Development of a Novel Experimental *In Vitro* Model of Isothiocyanate-induced Apoptosis in Human Malignant Melanoma Cells

THEODORA MANTSO[1*], ARISTEIDIS P. SFAKIANOS[1*], AITHNE ATKINSON[1], IOANNIS ANESTOPOULOS[2], MELINA MITSIOGIANNI[3], SOTIRIOS BOTAITIS[4], SEBACHEDIN PERENTE[4], CONSTANTINOS SIMOPOULOS[4], STAVROS VASILEIADIS[2], RODRIGO FRANCO[5,6], AGLAIA PAPPA[2] and MIHALIS I. PANAYIOTIDIS[1,3]

[1]*School of Life Sciences, Heriot Watt University, Edinburgh, Scotland, U.K.;*
[2]*Department of Molecular & Cell Biology, Democritus University of Thrace, Alexandroupolis, Greece;*
[3]*Department of Applied Sciences, Northumbria University, Newcastle Upon Tyne, U.K.;*
[4]*Second Department of Surgery, Democritus University of Thrace, Alexandroupolis, Greece;*
[5]*Redox Biology Centre, University of Nebraska, Lincoln, NE, U.S.A.;*
[6]*School of Veterinary Medicine & Biomedical Sciences, University of Nebraska, Lincoln, NE, U.S.A.*

Abstract. *Background: Isothiocyanates are constituents of cruciferous vegetables which have been associated with reduced cancer risk partially through their ability to induce apoptosis in malignant cells including melanoma. Materials and Methods: We have utilized human malignant melanoma (A375), epidermoid carcinoma (A431) and immortalized keratinocyte (HaCaT) cells exposed to various isothiocyanates, under different experimental conditions. Results: An experimental in vitro model utilizing low isothiocyanate concentrations (0.1-5 μM for 48 h with all treatments being refreshed after 24h) was shown to be (i) most efficient in exerting an anti-cancer effect when compared to higher concentrations (5-100 μM for 24 or 48 h added as a single bolus) and (ii) specific to A375 cells while A431 and HaCaT cells remained unaffected. Such effect involved the activation of several caspases including (iii) initiator caspases 8, 9, 4 (indicating the involvement of intrinsic, extrinsic and endoplasmic reticulum-based pathways) and (iv) effector caspases 3, 7 and 6. Conclusion: Utilization of low isothiocyanate concentrations (under the conditions described*

herein) exerts an anti-cancer effect specific to human malignant melanoma cells thus providing a therapeutic basis for their utilization in management of the disease.

Malignant melanoma is the fifth most common cancer in UK with its incidence rates being continuously rising faster than any other malignancy (1). Epidemiological studies suggest that an increased dietary consumption of cruciferous vegetables can reduce cancer incidence. These effects can be attributed to the high levels of glucosinolates (GSLs) present which are sulphur-containing glycosides and precursors for a group of compounds called isothiocyanates (ITCs) (2). Briefly, upon mechanical disruption of the plant cell wall (*e.g.* by chewing), the enzyme myrosinase is released which then catalyses the hydrolysis of GSLs to ITCs, with subsequent release of HSO_4^- and D-glucose (3). Different GSLs can form different ITCs, since glucoraphanin acts as the precursor for sulforaphane (SFN), gluconasturtin for phenethyl isothiocyanate (PEITC) and glucotropaeolin for benzyl isothiocyanate (BITC) (4). There is much speculation as to how ITCs may exhibit their chemotherapeutic effects, but the likelihood is that multiple molecular events are responsible. Potential biochemical mechanisms include (i) inhibition of carcinogen activity *via* suppression of phase I enzymes in xenobiotic metabolism, (ii) stimulation of phase II enzymes and (iii) induction of apoptosis (5, 6). Despite many reports demonstrating ITCs' effectiveness against different cancers there have been a limited number of studies investigating their ability to induce apoptosis in human malignant melanoma cells (7) which their results are dependent on the utilization of high concentrations of ITCs.

In this study, we aimed to develop a novel experimental *in vitro* model based on the utilization of low ITC concentrations capable of inducing apoptosis in human malignant melanoma (A375) cells. For this reason, our objectives were to (i) establish an experimental model of exposure to various ITCs and, furthermore, to (ii) characterize ITCs' anti-cancer activities by determining the induction of a number of caspases descriptive of various apoptotic pathways including those of intrinsic, extrinsic and ER-dependent origin.

Materials and Methods

Chemicals. SFN was obtained from Abcam (Cambridge, UK) whereas PEITC and BITC from Sigma-Aldrich (St. Louis, MO, USA). All compounds were dissolved in DMSO (Sigma-Aldrich) and stored at –20˚C. DMEM medium, trypsin, PBS, FBS, L-Glutamine and Penicillin/Streptomycin were obtained from Labtech International Ltd (East Sussex, UK). Resazurin sodium salt was supplied by Sigma-Aldrich. All chemicals were of analytical grade and purchased from Sigma-Aldrich, Applichem (Darmstadt, Germany) and Invitrogen (Carlsbad, CA, USA). BSA was obtained from Biosera (Boussens, France). Protease inhibitor cocktail was obtained from Roche (Basel, Switzerland). PVDF membranes (0.45 and 0.2 μm) were purchased from Millipore (Bedford, MA, USA). The pre-stained pink protein ladder was from Nippon Genetics Europe (Dueren, Germany). Primary (anti-PARP, anti-procaspase 3, anti-procaspase 6, anti-procaspase 7 and anti-lamin A/C) and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA) whereas anti-β-actin from Sigma-Aldrich and enhanced chemi-luminescence western blotting substrate kit from Pierce Thermo Scientific (Waltham, MA, USA).

Cell culture and treatment protocols. A375 and A431 cells were purchased from Sigma-Aldrich (St. Louis, MO, USA). HaCaT cells were a kind gift from Dr Sharon Broby (Dermal Toxicology and Effects Group; Centre for Radiation, Chemical and Environmental Hazards; Public Health England, UK). All cell lines were cultured in DMEM medium (10% FBS, 2 mM L-Glutamine and 1% penicillin/streptomycin) and maintained in a humidified atmosphere at 37˚C and 5% CO_2. SFN, PEITC, BITC or vehicle were added either as (i) a single bolus concentration ranging between 5-100 μM for 24 to 48h or (ii) at 0.1-5 μM for 48 h with all treatments being refreshed after 24h.

Cell viability assay. Cells (5×10^3) were seeded in 100 μl of complete medium into 96-well plates and kept overnight in the incubator before they were treated with either one of the treatment protocols. Cell viability was assessed by using the Alamar blue assay where in brief, resazurin sodium salt was dissolved in PBS (1mg/ml final concentration) and 10 μl of reagent were added into each well of the 96-well plates. After 4h of incubation, fluorescence intensity was measured at $560_{Exc}/590_{Em}$ nm by using a Spectramax M5 multi-mode plate reader (Sunnyvale, CA, USA).

Caspase activity assay. The caspase family fluorometric substrate kit II plus from Abcam (Cambridge, UK) was used according to the manufacturer's instructions. Cell lysates were prepared and 150-200 μg of protein extracts were incubated with each respective substrate for 1 h at 37˚C. Fluorescence intensity was monitored at $400_{Exc}/505_{Em}$ nm by using a Spectramax M5 multi-mode plate reader.

Preparation of cell lysates and protein determination. Cells (0.8×10^6) were plated in 100-mm dishes and cultured overnight at 37˚C. Next day, cells were treated with 0.1-5 μM of each ITC for 48 h (with all treatments being refreshed after 24 h) and then trypsinized, collected in micro-centrifuge tubes and washed twice with PBS. Cell pellets were lysed in lysis buffer (10 mM HEPES at pH 7.9, 10 mM KCl, 0.1mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP40) and supplemented with a protease inhibitors' cocktail (20 mM β-glycerophosphate and 0.2 mM Na_3VO_4). Then, they were left on ice while periodically vortexed over a 30 min period. Cell lysates were centrifuged at $14,000 \times g$ for 15 min at 4˚C and supernatants were transferred in fresh tubes. Protein content was determined by utilizing the BCA protein assay from Thermo Scientific (Waltham, MA, USA) according to manufacturer's instructions. Protein extracts were stored at –20˚C until usage.

Western immunoblotting. Forty micrograms (40 μg) of cellular protein extracts were separated by SDS-polyacrylamide gels and transferred electrophoretically onto PVDF membranes (either 0.45 or 0.2 μm). The blots were blocked in 5% non-fat milk powder in TBST buffer (50 mM Tris-HCl, 150 mM NaCl at pH 7.6 and 0.1% Tween-20) for 2h at room temperature. The membranes were incubated with the appropriate primary antibody (1:1,000 for anti-PARP, anti-procaspase 3, anti-procaspase 6, anti-procaspase 7, anti-lamin A/C and 1:2,000 for anti-β-actin) overnight at 4˚C under agitation. Next day, the membranes were incubated with the appropriate (mouse or rabbit) horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1h at room temperature under agitation, after being washed three times with TBST. Membranes were washed three times with TBST and once with distilled water. Labeled protein bands were detected by utilizing enhanced chemi-luminescence.

Statistical analysis. Measurements for cell viability assays were performed in quintuplicate (n=5) while those for caspase activity assays in triplicates (n=3) and all values are expressed as mean±standard error. Statistical analysis was performed by means of a Student *t*-test. A value of *p*<0.05 was considered statistically significant.

Results

The effect of three different ITCs (SFN, PEITC and BITC) was evaluated at a range of single bolus concentrations (5-100 μM) in both human A375 malignant melanoma (Figure 1A-C) and HaCaT immortalized keratinocyte cells (Figure 1D-F) after 24 h and 48 h of treatment. Addition of a single bolus of each ITC concentration statistically significantly decreased (*p*<0.05) both A375 and HaCaT cell viability levels in a concentration-dependent manner. More specifically, SFN decreased cell viability at 10 μM onwards (24-48h) in A375 (Figure 1A) and HaCaT (Figure 1D) cells. PEITC also decreased cell viability levels but at a concentration of 25 μM onwards (24-48h) for A375 (Figure 1B) and 10 μM onwards (24-48h) for HaCaT (Figure 1E) cells. BITC exerted the
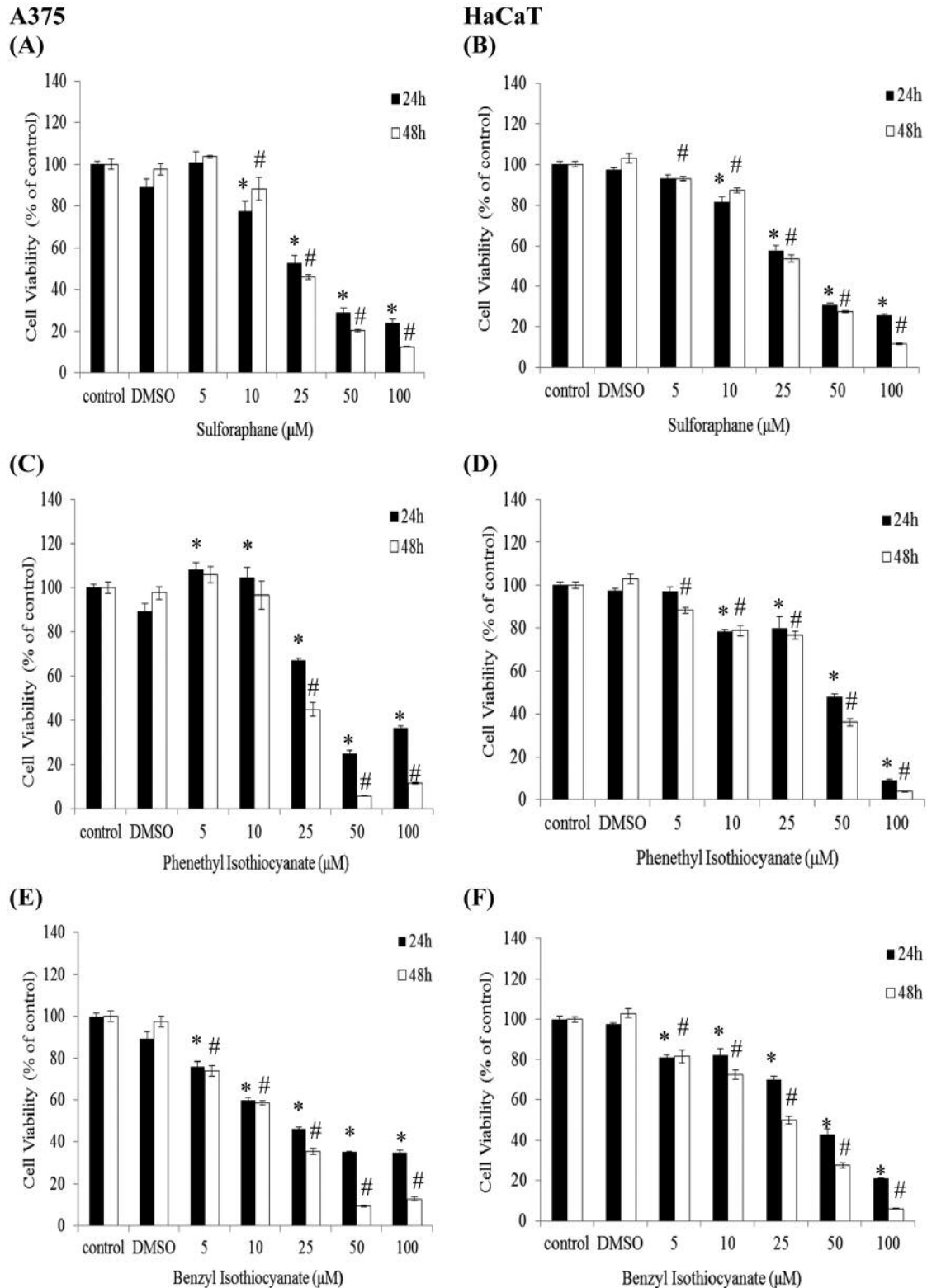
A375
HaCaT

Figure 1. *The effect of different concentrations of ITCs in A375 and HaCaT cells after 24 and 48 h of treatment. Cells were subjected at a single bolus concentration ranging between 5-100 μM of sulforaphane (A; A375 cells and B; HaCaT cells), phenethyl isothiocyanate (C; A375 cells and D; HaCaT cells) and benzyl isothiocyanate (E; A375 cells and F; HaCaT cells) for 24 and 48 h. Data are representative of two independent experiments. An asterisk (*) or hashtag (#) indicates statistical significance when compared to their respective controls (DMSO) for 24 or 48 h (p<0.05).*

highest potency, as it induced a significant reduction in cell viability levels at 5 μM onwards (24-48 h) in both A375 (Figure 1C) and HaCaT (Figure 1F) cells.

When we utilized an *in vitro* model of ITC treatment by subjecting human malignant melanoma (A375) and non-melanoma (A431) cells as well as human normal keratinocyte (HaCaT; control) cells to a range of low ITC concentrations (0.1-5 μM for 48 h but being refreshed after an initial 24 h of treatment) our data revealed that SFN and PEITC inhibited in a statistically significant manner ($p<0.05$) cell viability levels at 5 μM in A375 cells only (Figure 2A and B respectively). However, at this concentration, cells exposed to SFN showed a more profound inhibitory effect on viability levels compared to PEITC (approximately 60% *vs.* 80% respectively). Finally, treatment with BITC also, statistically significantly decreased ($p<0.05$) the viability levels at concentrations as low as 2.5 μM onwards in A375 and A431 cells only. However, this effect was more profound in A375 cells where a 2.5 μM inhibition of cell viability was recorded to levels of ~70% which was further decreased to ~30% after treatment with 5 μM of BITC (Figure 2C).

Finally, in order to investigate whether treatment with ITCs induces an apoptotic response in A375 cells, the activity levels of the initiator caspases 4, 8 and 9 were evaluated. Activation of these caspases is indicative of the involvement of various apoptotic pathways in a manner where caspase 8 involves the extrinsic (29), caspase 9 the intrinsic (30) and caspase 4 the endoplasmic reticulum (ER)-dependent (31) pathways. Our data showed that treatment with BITC statistically significantly increased ($p<0.05$) the activity levels of all initiator caspases whereas this was the case only for caspase 9 in PEITC-treated cells. Alternatively, treatment with SFN showed no statistically significant changes in the activity levels of any of the above-mentioned caspases (Figure 3A-C). Similarly, western blot analysis data indicate that each of the SFN, PEITC and BITC were responsible for inducing a statistically significant decrease ($p<0.05$) in protein expression levels of the effector procaspases 3 (Figure 3D) and 6 (Figure 3E) while for procaspase 7 this was the case only for BITC treatment (Figure 3F). Consequently, when these procaspases become activated they cleave specific cellular proteins including poly(ADP-ribose) polymerase (PARP) (Figure 3D) and lamins A and C (Figure 3E) such as in the case of procaspase 3 and 6, respectively. Overall, our results demonstrate that ITCs induce caspase activation in a manner which treatment with BITC results in the largest decrease in procaspase levels, followed by PEITC and SFN.

Discussion

The majority of studies have utilized non-melanoma (8-14) and melanoma (15-19) experimental models based on a single bolus addition of large ITC concentrations where a wide range of biological effects were documented but without provision of appropriate control (non-tumorigenic) cells. Similarly, we have been able to document such anti-cancer activity in melanoma cells but also in control keratinocyte cells suggesting a non-specific potency towards any type of cell line (tumorigenic or not).

To overcome the barrier of non-specific potency, we have developed a novel experimental *in vitro* model of ITC treatment by subjecting human malignant melanoma (A375) and non-melanoma (A431) cells as well as human normal keratinocyte (HaCaT; control) cells to a range of low ITC concentratios (0.1-5 μM) for 48 h, that were refreshed after an initial 24 h of treatment. Overall, our results highlight the novelty of the *in vitro* experimental model developed not only of being capable in exerting a potent anti-cancer activity (at low ITC concentrations) but also of being specific to human malignant melanoma (A375) cells while leaving unaffected human non-melanoma (A431) and normal keratinocyte (HaCaT) cells. Moreover, our results indicate that BITC (among all the three ITCs) exerts the highest anti-cancer potency and thus highlights its effectiveness as a potential anti-cancer strategy.

Finally, since ITCs have shown to exert a plurality of anti-cancer effects by targeting various cellular processes such as, apoptosis, autophagy, cell cycle, angiogenesis, epigenetic pathways, antioxidant defenses, *etc.* (20-28), we focused on apoptosis in order to elucidate which underlined pathways were involved in accounting for the observed reduction of viability levels in A375 cells. Overall, ITCs induced apoptosis by activating a number of initiator (8, 9, 4) and effector (3, 6, 7) caspases indicating the involvement of various apoptotic pathways including but not limited to, the extrinsic (caspase 8), intrinsic (caspase 9) and ER-dependent (caspase 4) pathways. To the best of our knowledge, this is the first report that (i) describes a novel experimental *in vitro* model based on the utilization of low ITC concentrations in a manner described herein and (ii) how it exerts an anti-cancer effect uniquely to melanoma cells (without affecting other non-melanoma and normal keratinocyte cells) by inducing apoptosis at concentrations substantially lower than those utilized in current bibliography.
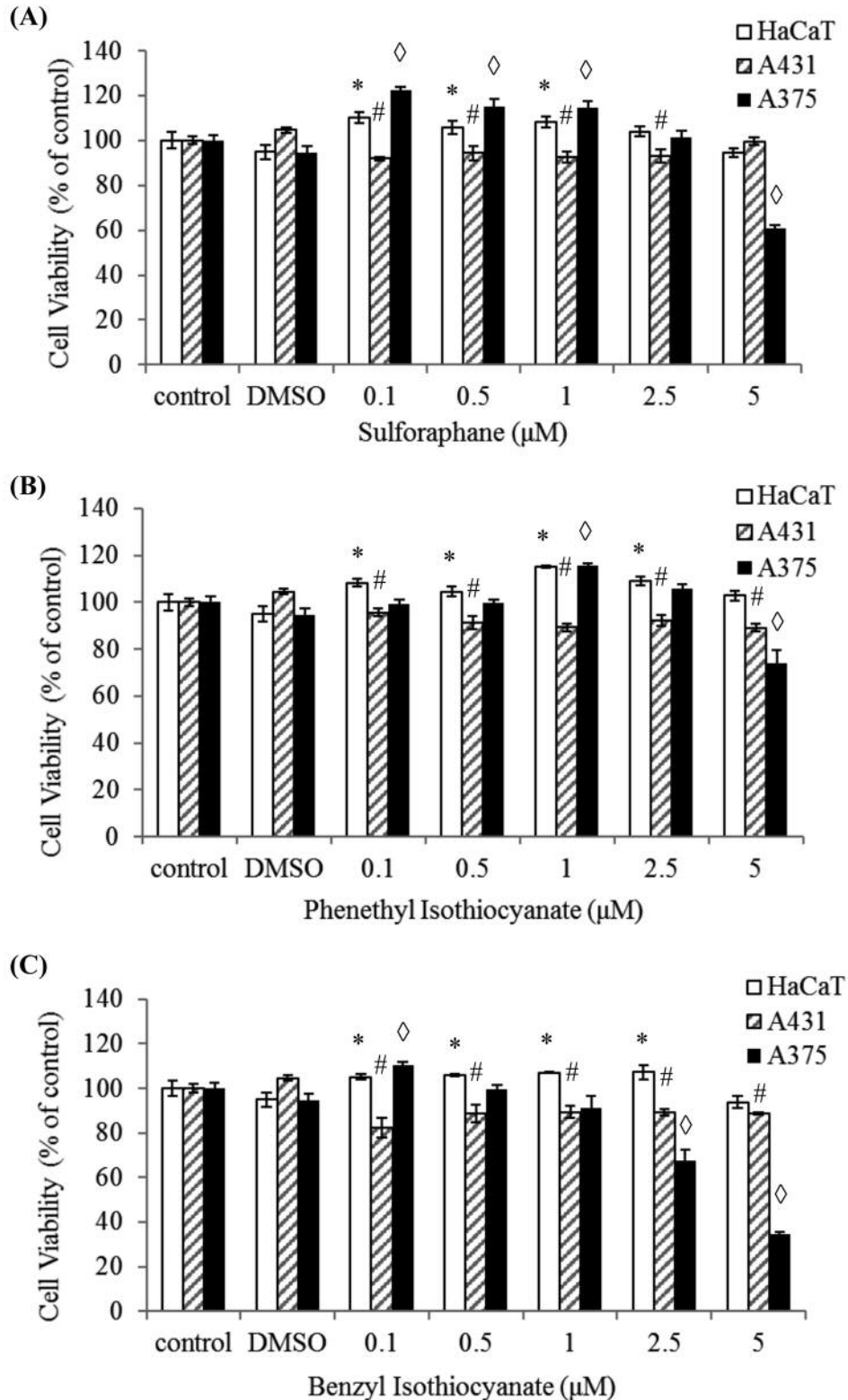
Acknowledgements

Figure 2. *The effect of different concentrations of ITCs on HaCaT, A431 and A375 cells after 48 h of treatment. In general, cells were subjected to 0.1-5 μM of sulforaphane (A), phenethyl isothiocyanate (B) and benzyl isothiocyanate (C) at 0.1-5 μM for 48 h with all treatments being refreshed after 24h. Data are representative from two independent experiments. An asterisk (*), hashtag (#) or diamond (◊) indicates statistical significance for HaCaT, A431 or A375 treated cells, respectively when compared to their corresponding controls (DMSO) (p<0.05).*
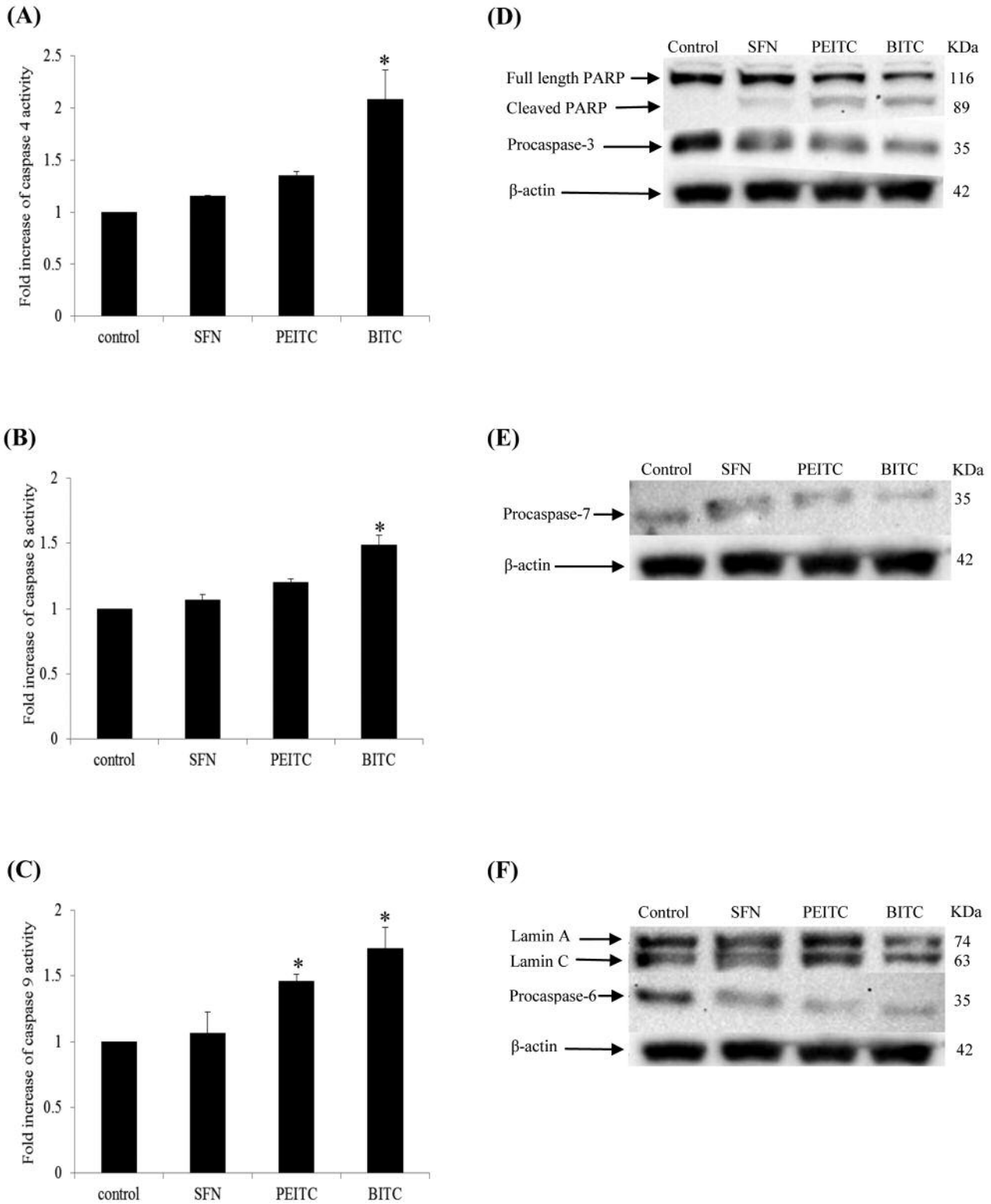
Figure 3. *The effect of ITCs on enzymatic activity and protein expression levels of various caspases. A375 cells were subjected to 5 μM of ITC (for 48 h with all treatments being refreshed after 24 h) and enzymatic activity levels were recorded for caspase 4 (A), caspase 8 (B) and caspase 9 (C). Additionally, under the same treatment conditions, protein expression levels were assessed for procaspase-3 (D), procaspase-6 (E) and procaspase-7 (F). Data are representative from two independent experiments. An asterisk (*) indicates statistical significance when compared to their respective controls (p<0.05).*

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