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Isothiocyanate-induced Cell Cycle Arrest in a Novel *In Vitro* Exposure Protocol of Human Malignant Melanoma (A375) Cells

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Abstract. *Background/Aim: Several studies have documented the effects of isothiocyanates (ITCs) on cancer prevention by inducing oxidative stress, activating apoptosis, affecting cell-cycle regulation, etc. Previously, we have shown that ITCs, administered at low concentrations by the means of double-bolus are capable of potentiating cytotoxicity in human malignant melanoma (A375) cells by inducing apoptosis. The aim of the present study was to further investigate the effect of the treatment of A375 cells with ITCs on cell-cycle progression and the levels of various cell cycle regulators. Materials and Methods: Cell-cycle analysis was performed by means of flow cytometry whereas western immunoblotting was used to determine the expression levels of these protein regulators. Results: Our data showed an increase in the number of cells in the G_2/M phase accompanied by a decrease in the G_0/G_1 phase, while several cell-cycle regulators were shown to be differentially expressed upon exposure to ITCs. Conclusion: ITCs induced cell-cycle arrest in A375 cells.*

Malignant melanoma is the most aggressive and lethal form of skin cancer due to its high mortality and continuously increasing incidence rates (1, 2). On the other hand, consumption of cruciferous vegetables has been suggested to play a beneficial role in cancer prevention. Their anti-cancer effects appear to be attributed to isothiocyanates (ITCs), a group of compounds derived from the hydrolysis of glucosinolates (GSLs) which are sulfur- and nitrogen-containing glycosides, by the enzyme myrosinase (3-6). In recent years, various studies have focused on investigating the diverse pathways induced by ITCs including (i) activation of phase I and II detoxification, (ii) generation of oxidative stress, (iii) induction of cell-cycle growth arrest and apoptosis, (iv) inhibition of tumor cell invasion, metastasis and angiogenesis, (v) suppression of inflammatory response and (vi) regulation of epigenetic cascades (7-11). To this end, we have previously published on the ability of different ITCs (*e.g.* sulforaphane, SFN; phenethyl isothiocyanate, PEITC; and benzyl isothiocyanate, BITC) to trigger apoptosis in human malignant melanoma (A375) cells by utilizing a previously published means of administering low concentrations of ITCs as a refreshed double-bolus (12). This study aimed to investigate further the effects of ITCs on the cell cycle, as an additional mechanism contributing to the cytotoxicity observed in our previous study in A375 cells.

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Materials and Methods

Chemicals and reagents. Dulbecco's Modified Eagle Media (DMEM), Phosphate Buffer Saline (PBS), Fetal Bovine Serum (FBS), trypsin, penicillin/streptomycin and L-glutamine were obtained from Labtech (Sussex, UK). Bovine Serum Albumin (BSA) was purchased from Biosera (Boussens, France). Polyvinylidene Difluoride (PVDF) membranes, page ruler plus protein marker, ECL and FxCycle PI/RNase staining solution were supplied by Thermo Fisher Scientific (Waltham, MA, USA). All primary and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling (Danvers, MA, USA) apart from β-actin (Sigma-Aldrich; St. Louis, MO, USA). SFN was purchased from Abcam (Cambridge, UK) while PEITC and BITC from Sigma-Aldrich. Stock solutions were prepared in DMSO (Sigma-Aldrich) at 100 mM and stored at –20˚C. All chemicals were of analytical grade and obtained from Invitrogen (Carlsbad, CA, USA), Applichem (Darmstadt, Germany) and Sigma-Aldrich.

Cell lines and treatments with isothiocyanates. The A375 cell line was purchased from Sigma-Aldrich and maintained in high glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% pen/strep (100 U/ml penicillin, 100 μg/ml streptomycin). Cells were

cultured in a humidified atmosphere at 37˚C and 5% CO_2. They were grown as monolayer cultures and sub-cultured when reaching 80-90% confluence. SFN, PEITC and BITC were added twice as a refreshed bolus concentration of 5 μM (each added at t0 and refreshed after 24 h) over a 48-h incubation period.

Protein extraction and quantification. Cells were treated with 5 μM of ITC or vehicle (for 48 h), harvested *via* trypsinization, washed twice with cold PBS and then stored at –20˚C. Cell pellets were lysed in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP-40) supplemented with protease inhibitors. Samples were incubated on ice while periodically vortexed over a 30 min period. Cell lysates were centrifuged (14,000 × *g*) at 4˚C for 15 min and supernatants were collected. Protein concentration was determined by the BCA assay (Thermo Fisher Scientific) according to the manufacturer's protocol. Protein extracts were stored at –20˚C until usage.

Western immunoblotting. Proteins (40 μg) were separated by using gradient SDS-polyacrylamide gels (8-20%) and then were transferred onto either 0.2 or 0.45 μm PVDF membranes (depending on their molecular weight) by wet transfer (in 1X transfer buffer) at predetermined running conditions. The blots were blocked with 5% (w/v) non-fat milk powder in Tris Buffered Saline, with Tween 20 (TBST) buffer, for 1 h at room temperature (RT) and then incubated with specific primary antibodies (overnight at 4˚C) under gentle agitation. Next day, the blots were washed (three times) in TBST buffer for 10 min and then incubated with an appropriate secondary antibody (for 1 h at RT) under agitation. Blots were incubated with the super signal west pico chemiluminescent substrate (Thermo Fisher Scientific) according to the manufacturer's instructions before being imaged by using a ChemiDoc XRS+ system (Bio-Rad, Perth, UK).

Cell cycle analysis by flow cytometry. Cells were treated with 5 μM of ITC or vehicle (for 48 h), harvested *via* trypsinization and washed twice with PBS. Then, cells (0.5×10^6) were fixed in cold 70% ethanol (for 1 h) at 4˚C until further processed. Cells were washed twice with PBS to remove ethanol, and then were suspended in FxCycle PI/RNase staining solution for 30 min (at RT) in dark. Samples were analyzed by FACS Calibur Flow Cytometer (BD Biosciences) with 10,000 events being recorded under each condition and analyzed using the FlowJo V10 software (BD Biosciences).

Statistical analysis. Statistical analyses were performed by one-way ANOVA with Tukey's test for multiple comparisons after using the SPSS v.22 software. Statistical significance was set at $p<0.05$.

Results

Human malignant melanoma (A375) cells were exposed to a refreshed, double-bolus low concentration (5 μM) of SFN, PEITC or BITC according to our previous findings (12). Determination of the distribution of cells to cell-cycle phases by flow cytometry, revealed an induction of the G_2/M cell cycle phase by all ITCs (Figure 1A and B). Exposure to BITC induced the highest increase in the number of cells in the G_2/M phase followed by SFN and PEITC which had the same effect. This pattern was followed by a reduction in the number of cells in the G_0/G_1 phase, by all three ITCs, while only BITC induced a significant change in the number of cells in the S phase of the cell cycle (Figure 1A and B). Finally, our data showed a profound elevation, to a variable degree, in the percentage of cells in the sub-G_1 phase (which accounts for a necrotic and/or apoptotic cell population) by all three ITCs (Figure 1A and B). The underlying mechanism(s) contributing to the observed induction of cell-cycle growth arrest in A375 cells was examined by monitoring alterations in expression levels of various cell cycle protein regulators. According to our results, the levels of cyclin dependent kinase inhibitors p21 and p27 as well as phospho-p53 (Ser15) were up-regulated in contrast to those of p18 which were markedly reduced (Figure 2A). Furthermore, the levels of cyclins D1 and D3 were increased independently of the type of ITC utilized (Figure 2B). In addition, cyclin-dependent kinase 6 (CDK6) was shown to decrease while cyclin-dependent kinase 2 (CDK2) significantly increased in response to ITCs exposure. Finally, the protein expression levels of cyclin-dependent kinase 4 (CDK4) were shown to be up-regulated, in the case of SFN and PEITC, as opposed to exposure to BITC, which caused a marked reduction (Figure 2C). Overall, our data highlight the involvement of various cell cycle-regulating proteins in inducing cell cycle arrest as a consequence of the exposure of A375 cells to low-ITC concentrations administered as refreshed, double-bolus (12).

Discussion

Our findings revealed an increase in the number of cells in the G_2/M phase accompanied by a decrease in the number of cells in the G_0/G_1 phase following exposure of A375 cells to ITCs. Furthermore, a reduction in the S phase population was also observed but only after BITC exposure. Moreover, in agreement with our previous findings of ITC-induced apoptosis in A375 cells, a profound elevation in the percentage of cells in the sub-G_1 phase fraction was detected (12, 13). However, it is of significance to note that the levels of cytotoxicity were comparable with those in other experimental platforms using rather higher concentrations of ITCs, administered as a single bolus (14-18). In order to study the mechanism of action of ITCs, the expression levels of various cell cycle regulators were examined. Treatment with ITCs resulted in an induction of p21 and p27 expression levels, a finding consistent with the role of both proteins in binding to cyclin-CDK complexes and thus suppressing cell cycle progression (19). In support, several studies have demonstrated the ITC-induced up-regulation of p21 in various human cancer cell lines (20, 21). Similarly, increased p27 expression has been documented in both *in vitro* and *in vivo* models treated with various ITCs (22, 23). In addition, the levels of phospho-p53 (Ser15) protein were also found to be upregulated. This finding is in accordance with the

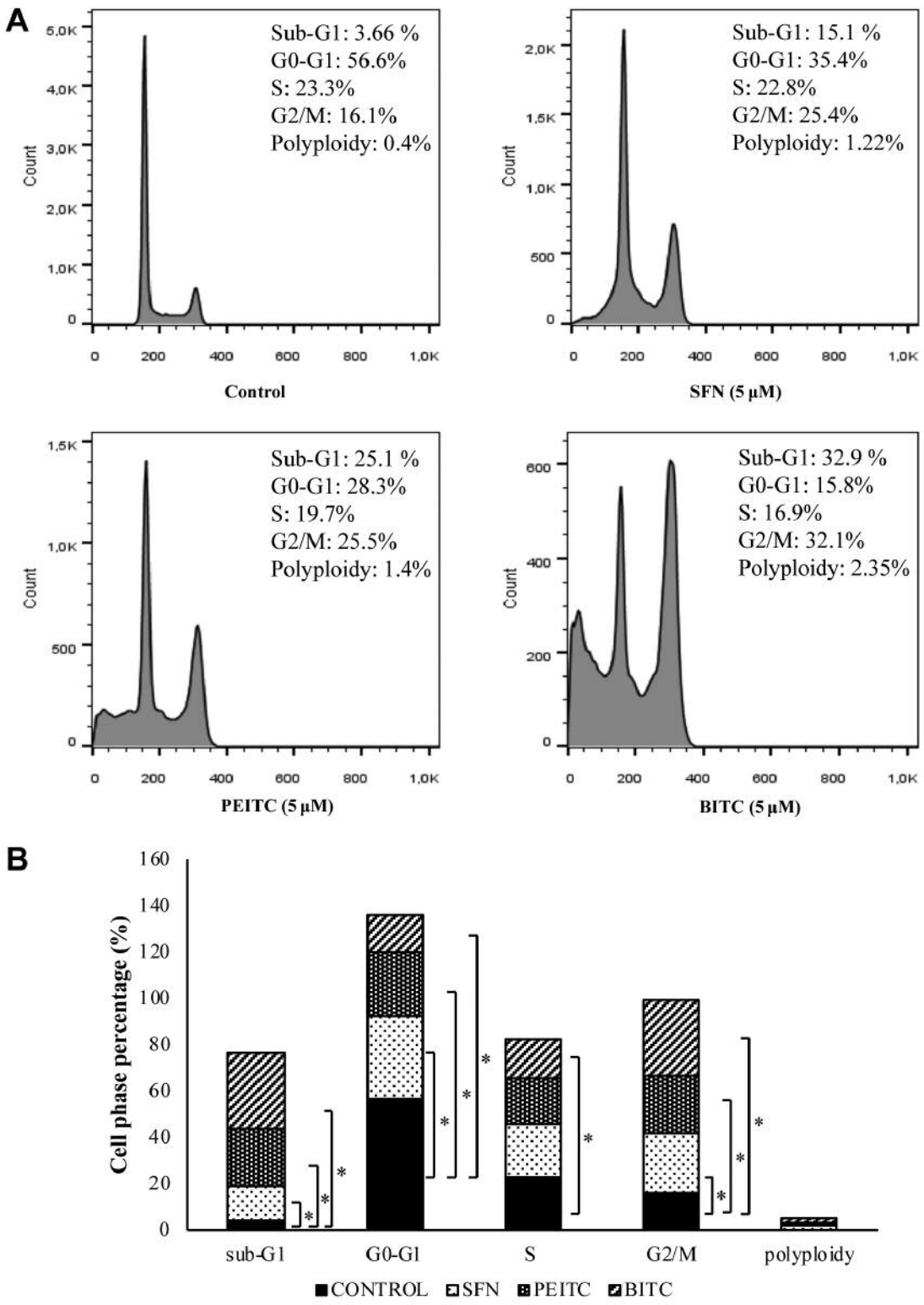

Figure 1. *The effect of ITCs exposure on the distribution of human malignant melanoma (A375) cells in cell-cycle phases. (A) Representative histograms of the distribution of cells in cell-cycle phases following exposure to 5 µM of SFN, PEITC or BITC for an initial 24 h after which exposures were refreshed for a further 24 h incubation (for a total exposure period of 48 h). (B) Diagram showing the distribution of cells in sub-G_1, G_0/G_1, S, G_2/M cell-cycle phases and polyploidy. Results are expressed as percentage of total cells and mean±SD were calculated. Asterisk (*) indicates statistical significance at $p<0.05$ when compared to control. Brackets denote comparisons between each cell-cycle phase under each individual ITC exposure.*

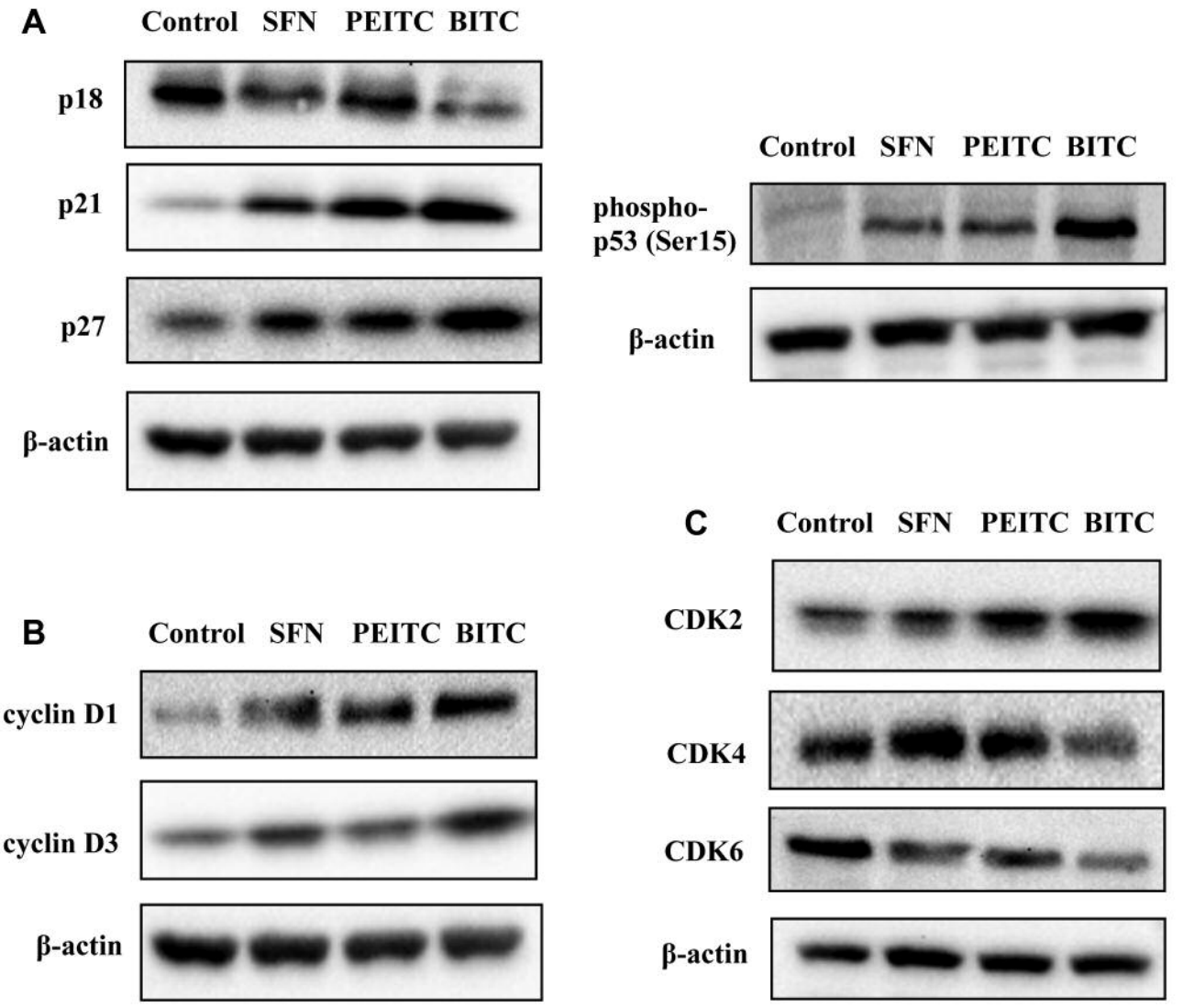

Figure 2. *The effect of ITCs on the levels of various cell-cycle regulators in human malignant melanoma (A375) cells. A375 cells were exposed to 5 μM of SFN, PEITC or BITC for an initial 24 h after which exposures were refreshed for a further 24 h incubation (for a total exposure period of 48 h). Cell extracts were analysed by western blot using antibodies against p18, p21, p27 and phospho-p53 (Ser15) (A), cyclins D1 and D3 (B) and cyclin dependent kinases 2, 4, and 6 (C) β-actin was used as a loading control.*

findings of other studies showing that ITC-induced apoptosis is associated with p53 up-regulation in melanoma cells (16, 24). On the contrary, a reduction in p18 levels (more evident in BITC exposure) was observed. This effect can be attributed to the interaction of this protein with CDK4 and CDK6 resulting in their inhibition and consequently cell cycle halt (25). Furthermore, increased levels of CDK2 (under exposure to each ITC) were also observed suggesting its interaction with cyclin E which may lead to cell-cycle progression and/or apoptotic induction (26, 27). Finally, when examining expression levels of cyclins D1 and D3, it was apparent that both proteins were up-regulated in response to each ITC exposure. These findings are also consistent with those of other studies where exposure of melanoma cells to ITCs was shown to be associated with the constitutive activation of the BRAF-MEK-ERK1/2 cascade as well as cell cycle progression (28, 29). Overall, the present study provides further evidence in support of our previously published means of treatment with ITCs in the context of halting cell-cycle progression by affecting the expression of several cell-cycle regulators in human malignant melanoma (A375) cells. These observations, along

with our previously published apoptotic induction, highlight the importance of this novel means of delivering low-ITC concentrations as refreshed, double-bolus as a more efficient and potent therapeutic strategy against malignant melanoma.

Authors' Contributions

Conceptualization, M.I.P.; methodology, T.M., E.L., I.K., M.I.P.; analysis, T.M., I.A., E.L., I.K., A.P., M.I.P.; investigation, T.M., I.A., E.L.; resources, A.P., I.K., M.I.P.; writing-original draft preparation, T.M., M.I.P.; writing-review and editing, T.M., I.A., E.L., I.K., A.P., M.I.P.; supervision, M.I.P.; funding acquisition, A.P., M.I.P.

Conflicts of Interest

The Authors declare no competing financial interests regarding this study.

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