Biomaterials
Science

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Journal:	*Biomaterials Science*
Manuscript ID:	BM-COM-07-2014-000245.R3
Article Type:	Communication
Date Submitted by the Author:	17-Dec-2014
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Communication

The effect of photoinitiators on intracellular AKT signaling pathway in tissue engineering application

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Abstract

Free-radical photopolymerization initiated by photoinitiators is an important method to make tissue engineering scaffolds. To advance understanding of photoinitiator cytocompatibility, we examined three photoinitiators including 2,2-dimethoxy-2-phenylacetophenone (DMPA), Irgacure 2959 (I-2959), and eosin Y photoinitiating system (EY) in terms of their effects on viability of HN4 cells and expression levels of intracellular AKT and its phosphorylated form p-AKT. Our results show that the photoinitiators and their UV-exposed counterparts affect intracellular AKT signaling, which can be used in conjunction with cell viability for cytocompatibility assessment of photoinitiators.

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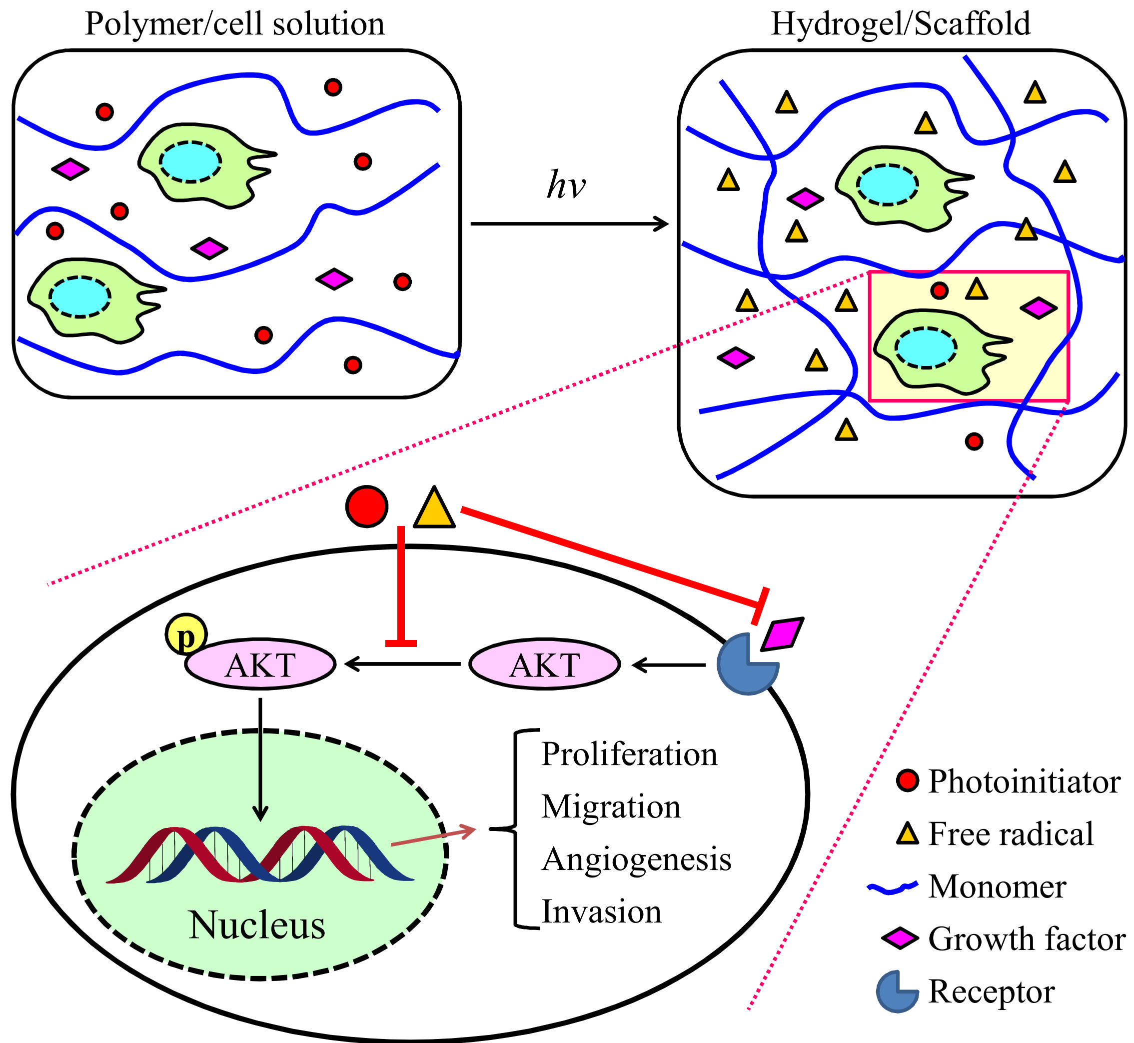

Introduction

A variety of biomedical materials have been successfully synthesized using photopolymerization chemistry, which allows rapid conversion of photoreactive solutions into gels or solids in situ under physiological conditions with minimal heat production.[1-5] Photopolymerization is also an important strategy to make tissue engineering scaffolds as bioactive compounds, drugs, and even cells can be readily loaded into the gel matrix during photoinitiated polymerization. Since the Anseth group first reported the effects of photoinitiators on cell survival 15 years ago, the effects of photoinitiators on cell enzymatic activity, cell membrane permeability, cell adherence, cell cycle, apoptosis, stem cell differentiation, protein production and function as well as plasmid DNA have been examined.[6-10] Nonetheless, the effect of photoinitiators on intracellular signaling pathways has not been fully elucidated but would be an important aspect to understand photoinitiator cytocompatibility given that cells utilize diverse signaling pathways to regulate their biological activities and transmit information from the microenvironment.

AKT, known as protein kinase B, is a serine/threonine-specific protein kinase that regulates cell proliferation, survival, and motility.[11] It has been well-documented that AKT plays a central role in the downstream of activated growth factor receptor signaling.[12] First, AKT enhances cell survival by blocking the function and expression of several B-cell lymphoma 2 (Bcl-2) homology domain-only proapoptotic proteins, which bind and inactivate prosurvival Bcl-2 family members.[13] Second, AKT promotes cell growth by activating mammalian target of rapamycin complex 1 (mTORC1), a critical regulator of translation initiation and ribosome biogenesis.[14] Third, AKT stimulates cell proliferation through multiple downstream targets such as glycogen synthase kinase 3 (GSK3), tuberous sclerosis 2 (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40).[12] Given the fundamental roles the AKT signaling plays, it is of importance to understand how AKT signaling is affected by photopolymerization, which, in turn, sheds light into understanding of photoinitiator

cytocompatibility at the molecular level. Photopolymerization is initiated by free radicals resulting from UV or visible light-induced photoinitiator decomposition. Free radicals may react with cell membrane and intracellular components (e.g., proteins and DNA) or induce formation of reactive oxygen species (ROS), thereby causing unwanted cellular damages.[6] In this work, we studied three widely used photoinitiators: 2,2-dimethoxy-2-phenylacetophenone (DMPA), 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (commonly known as Irgacure 2959 or I-2959), and eosin Y photoinitiating system (EY) by examining their effects on cell viability and the intracellular AKT signaling along with their UV-induced decomposed counterparts.

Experimental Section

Materials

1-vinyl-2 pyrrolidinone (NVP), 2,2-dimethoxy-2-phenylacetophenone (DMPA), eosin Y, ethanol, phosphate buffered saline (PBS), and triethanolamine (TEOA) were purchased from Sigma-Aldrich (St. Louis, MO). I-2959 was provided by Ciba Corporation (Newport, DE). Cell proliferation reagent WST-1 and protease and phosphatase inhibitors were purchased from Roche Applied Science (Indianapolis, IN). Phospho-AKT (Ser473) (p-AKT) antibody was purchased from Cell Signaling Technology (Danvers, MA). AKT1 (559028) antibody was purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). β-actin (ACTBD11B7) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit antibody conjugated to horseradish peroxidase and goat anti-mouse antibody conjugated to horseradish peroxidase were purchased from Bio-Rad (Hercules, CA).

Cell culture

HN4 cells, derived from a primary squamous cell carcinoma of the head and neck, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Cosmic calf serum at 37 ºC in 95% air/5% CO_2.[15]

Preparation of cell culture media conditioned with photoinitiators

Photoinitiator DMPA and I-2959 stock solutions (0%, 2.5%, 5%, 10% and 25% (w/v) in ethanol) were prepared based on weight per volume calculations per industry standard. EY stock solution was prepared by mixing 0.1% eosin Y, 4% NVP, and 40% TEOA in PBS according to a previous report.[16] The prepared stock solutions were stored in dark at room temperature until use.

Cell culture media containing DMPA or I-2959 at various final concentrations (i.e., 0%, 0.05%, 0.1%, 0.2% and 0.5% w/v) were prepared by mixing 2 ml of fresh culture medium with 40 µl of stock solution. To prepare EY-containing cell culture media, 0, 5, 10, 20 or 50 µl of EY stock solution was added to 2 ml of fresh culture medium to obtain final concentrations of 0%, 0.0025%, 0.005%, 0.01% and 0.025 % (v/v) of EY.

UV-exposed photoinitiator-containing media were prepared by subjecting photoinitiator-containing media mentioned above to UV light at 365 nm with an intensity of 1 W/cm^2 at a distance of 14 cm for 30 min (UVP Blak-Ray Long Wave Lamp)[17, 18] and used immediately. The media without photoinitiator subjected to UV light under the same condition were used as an experimental control.

Cell proliferation assay

HN4 cells pre-seeded in 96-well plates were incubated in the conditioned media for 24 h. Cell proliferation was then determined by using WST-1 assay following the standard manufacturer's protocol as described previously.[19]

Western blotting

HN4 cells pre-seeded in 6-well plates were treated with the conditioned media for 30 min. Total protein lysates were then harvested with total cell lysate buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). The total cellular p-AKT and AKT1 expression levels were evaluated by Western blotting following the standard procedures as described previously.[20]

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Western blotting results were repeated at least three times. One way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed by using GraphPad Prism 5. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

In the state-of-the-art tissue engineering scaffolding approaches, cell seeding relies extensively on photopolymerization, which is quick and efficient. A good understanding of toxicity of photoinitiating agents is important to the success of tissue engineering. During photopolymerization, besides UV exposure, free radicals and photoinitiators themselves present in those hydrogels or engineering scaffolds may cause toxicity to the seeded cells. Cytotoxicity profiles of several photoinitiating systems have been well reported.[4, 6, 21] However, the effects of photoinitiators on intracellular signaling have not been fully documented. In this work, we mimicked the microenvironment in the cell encapsulated hydrogels or tissue engineering scaffolds by using a simple 2-D cell culture model. First, most growth factors bind to receptors and then activate intracellular AKT signaling pathway. Therefore, we chose HN4 cells as our model cell line because they constitutively express a high level of AKT activity.[15] Although HN4 cells are not a standard cell type utilized in tissue engineering, the hyperactivity of AKT in HN4 cells can be used to mimic intracellular response to growth factors. Second, in tissue engineering, the encapsulated cells and cell culture media components such as growth factors will be directly exposed to the free radicals during photopolymerization. These free radicals not only trigger polymerization of monomers but also may cross-react with proteins, fatty acids and even cells. Although radicals may be immediately quenched following UV or visible light exposure, the photoinitiators remain in the hydrogels or scaffolds until they are diminished. Therefore, we treated cells with photoinitiator- or UV-exposed photoinitiator-containing media for 30 min and 24 h to mimic the microenvironment for cell encapsulation.

The study presents the cytotoxicity profiles of DMPA, I-2959 and EY on HN4 cells following 24 h treatment (**Fig. 1**). Cells treated with DMPA at 0.05% (w/v) and above lost viability significantly. In contrast, cells treated with I-2959 showed decreased cell viability in a dose-dependent manner. This finding supports previous reports that I-2959 is relatively nontoxic within the range of 0.03-0.1% (w/v) to a variety of cell types.[4] Similarly, cells treated with EY decreased viability in a dose-dependent manner. Particularly, toxicity of EY at 0.01% (v/v) or below was negligible. This finding agrees well with our previous reports showing good cytocompatibility of dendrimer hydrogel formulations prepared with EY.[5, 16]

Photopolymerization has been widely explored in tissue engineering scaffold and hydrogel formulations. In this application, cell invasion, migration, proliferation and differentiation as well as angiogenesis are key factors for the success of photopolymerized scaffolds and hydrogels.[22] To foster various cellular activities, growth factors are incorporated into the scaffolds or hydrogels. For instance, transforming growth factor-β1 (TGF-β1) was loaded into poly(ethylene oxide)-terephtalate and poly(butylene terepthalate) scaffold to promote the chondrogenesis of human bone marrow mesenchymal stem cells.[23] To stimulate vascularization in bone regenerative constructs, VEGF was loaded in gelatin microparticles at the preferred location within 3D bioprinted scaffolds.[24] Besides TGF and VEGF, a number of growth factors have been investigated in tissue engineering, including Ang, angiopoietin; BMP, bone morphogenetic protein; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor, etc.[25] Despite their different functions, these growth factors can trigger AKT signaling pathway activation. As a master regulator, AKT transmits signals from the extracellular environment and regulates cell viability, migration and proliferation as well as angiogenesis.[11, 12]

The effects of photoinitiators on intracellular AKT signaling in HN4 cells were examined. After 30 min treatment, DMPA rapidly diminished p-AKT expression in a dose-dependent manner (**Fig. 2A**), suggesting that DMPA inhibited intracellular AKT signaling

pathway. The rapid inhibition of AKT activity could lead to several downstream effects, including reduction in cell viability, motility and proliferation as well as angiogenesis. Similar observation was achieved in I-2959 but with higher concentrations (**Fig. 2B**). The inhibition of intracellular AKT signaling by I-2959 was closely correlated to the cytotoxicity of I-2959 (**Fig. 1A**). It was noticed that I-2959 at low concentrations (up to 0.2% w/v) had no effect on AKT signaling. In contrast, EY had no significant rapid effect on AKT signaling up to 0.025% (v/v) (**Fig. 2C**). This data suggested that EY rarely induced rapid effect on AKT signaling, which supported our finding that EY exhibited relatively moderate cytotoxicity (**Fig. 1B**).

DMPA and I-2959 are UV-photoinitiators, and they can decompose into free radicals at 365 nm to trigger polymerization of monomers or macromonomers.[6, 18] Eosin Y is a visible light photoinitiator. In the presence of electron donor TEOA serving as a co-initiator, eosin Y can trigger polymerization upon UV irradiation at 365 nm.[17, 26] It is of interest to investigate the long-term side-effects of these free radicals on cell viability and intracellular AKT signaling. HN4 cells were treated with three UV-exposed photoinitiators, i.e., UV-DMPA, UV-I-2959 and UV-EY for 24 h. UV-DMPA at 0.1% (w/v) and higher exhibited strong toxicity and invariably led to most pronounced cell death (**Fig. 3A**), consistent with its precursor toxicity profile. UV-I-2959 (**Fig. 3A**) and UV-EY (**Fig. 3B**) induced cytotoxicity in a dose-dependent manner but showed stronger toxicity than their non-UV-exposed precursors. These findings are in agreement with previous reports that free radicals generated during the photopolymerization process are potentially more cytotoxic than photoinitiators themselves.[4] It is widely acknowledged that free radicals are toxic in that they can induce formation of ROS, oxidizing cells and causing damages to cellular proteins, nucleic acids, and lipids.[27] To alleviate oxidative stress within cells, exogenous defenses against oxidative damage and intracellular anti-oxidants are commonly used to quench ROS, such as ascorbic acid (vitamin C), tocopherol (vitamin E), glutathione, and lactic acid.[28-30] Generally, the lifetime of a radical,

especially in a high volume of solvent, is shorter than a second.[19] Therefore, the media conditioned with UV-exposed photoinitiator may have a limited amount of free radicals when they were applied to treat cells in our experiments. The further inhibition of AKT signaling pathway and cell viability indicates that free radicals may react with proteins in the media, hence altering their structure and function. Growth factors were most likely influenced because they are involved in the intracellular AKT signaling pathway. Therefore, frequently changing media after photopolymerization may facilitate rapid removal of photoinitiators and denatured growth factors to alleviate free radical-induced side effects.

In this work, we demonstrated both DMPA and I-2959 inhibited intracellular AKT signaling pathway (**Fig. 2A-B**). Such inhibition occurred in short time (30 min) suggests that photoinitiators interact with cell immediately. A longer exposure (24 h) of photoinitiators could lead to cell death (**Fig. 1**). Therefore, exposure time of photoinitiators to cells is critical for successful cell encapsulation in photopolymerization. Our results showed that all three UV-exposed photoinitiators rapidly decreased the phosphorylation of AKT after 30 min treatment (**Fig. 3C**), which was closely correlated to their cytotoxicity profile. These results suggest that free radicals may interact with proteins in the cell culture media, which can further affect intracellular AKT signaling pathway. The rapid reduction in AKT phosphorylation has the potential to intervene numerous intracellular signaling pathways, such as mTOR signaling in cell proliferation, ROS signaling pathway in ER stress and caspase 9 signaling in apoptosis.[31]

Besides, it is important to increase polymerization efficiency by engaging macromonomers in the reaction.[32] Unreacted photoreactive macromonomers that remain in the resulting scaffolds may affect encapsulated cells. For example, although deemed cytocompatible in general, short-chained polyethylene glycol-diacrylate (PEG-DA) has been found to be less cytocompatible than long-chained PEG-DA presumably due to a higher diffusion rate across the cell membrane.[33] Our recent publication further confirmed that PEG-

DA macromonomers alone affect cell viability and ratio of macromonomer to photoinitiator can be adjusted to improve cytocompatibility of the scaffolds made via photoinitiated polymerization.[17] It is necessary to develop an efficient post-polymerization purification procedure to remove residues of photoinitiator, free radical and macromonomer from the scaffold to provide an environment to maintain cell viability and foster desirable cellular activities.

Conclusions

The present work revealed the effects of DMPA, I-2959 and EY and their UV-induced decomposed counterparts on cell viability and intracellular AKT signaling in HN4 cells. The cytotoxicity profiles of the photoinitiators were closely correlated to intracellular AKT signaling in these three photoinitiating systems with or without UV-exposure. These findings provided a new way to examine the interaction between cells and photoinitiators for in-depth understanding of the cytocompatibility of photoinitiating systems in tissue engineering application.

Acknowledgements

This work was supported, in part, by the National Science Foundation (CAREER Award CBET0954957) and National Institutes of Health (R01EY024072).

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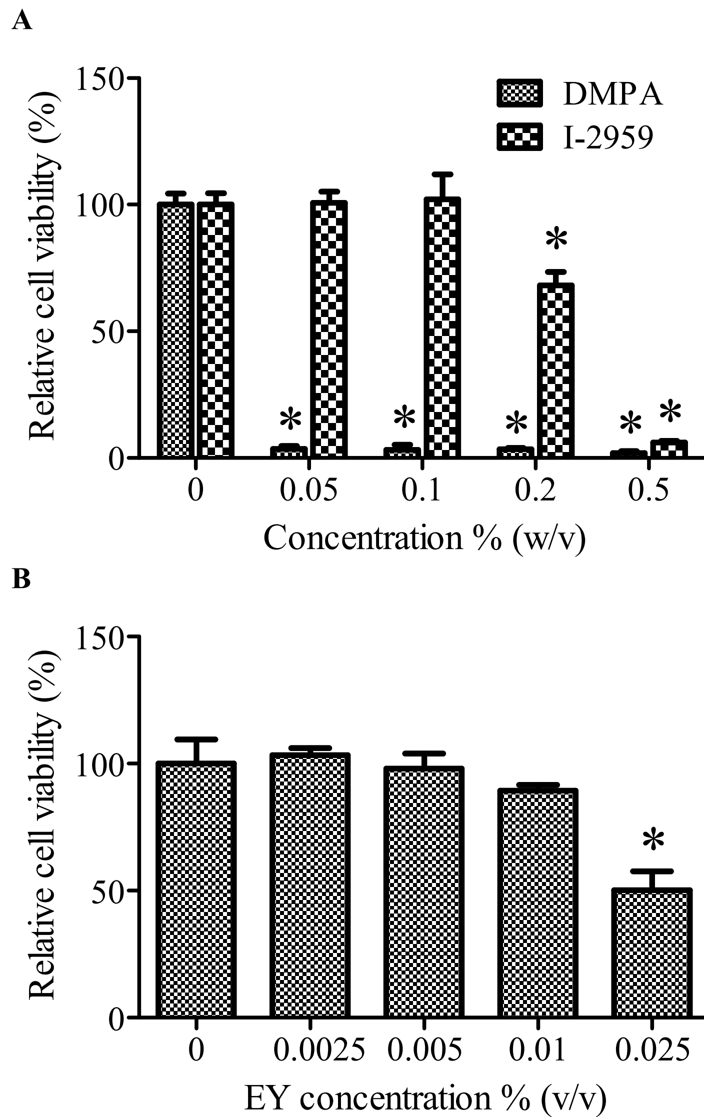


Fig. 1 Dose-dependent toxicity of DMPA, I-2959 (A) and EY (B) to HN4 cells. Cell viability of HN4 cells following treatment was determined by using WST-1 cell proliferation assay. The relative cell viability was normalized with respect to the viability of the control group. The data are expressed as mean ± SD (n = 3). * = $p < 0.05$.

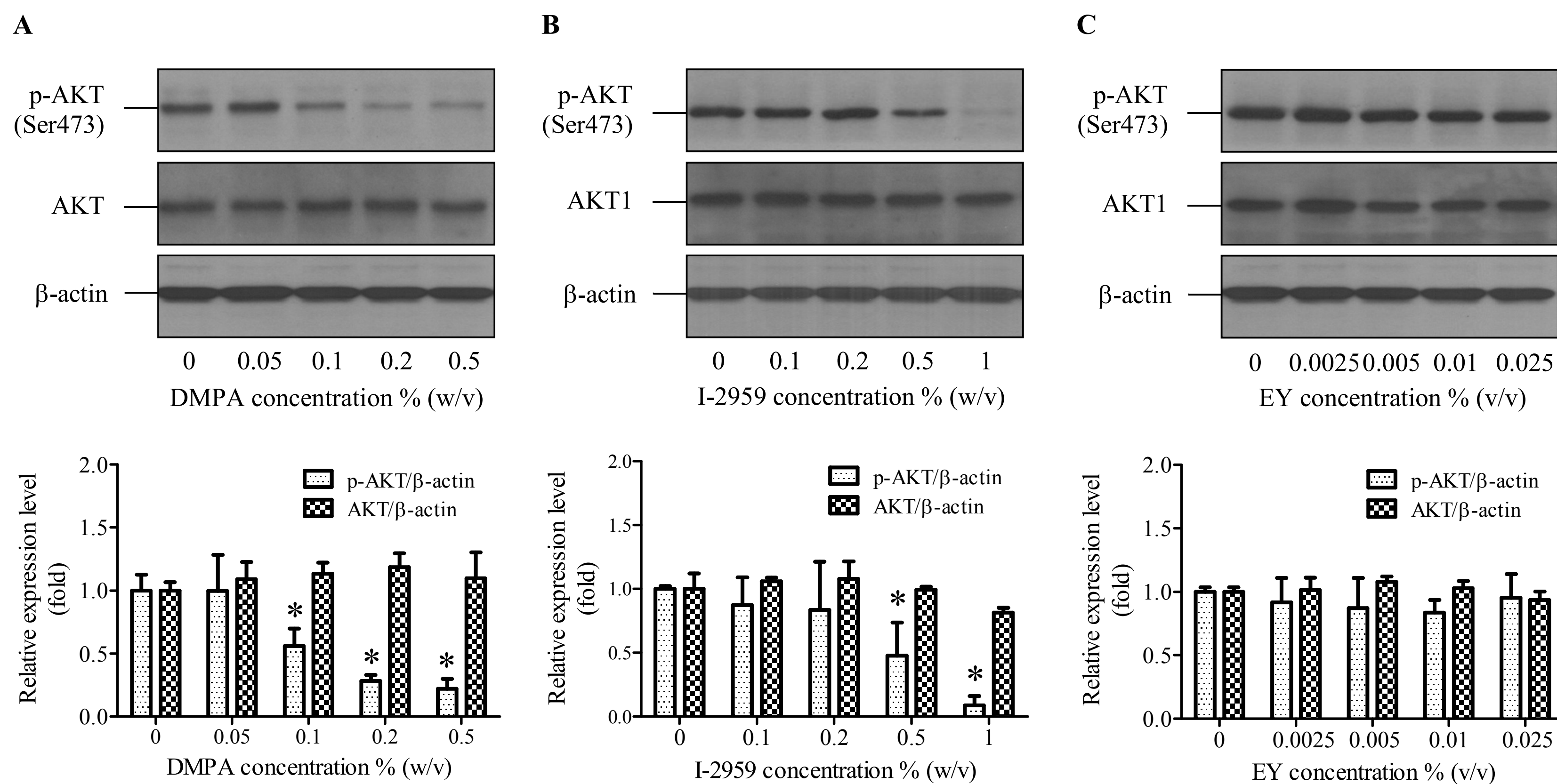
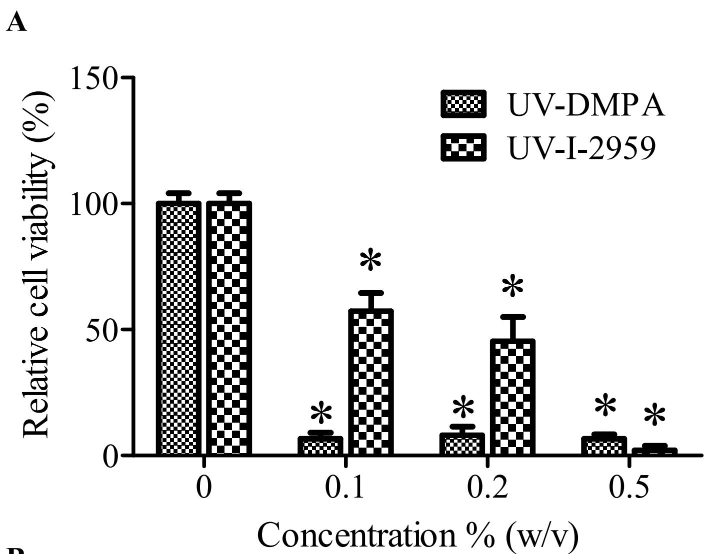


Fig. 2 Effects of DMPA (A), I-2959 (B) and EY (C) on intracellular AKT signaling in HN4 cells. Signaling molecule AKT and its phosphorylated form p-AKT expression levels were determined by using Western blotting. Quantitative analysis of the bands was made by densitometry using NIH ImageJ and expression levels normalized to β-actin are presented. The data are representative of one of three independent experiments and expressed as mean ± SD (n = 3). * = $p < 0.05$.


A
Relative cell viability (%)
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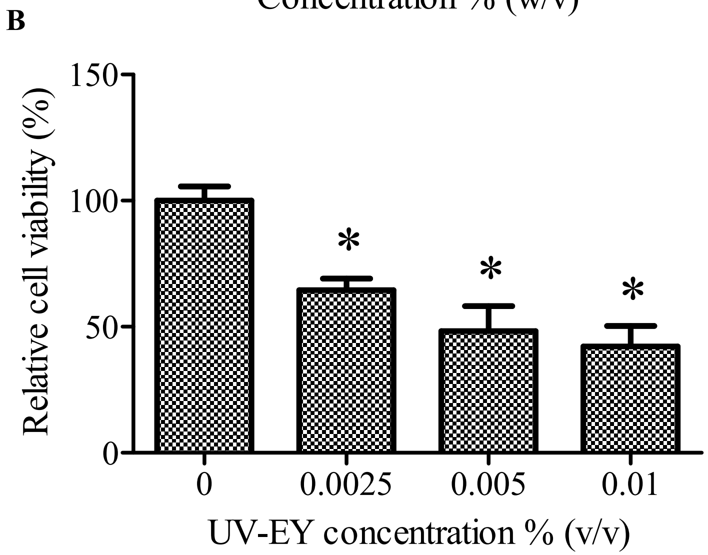
100
50
0
UV-DMPA
UV-I-2959
*
0
0.1
0.2
0.5
Concentration % (w/v)


B
Relative cell viability (%)
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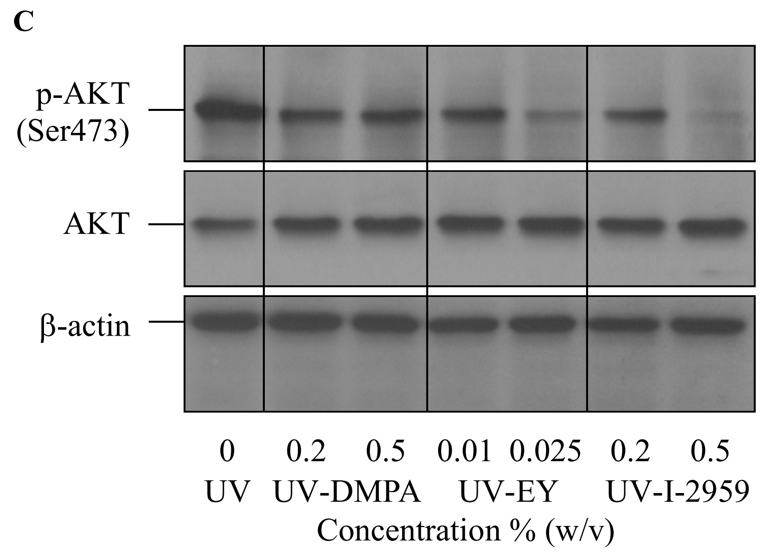
100
50
0
*
0
0.0025
0.005
0.01
UV-EY concentration % (v/v)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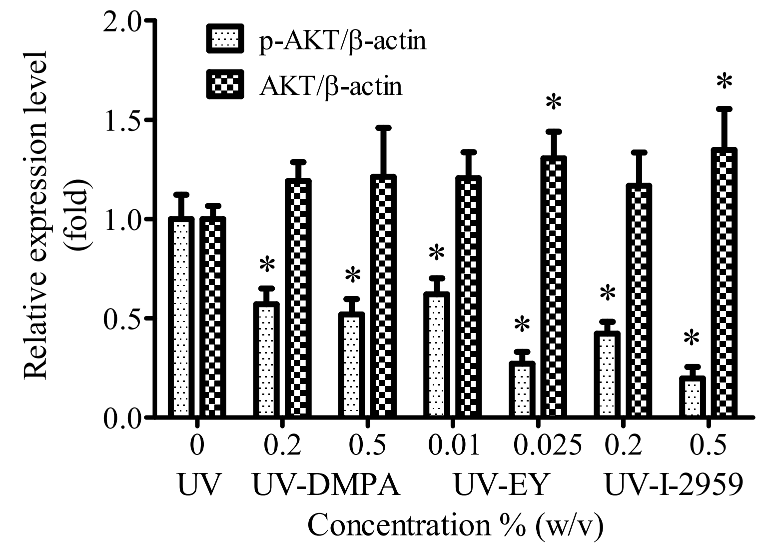
UV-EY
UV-I-2959
Concentration % (w/v)


Relative expression level
(fold)
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1.0
0.5
0.0
p-AKT/β-actin
AKT/β-actin
*
0
0.2
0.5
0.01
0.025
0.2
0.5
UV
UV-DMPA
UV-EY
UV-I-2959
Concentration % (w/v)

Fig. 3 Effects of UV-exposed photoinitiators on cell viability and AKT signaling. Following treatment of UV-DMPA, UV-I-2959 and UV-EY, cell viability of HN4 cells was determined by using WST-1 cell proliferation assay (A, B). The relative cell viability was normalized with respect to the viability of the control group. The signaling molecule AKT and p-AKT expression levels were determined by using Western blotting (C). Quantitative analysis of the bands was made by densitometry using NIH ImageJ and expression levels normalized to β-actin are presented. The data are expressed as mean ± SD (n = 3). * = $p < 0.05$.