%-air atmosphere. The medium was enriched with 10% (vol/vol) fetal calf serum, penicillin G (100 μg/mL) and streptomycin (100 μg/mL). Before a uniform monolayer of BHNF-1 cells was formed, cells were freed from the surface of the culture dish by a 0.25% solution of trypsin and were subcultivated two to three times a week. The cells were plated on Petri dishes (diameter 60 mm) at a density of 3×10^5–5×10^4 cells per mL of medium and incubated for 24 h prior to the experiments. Cell viability was determined by trypan blue exclusion tetradiazoloquinolone on fibroblasts and skin model.

3D human skin model

Reconstructed human three-dimensional skin constructs EpiDerm™ (EPI-200) were used for the toxicity test. The reconstructed human epidermis tissues (EpiDerm™) were obtained from MatTek Corp. (Bratislava, Slovakia). This *in vitro* model consists of normal, human-derived epidermal keratinocytes (NHEK) cultured at the air-liquid interface on a semi-permeable tissue culture insert. The NHEK cells form a multilayered, highly differentiated model of the human epidermis that consists of organized basal, spinous, granular, and cornified layers (stratum corneum), closely resembling native human epidermis.

Upon arrival, the EpiDerm™ tissues were transferred to 6-well plates (Sarstedt, Slovakia) containing 0.9 mL Maintenance Media (proprietary media provided by MatTek Co., and included with the tissues). The tissues were then equilibrated overnight at 37 °C, 5% CO_2 in a humidified incubator prior to exposure to the test substances.

Growth inhibition assay

The inhibitory effects of cell proliferation by E2*h* were investigated by direct counting of the cell number in Bürker chamber, as described by Jantová *et al.* (2007). Before counting, the cells were stained with trypan blue dye (diluted 1:1 with cell suspension) to detect cell viability. The cells at a final density of 5×10^4 cells/mL were placed in Petri dishes (diameter 60 mm) overnight and treated with different concentrations of E2*h* (Scheme) for 24, 48 or 72 h. Final concentrations of the derivative added to the cells were in the range from 14 to 143 μM. Control cells were treated with DMSO, its final concentration never exceeding 0.1% (vol/vol). Negative control (NC) experiments were performed using DMSO-free cell systems.

The relative inhibition of cell proliferation or degeneration of a cell population was calculated as described by Letašiová *et al.* (2006).

After curve fitting, using nonlinear regression (Origin 7.0, Microcal), the IC_{50} values (the concentration resulting in 50% inhibition of the cell proliferation recorded in the control experiments) were determined separately for each experiment. The values were calculated from at least three independent experiments.

Chemical exposure of tissue model EpiDerm™

EPI-200 culture inserts were placed in 6-well plates and equilibrated with 0.9 mL of EPI-200-MM medium at 37 °C. Following 1 h pre-incubation, the culture medium was replaced with fresh 0.9 mL of medium and skin cultures were placed on top of two stainless steel washers in 24-well plates. Tissues were treated by topically applying 50 µL of E2*h* derivative concentrations of 143 µM for 24, 48 and 72 h and at each time interval the culture medium and tissues were collected for analysis. Non-treated tissues were used as negative control, the tissue treated with 0.1% DMSO was used as control with dissolvent. Tissue samples were either used for the MTT tissue viability assay or harvested and stored in buffered formalin for histological analyses.

MTT tissue viability assay

The MTT assay (MTT-100, MatTek Corporation) was carried out as per manufacturer's instructions. In brief, at the end of 24,48 and 72 h of treatment, EPI-200 tissue samples were washed twice with PBS and placed in a fresh 24-well plate containing 300 µL/well of MTT solution.

After 3 h of incubation at 37 °C, each insert was removed carefully, the bottom was blotted with paper wipes and the insert was transferred into a fresh 24-well plate. The culture inserts were then immersed in 2 mL/well of extraction solution (isopropanol). The plates were covered to reduce evaporation and incubated and shaked for 2 h at room temperature in the dark. After 2 h extraction, inserts were discarded and the contents of each well were mixed thoroughly before transferring 200 µL of the sample into 96-well plates. The optical density of the samples was read at 540 nm. Background readings for all the samples were determined at 740 nm and were subtracted to obtain the correct O.D. The % viability was determined for each tissue using the equation:

% Viability = 100 × [OD(sample)/OD(negative control)], where OD is optical density.

Table 1. Concentration of E2h inducing 50% inhibition of growth (IC_{50}) of B-HNF-1, HeLa, HL-60 and A549. The values are in µM.

Cell line	24 h	48 h	72 h
BHNF-1	>143.0	>143.0	>143.0
HeLa*	41.7±8.2	43.2±3.0	29.0±2.9
HL-60*	55.0±5.0	28.0±1.8	27.0±2.1
A549*	99.0±6.5	101.0±6.0	102.0±8.5

*In: Jantová *et al.*, 2016a,b

Histological studies of tissue model

The EPI-200 cultures were collected at the end of the study and fixed in 10% neutral phosphate buffered formalin for at least 24 h at room temperature. Following fixation, the samples were dehydrated and embedded in paraffin. Five micrometer microtomed sections of the skin tissue samples were stained with hematoxylin and eosin according to common histological procedures (MatTek Corporation Protocol). The stained slides were examined under a Motic BA410 and assessed for histo-pathological changes associated with derivative exposure.

Statistical analysis

The individual data points from cell culture and 3D human skin model are presented as the means ± standard deviation (SD) of the mean of three separate experiments (each experiment was done with five parallels). The statistical significance of the results obtained from *in vitro* studies was evaluated by Student's t-test, with probability values of 0.05 being considered significant.

Results

The results of the cytotoxic effects of selenaquinolone E2*h* (Scheme) measured by direct cell counting are shown in Figure 1. Different derivative concentrations in the range of 14.0–143 µM were added to the fibroblast BHNF-1 cells and cultivated for 24–72 h. As shown in the figure, derivative E2*h* did not manifest massive cytotoxic effect on normal fibroblast cells. We found only 25.2% growth inhibition after 72 h of BHNF-1 cells treated with the highest concentration tested (143 µM). The cells treated with the other concentrations tested (109, 71, 29 and 14 µM) grew comparably to non-treated control cells.

Table 1 shows the values of growth inhibitory concentrations IC_{50} of E2*h*. The values were obtained from the

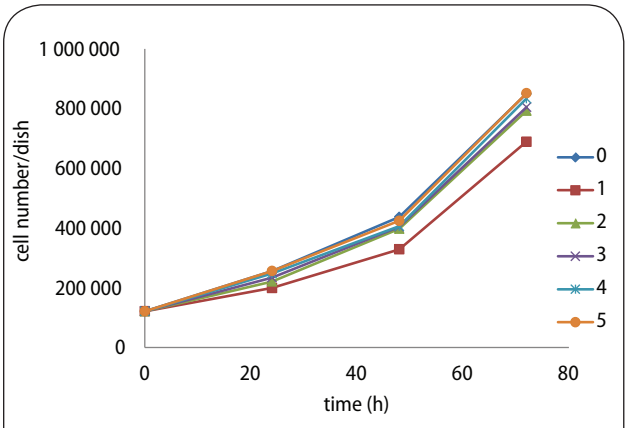


Figure 1. Effect of E2*h* on proliferation of B-HNF-1 cells. The cells were cultured during 72 h in the absence or presence of E2*h* (concentrations in the range 14 - 143 µM). C – control cells treated with 0.1% DMSO. Cell proliferation was determined by direct counting in a counting chamber. Results were expressed as proliferation (cell number per dish) and represent mean ± SEM of three independent experiments, performed by triplicate.

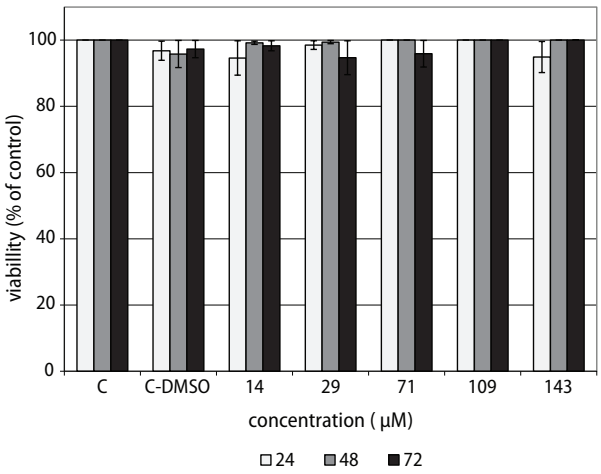


Figure 2. Assessment of E2*h* skin toxicity. Toxicity of E2*h* (concentrations in the range 14 - 143 μM) was evaluated in the EpiDerm™ by the MTT assay after 24, 48 and 72 h incubation at 37°C. The fraction of epidermal cells remaining highly viable after 24, 48 and 72 h at 37°C was measured as the absorbtion of formazan at 570 nm, subtracting a background reading for all samples at 650 nm with UV-visible spectrometer. Percentage of cell viability was determined at each of the concentration doses using the following formula: % viability = 100× ([Abs570 - Abs650] of sample)/([Abs570 – Abs650] of a control). Control was non-treated tissue and all data were normalized using data of control at 24, 48 and 72 h, and reported as mean ± SD. C-DMSO – tissue treated with 0.1 % DMSO.

growth curve of non-cancer BHNF-1 and cancer cell lines HeLa, A549 and HL-60 treated for 72 h with E2*h*. As seen from Table 1, the IC_{50} values measured in the experiment with the cancer cell lines were lower than for the non-cancer fibroblast cell line. Further, the effects on viability and morphology of the skin tissue model EpiDerm™ were evaluated. The results are documented in Figures 2 and 3. Initial experiments with the EpiDerm™ model indicated that a dose volume of 50 μL of E2*h* concentrations tested (14, 29, 71, 109 and 143 μM) per EPI-200 tissue did not result in any loss of tissue viability (Figure 2).

Even the highest concentration tested, *i.e.* 143 μM, did not cause reduction of tissue viability. Figure 3 presents the morphological changes in EPI-200 skin following exposure to E2*h* concentration of 143 μM for 24, 48 and 72 h. After 24, 48 h and 72 h of exposure, E2*h* did not induce significant structural changes to the tissue (Figure 3).

Discussion

Quinolone derivatives are condensed nitrogen heterocyclic compounds known as antibacterial agents, which have been the subject of many research interests. Quinolone antibiotics come close to be ideal chemotherapeutic agents administered orally, parenterally and topically, concentrated in cells and tissue, readily available, relatively safe and exhibiting increased activity against bacteria and tumor cells both *in vitro* and *in vivo*. Some quinolone derivatives are currently explored clinically as potential drugs for different hematologic malignancies, acute myeloid leukemia, colon cancer or melanoma therapy (Thadepalli *et al.*, 2005).

On the other hand, different sensitivities of non-cancer cells were found on quinolone treatment by some authors (Aranha *et al.*, 2003; Xiao *et al.*, 2008; Wang *et al.*, 2008; Al-Trawneh *et al.*, 2010; Huang *et al.*, 2011; Forezi *et al.*, 2014).

With the aim of obtaining new anticancer agents, a series of substituted quinolones, fluoroquinolones and selenadiazoloquinolones were prepared (Bella *et al.*, 2014). Our previous experiments revealed some of these agents to be potent drugs towards microbial and cancer cells. The highest biological activity was caused by 7-ethyl 9-ethyl-6-oxo-6,9-dihydro [1,2,5]selenadiazolo[3,4-h] quinoline-7 carboxylate (E2*h)* on cancer cells.

Cytotoxic tests showed that derivative E2*h* evoked significant cytotoxic activity towards leukemia cell lines (L1210 and HL-60 cells), adenocarcinomic human alveolar basal epithelial cells A549 and cervical cancer cells HeLa (Jantová *et al.*, 2016a,b). On the other hand, this agent did not display massive cytotoxicity on mouse non-cancer fibroblast cells NIH-3T3 (Jantová *et al.*, 2010; Jantová *et al.*, 2011; Barbieriková *et al.*, 2011a; Barbieriková *et al.*, 2011b; Barbieriková *et al.*, 2013). Further we found that selenadiazoloquinolone E2*h* is inducer of apoptotic death of leukemia cells with immunomodulate properties on murine RAW 264.7 macrophages. E2*h* induced time- and concentration-dependent effective immunomodulation of pro- and anti-inflammatory cytokine release and antiproliferative/cytotoxic effect on RAW 264.7 cells (Jantová *et al.*, 2016b). Our data show the E2*h* anticancer potential, suggesting its eventual application as chemotherapeutic drug.

This finding prompted us to study the effects of E2*h* on growth and viability of human non-cancer BHNF-1 cells and human derived non-transformed tissue model EpiDerm™. 3-D human skin culture (EpiDerm™) is a living reconstructed human epidermis used to provide information regarding the cytotoxicity, irritant potential and immunotoxicity of various compounds. In our study, we used the 3D skin model for toxicity observation of E2*h*, which could be topically administered in cervix adenocarcinoma and melanoma treatment.

As follows from the growth curves of fibroblast BHNF-1 (Figure 1), derivative E2*h* induced low antiproliferative effect, which even decreased with time. The cytotoxicity of the highest concentration of E2*h* tested was only 25.2% after 72 h of treatment.

On comparing the IC_{50} values as determined by the cell growth inhibition assay (Table 1) for non-cancer and cancer cell lines, it can be summarized that the least sensitive to E2*h* were human fibroblast BHNF-1 cells.

Our results are in accordance with the findings of many authors (Aranha *et al.,*2003; Xiao *et al.,*2008; Wang *et al.,*2008; Al-Trawneh *et al.,*2010; Huang *et al.,*2011; Forezi *et al.,*2014). Aranha *et al.* (2003) reported that ciprofloxacin showed antiproliferative and apoptosis inducing activity on prostate cancer cells but not on non-tumorigenic prostate epithelial cells. Xiao *et al.* (2008)

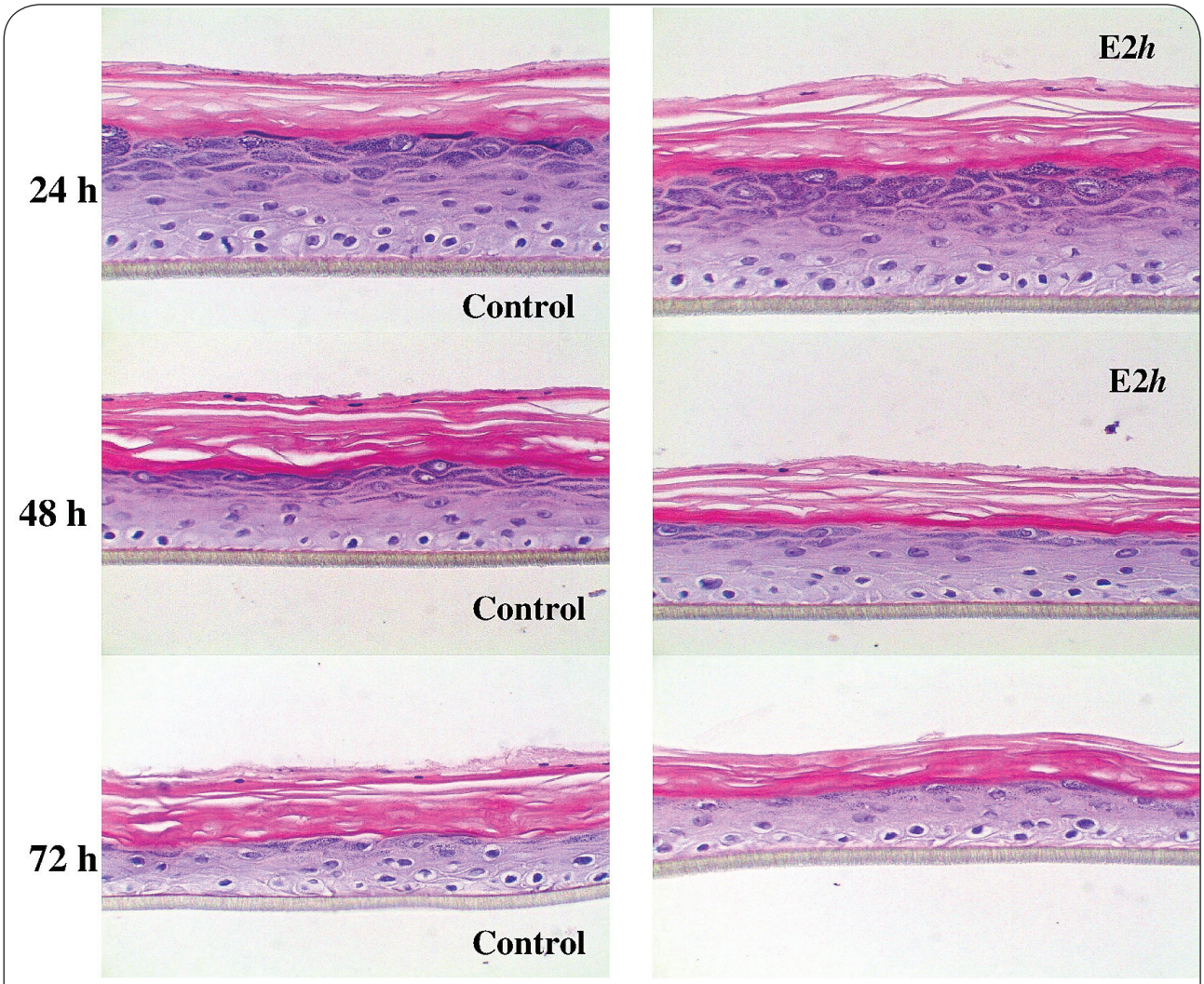


Figure 3. Effect of E2*h* (143 μM) on EPI-200 tissue morphology. Control and treated EPI-200 cultures were fixed in 10% neutral phosphate buffered formalin for at least 24 h at room temperature. Then the samples were dehydrated and embedded in paraffin. Five micrometer microtomed sections of the skin tissue samples were stained with hematoxylin and eosin and the stained slides were examined under microscope and photographed. Images were obtained at 400× magnification.

found that most of 3-aryl-1H-quinolin-4-one derivatives showed good cytotoxic activity against human cancer cell lines, but no cytotoxicity against a human normal cell line (L02). Selective cytotoxic activity of 2'-fluoro-6,7-methylenedio- xy-2-phenyl-4-quinolone was reported by Wang *et al.* (2008). Quionolone induced growth inhibition of cancer HA22T, Hep3B, and HepG2 cells in a concentration-dependent manner but did not impair the viability of normal cells. Al-Trawneh *et al.* (2010) reported that five of 6-fluoro-4-oxopyrido[2,3-a]carbazole-3-carboxylic acids displayed growth inhibition against MCF-7 breast tumor and A549 non-small cell lung cancer cells coupled with the absence of cytotoxicity toward normal human-derm fibroblasts (HuDe). Huang *et al.* (2011) found that 2-(3-(methylamino) phenyl)-6-(pyrrolidin-1-yl) quinoline-4-one inhibited cell growth of leukemia cells in a dose-dependent manner. The derivative showed a much lower cytotoxic effect on normal HUVEC cells than on HL-60 cells. Also Forezi *et al.* (2014) reported that two derivatives from a series of 4-oxoquinolines displayed cytotoxicity against gastric cancer cell line but they did not display cytotoxicity at 20 μM on normal fibroblasts MRC-5.

3-D human skin culture (EpiDerm™) is a living reconstituted human epidermis used to provide information regarding the cytotoxicity, irritant potential and immunotoxicity of different compounds. In our study, we used this 3D skin model for assessing E2*h* toxicity. As seen in Figure 2, selenadiazoloquinolone E2*h* did not manifest toxic effects, with the percentage of cell viability in the range of 95-100.

EpiDerm™ tissue (EPI-200) obtained from MatTek Corporation is a multilayered, differentiated tissue consisting of basal, spinous, granular and cornified layers

resembling normal human epidermis. The tissue is constructed from normal epidermal keratinocytes (foreskin-derived), which are cultured on chemically modified, collagen-coated, 9-mm (i.d.) cell culture inserts (*e.g.* Millicell CM or Nunc polycarbonate cell-culture inserts). As seen in Figure 3, the control tissue and tissue treated with E2*h* (143 μM) consisted of non-damaged layers and no structural and morphological changes were found.

As follows from Table 1 and Figures 3 and 4, quinolone E2*h* did not manifest toxicity on human three-dimensional skin constructs EpiDerm™ and the highest derivative concentration of 143 μM did not induce significant morphologic damage in tissues.

In summary, we can conclude that the novel anticancer 7-ethyl 9-ethyl-6-oxo-6,9-dihydro[1,2,5] selenadiazolo [3,4-*h*] quinoline-7-carboxylate (E2*h*) which induces cytotoxic effects and apoptosis on human cancer cells is not toxic on human normal fibroblast cells BHNF-1 and human three-dimensional skin constructs EpiDerm™ and can thus be considered a potent chemotherapeutic drug.

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