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ORIGINAL CONTRIBUTION

Sulforaphane and iberin are potent epigenetic modulators of histone acetylation and methylation in malignant melanoma

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Abstract

Objective(s) Growing evidence supports that isothiocyanates exert a wide range of bioactivities amongst of which is their capacity to interact with the epigenetic machinery in various cancers including melanoma. Our aim was to characterise the effect of sulforaphane and iberin on histone acetylation and methylation as a potential anti-melanoma strategy.

Methods We have utilised an in vitro model of malignant melanoma [consisting of human (A375, Hs294T, VMM1) and murine (B16F-10) melanoma cell lines as well as a non-melanoma (A431) and a non-tumorigenic immortalised keratinocyte (HaCaT) cell line] exposed to sulforaphane or iberin. Cell viability was evaluated by the Alamar blue assay whilst total histone deacetylases and acetyltransferases activities were determined by the Epigenase HDAC Activity/Inhibition and EpiQuik HAT Activity/Inhibition assay kits, respectively. The expression levels of specific histone deacetylases and acetyltransferases together with those of lysine acetylation and methylation marks were obtained by western immunoblotting.

Results Overall, both sulforaphane and iberin were able to (1) reduce cell viability, (2) decrease total histone deacetylase activity and (3) modulate the expression levels of various histone deacetylases as well as acetyl and methyl transferases thus modulating the acetylation and methylation status of specific lysine residues on histones 3 and 4 in malignant melanoma cells.

Conclusions Our findings highlight novel insights as to how sulforaphane and iberin differentially regulate the epigenetic response in ways compatible with their anticancer action in malignant melanoma.

Keywords Isothiocyanates · Sulforaphane · Iberin · Melanoma · Epigenetics · Acetyl transferases · Deacetylases · Methyl transferases · Lysine methylation · Lysine acetylation

Introduction

Epigenetics refer to hereditary, stable and reversible changes in gene expression that do not depend on the DNA sequencing itself. The two most important epigenetic mechanisms include DNA methylation and histone modifications both of which interact with each other to regulate gene expression [1]. The most extensively studied histone modifications are those of methylation and acetylation as both have the capacity to regulate access to transcription factors. Histone acetylation is catalysed by histone acetyl transferases (HATs) and contributes to an 'open' chromatin state thereby promoting gene activation whilst histone deacetylation is catalysed by histone deacetylases (HDACs) and results in gene silencing

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[2, 3]. Finally, apart from histones, another important target of HATs and HDACs is non-histone proteins, a key aspect of their function [4]. On the contrary, histone methylation is catalysed by histone methyl-transferases (HMTs) and results in either gene activation or silencing. Examples of lysine methylation-induced gene activation are those of histone 3 (H3) lysines 4 (H3K4), 36 (H3K36) and 79 (H3K79) whereas those leading to gene silencing include lysines 9 (H3K9) and 27 (H3K27) [5–7]. Finally, modulation of any of these epigenetic alterations can have a major role in disease pathology including melanoma [8–10].

Melanoma is a very heterogeneous, aggressive, highly metastatic and treatment-resistant type of skin cancer with its incidence being significantly increased over the years [11]. Aberrant epigenetic modifications are associated with the disease initiation and progression, alongside with various genetic lesions, thereby leading to the design of new approaches aiming to target and reverse the landscape of the cancer epigenome towards more effective therapeutic options [12]. To this end, a wide range of naturally occurring agents have been shown to modify various constituents on the epigenetic pathway(s) in various cancer types including prostate [13–16] and leukaemia [17, 18] thus preventing cell growth, proliferation and motility. Amongst these agents, isothiocyanates (ITCs; the bioactive components of cruciferous vegetables) have been extensively studied for their anticancer properties. These compounds are derived from the hydrolysis of glucosinolates, by myrosinase, and have been shown to exert a plurality of bioactive properties including induction of growth arrest and apoptosis as well as modulation of detoxification enzymes and the epigenetic machinery [19, 20]. In specific, sulforaphane (SFN) has been documented to induce apoptosis and growth arrest [21–23], trigger autophagy [24, 25] and modulate key components of the epigenetic machinery [26–28] in order to promote cell death. Although a number of studies support the involvement of ITCs in influencing the epigenetic response, in various cancers, there is only a limited number of them documenting the ability of ITCs to modulate the epigenome specifically in malignant melanoma [29, 30]. Thus, we have aimed to investigate the potential of two structurally similar ITCs, namely SFN and iberin (IBN) (Fig. 1), to modulate the acetylation and methylation status of specific lysine residues on histones H3 and 4 (H4) thus potentially acting as epigenetic drug compounds towards a more efficient strategy for the therapeutic management in these patients.

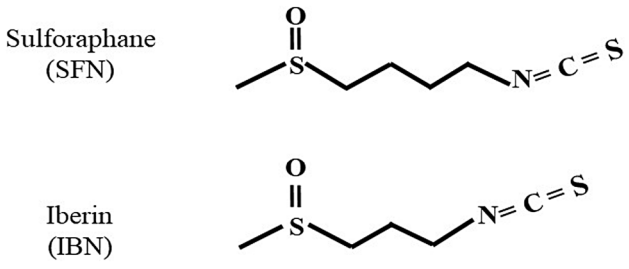


Fig. 1 The structure of sulforaphane (SFN) and iberin (IBN)

Materials and methods

Chemicals

SFN and IBN were obtained from Abcam (Cambridge, UK), dissolved in DMSO and ethanol (Sigma-Aldrich, St. Louis, MO, USA), respectively, and were stored at − 20 °C. Resazurin sodium salt was obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), Applichem (Darmstadt, Germany) and Thermo Fisher Scientific (Waltham, MA, USA). Bovine Serum Albumin (BSA) was supplied by Affymetrix (Santa Clara, CA, USA). Protease and phosphatase inhibitor cocktails were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and polyvinylidene difluoride (PVDF) membranes (0.45 and 0.2 µm) were obtained from Millipore (Bedford, MA, USA). All antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Dulbecco's Modified Eagle's (DMEM) medium, Roswell Park Memorial Institute (RPMI-1660) medium, trypsin, phosphate-buffered saline (PBS), Foetal Bovine Serum (FBS), L-Glutamine and Penicillin/Streptomycin were obtained from Labtech International Ltd. (East Sussex, UK).

Cell culture and exposure protocol

A375 human malignant melanoma and A431 non-melanoma epidermoid carcinoma cells were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brain (VMM1) and lymph node (Hs294T) metastatic melanoma cells were obtained from ATCC (American Type Culture Collection, Manassas, Virginia) and the murine malignant melanoma (B16F-10) cell line was purchased from LGC Standards (Middlesex, UK). HaCaT cells were kindly provided by Dr. Sharon Broby (Dermal Toxicology and Effects Group; Centre for Radiation, Chemical and Environmental Hazards; Public Health England, UK). A375, A431 and HaCaT cell lines were cultured in DMEM medium high glucose supplemented with 10% FBS, 2 mM L-Glutamine and 1% Penicillin/Streptomycin. Hs294T cells were cultured in DMEM high glucose (10% FBS, 4 mM L-Glutamine and 1% Penicillin/Streptomycin), whilst VMM1 cell line was cultured in RPMI-1640 high-glucose medium (10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin). All cell lines were maintained in a humidified atmosphere at 37 °C and 5% CO_2. SFN, IBN or vehicle were added as a single bolus concentration.

Determination of cell viability

All cell lines were seeded in 100 µl of complete medium into 96-well plates and kept in the incubator overnight. Next, they were exposed to either SFN or IBN at concentrations ranging between 2.5 and 50 µM for 24–48 h. Cell viability was assessed using the resazurin assay. In brief, resazurin sodium salt was dissolved in PBS at a final concentration of 1 mg/ml and added in an amount equal to 1/10 of the volume in each well. After 4 h of incubation at 37 °C, absorbance was measured at 570 nm using 600 nm as a reference wavelength using a Spark 10 M multi-mode plate reader (Tecan, Männedorf, Switzerland).

Preparation of cell lysates and protein determination

A375 cells were plated in 100-mm dishes and cultured overnight at 37 °C, before they were treated with 10 µM of SFN or IBN and incubated for 48 h. Then, they were trypsinised, washed twice with PBS and centrifuged at 4 °C. Pellets were kept at − 80 °C, until usage. Nuclear and cytosolic lysates were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Kit from Thermo Scientific (Waltham, MA, USA) and total histone extracts using the EpiQuik Total Histone Extraction Kit from Epigentek (Farmingdale, NY, USA). Protein content was determined by utilizing the BCA protein assay kit supplied by Thermo Fisher Scientific (Waltham, MA, USA). All extraction and assay kits were used according to the manufacturer's protocols. Protein extracts were stored at − 20 °C or − 80 °C (for estimation of HDAC/HAT activities) until usage.

Determination of HDAC and HAT activity

HDAC and HAT activity levels were determined using the Epigenase HDAC Activity/Inhibition Direct Assay kit and the EpiQuik HAT Activity/Inhibition Assay kit, respectively, according to the manufacturer's protocol. Both assay kits were supplied by Epigentek (Farmingdale, NY, USA). In brief, for HDAC activity determination, nuclear cell lysates were prepared and 10 µg of extracts were incubated with an acetylated substrate for 90 min at 37 °C. Optical density values were detected at 450 nm with an optional reference wavelength of 655 nm using a Spark 10 M multi-mode plate reader (Tecan, Männedorf, Switzerland). Accordingly, for HAT activity determination, 5 µg of nuclear extracts were incubated with a histone substrate for 60 min at 37 °C and optical density was monitored at 450 nm, using a Spark 10 M multi-mode plate reader.

Western immunoblotting

Twenty micrograms (20 µg) of nuclear and 10 µg of total histone protein extracts were separated by SDS–polyacrylamide gels using the mini gel tank from Invitrogen (Carlsbad, CA, USA). Proteins were next transferred electrophoretically onto a PVDF membrane (either 0.45 or 0.2 µm, according to protein target molecular weight) utilizing the Trans-Blot Turbo Transfer System, obtained from BioRad (Hercules, CA, USA). The blots were then blocked in 5% non-fat milk powder in TBST buffer consisting of 50 mM Tris–HCl, 150 mM NaCl and 0.1% Tween-20 (pH at 7.6) for 1 h at room temperature. After blocking, the membranes were washed three times with TBST and incubated with the appropriate primary antibody overnight at 4 °C, under agitation. All antibodies were diluted in 5% milk or BSA according to the manufacturer's instructions. Next day, the membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (mouse or rabbit at 1:1000) under agitation for 1 h at room temperature. After the incubation with the secondary antibody, the membranes were washed three times with TBST and labelled protein bands were detected using the SuperSignal West Pico PLUS Chemiluminescent Substrate supplied by Thermo Fisher Scientific (Waltham, MA, USA) according to the manufacturer's instructions. Protein bands were visualised utilizing the G:BOX Chemi XX6/XX9 gel imaging system (Syngene, Cambridge, UK).

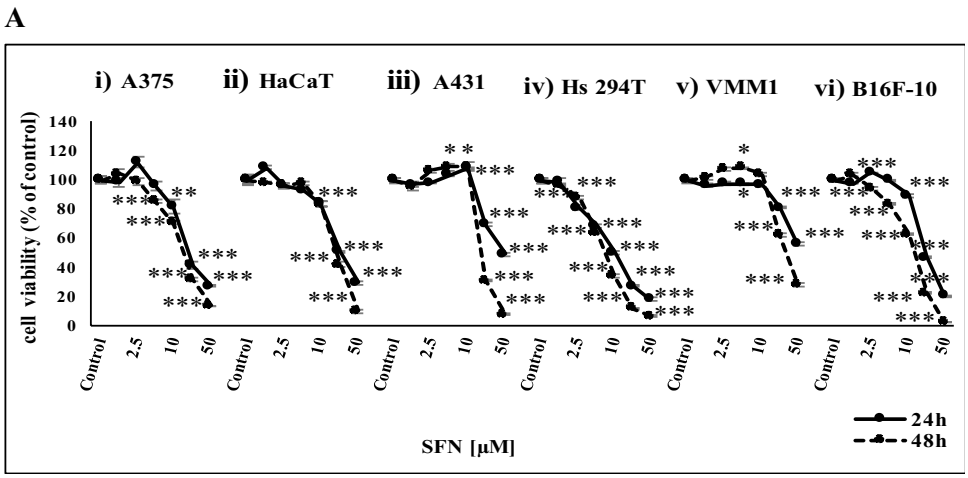
Statistical analysis

In all sets of experiments, data were expressed as mean values ± SEM (standard error of the mean). All calculations were performed on Microsoft Office Excel 2016 software, and comparisons and normalizations were made between the untreated control and exposure (treated) groups. Statistical significance for viability assays was tested by one-way analysis of variance (one-way ANOVA) with Tukey's test for multiple comparisons. Student's *t* test was used to compare the mean values for HDAC/HAT activity assays. IBM SPSS Statistics v.25 software was used for all statistical tests. $p < 0.05$ was considered statistically significant and results with levels of significance were reported as $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Results

SFN and IBN are more effective in reducing A375, Hs294T and B16-F10 levels of cell viability compared to those of VMM1, A431 and HaCaT

The anti-melanoma effect of SFN and IBN was assessed utilizing an in vitro model of malignant melanoma consisting of human (A375, VMM1, Hs 294T) and murine melanoma (B16F-10) as well as non-melanoma epidermoid carcinoma (A431) and non-tumorigenic immortalised keratinocyte (HaCaT) cell lines. In doing so, the resazurin assay was utilised to determine viability levels in each of the above-mentioned cell lines after exposure to a range of concentrations (2.5–50 μM) of SFN and IBN for 24 and 48 h. Overall, it was shown that addition of both SFN and IBN significantly reduced the viability of



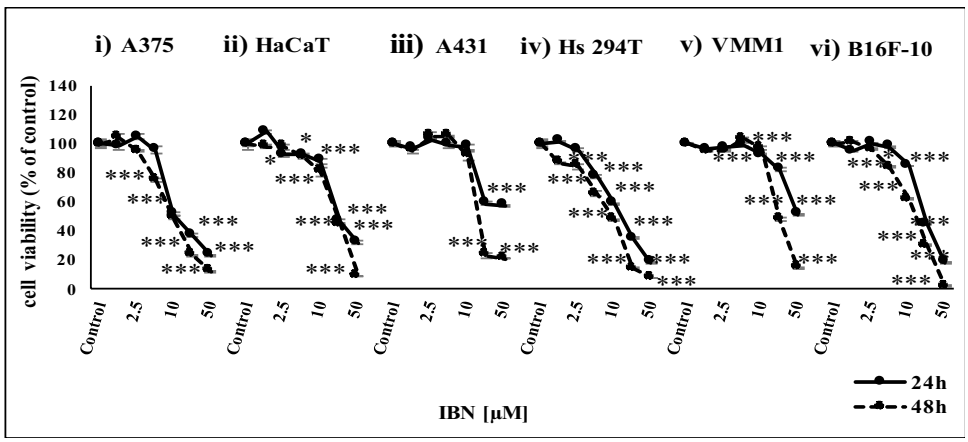


Fig. 2 Cytotoxic profile of SFN (**A**; i, ii, iii, iv, v, vi) and IBN (**B**; i, ii, iii, iv, v, vi) in an in vitro model of malignant melanoma. The experimental model consisted of (**A**i) and (**B**i) human malignant melanoma (A375); (**A**ii) and (**B**ii) immortalized keratinocyte (HaCaT); (**A**iii) and (**B**iii) non-melanoma epidermoid carcinoma (A431); (**A**iv) and (**B**iv) lymph node metastatic melanoma (Hs 294T); and (**A**v) and (**B**v) brain metastatic melanoma (VMM1), as well as (**A**vi) and (**B**vi) murine malignant melanoma (B16-F10) cells, exposed to a single bolus concentration of either SFN or IBN (2.5–50μM) at 24 and 48 h of exposure; **c** EC_{50} values were estimated for all cell lines at each exposure time point to SFN and IBN. Data are expressed as means ± SEM and are representative of three independent experiments. Statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to corresponding (DMSO or EtOH) controls

C

		A375	HaCaT	A431	VMM1	Hs294T	B16-F10
SFN	$EC50_{24h}$ (μM)	15.7 ± 0.15	21.6 ± 2.00	46.05 ± 12.04	35.3 ± 11.7	8.97 ± 0.8	20.88 ± 2.00
	$EC50_{48h}$ (μM)	15.6 ± 2.4	23.3 ± 2.4	20.87 ± 0.96	24.11 ± 1.3	6.5 ± 0.12	15.37 ± 0.5
IBN	$EC50_{24h}$ (μM)	8.3 ± 1.06	23.56 ± 4.4	48.51 ± 16.1	50.5 ± 9.5	11.3 ± 2.6	21.09 ± 0.9
	$EC50_{48h}$ (μM)	8.6 ± 0.7	23.6 ± 1.9	15.26 ± 1.8	23.05 ± 1.2	8.96 ± 1.6	19.97 ± 5.4

A375 (Fig. 2Ai, Bi), Hs294T (Fig. 2Aiv, Biv) and B16F-10 (Fig. 2Avi, Bvi) cells in a concentration- and time-dependent manner compared to VMM1 (Fig. 2Av, Bv), HaCaT (Fig. 2Aii, Bii) and A431 cells (Fig. 2Aiii, Biii) which were minimally affected and particularly at the highest concentrations. More specifically, SFN decreased viability at 10 μM onwards in A375 and B16-F10 cells and 5 μM onwards in Hs294T cells (at 48 h) in addition to IBN which also reduced viability levels, in all three cell lines, in a similar manner.

Taken together, IBN appears to be more potent against A375 melanoma cells ($EC_{50} = 8.6 \pm 0.7$, at 48 h) in contrast to SFN ($EC_{50} = 15.6 \pm 2.4$, at 48 h). On the contrary, SFN showed a more profound effect on the viability of Hs294T cells ($EC_{50} = 6.5 \pm 0.12$, at 48 h) compared to IBN ($EC_{50} = 8.96 \pm 1.6$, at 48 h). Finally, under the same experimental conditions, HaCaT, A431 and VMM1 cells were shown to be more resistant. Overall, A375, Hs294T and B16-F10 cells were more sensitive to the cytotoxic effect of both ITCs when compared to HaCaT, A431 and VMM1 ones (Fig. 2c).

SFN and IBN reduce protein expression of several HDACs and HATs in human malignant melanoma (A375) cells

The effect of SFN and IBN on the protein expression levels of various HDACs and HATs was evaluated in nuclear cell lysates of A375 cells treated with 10 μM of each of the two ITCs. More specifically, exposure to both SFN and IBN significantly decreased the expression levels of HDACs 1, 2 and 4 (Fig. 3a). Moreover, our data showed higher degree of reduction in protein levels of HDACs 4 and 6 by SFN whilst IBN exhibited a more potent inhibitory effect on the expression of HDACs 1 and 4, in A375 cells. Furthermore, treatment of A375 cells with both ITCs also reduced protein expression levels of CBP and acetyl CBP/p300 with SFN being more potent against CBP whilst IBN had a stronger effect on acetyl CBP/p300. Also, PCAF expression was diminished by SFN, but not by IBN, whilst GCN5L2 levels were similar to those of control under treatment with both ITCs (Fig. 4a, b). Next, we determined the effect of SFN and IBN treatment on total nuclear HDAC and HAT activity levels, on A375 cells subjected to 10 μM of each ITC for 48 h. Exposure to SFN and IBN reduced total HDAC activity levels in SFN- but not IBN-treated cells (Fig. 3b) whilst an increase in total HAT activity levels was observed under treatment with both SFN and IBN (Fig. 4c).

SFN and IBN affect the expression profile of specific histone lysine acetylation marks in human malignant melanoma (A375) cells

To further elucidate the effect of the observed changes in the expression and activity levels of HDACs and HATs, we focused on the acetylation patterns of specific lysine

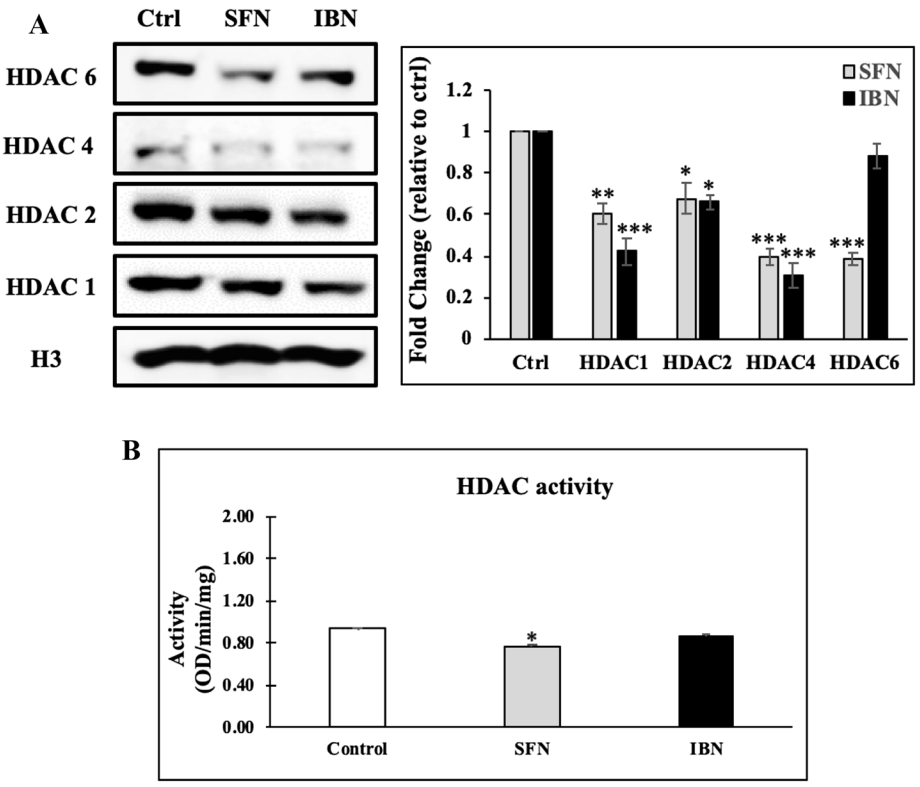


Fig. 3 The effect of SFN and IBN on protein expression and activity levels of HDACs. A375 cells were treated with 10 μM of either SFN or IBN for 48 h. **a** Western immunoblotting was used, in nuclear extracts, to determine the expression levels of HDACs 1, 2, 4 and 6. Western blots are representative of three independent experiments. Protein expression levels were quantified by densitometry relative to H3. Statistical significance was set at *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ relative to corresponding control; **b** total HDAC activity was evaluated using the Epigenase HDAC activity/inhibition direct assay kit and data were normalised to the corresponding untreated control (Ctrl). Western blots and HDAC activity assay are representative of three independent experiments

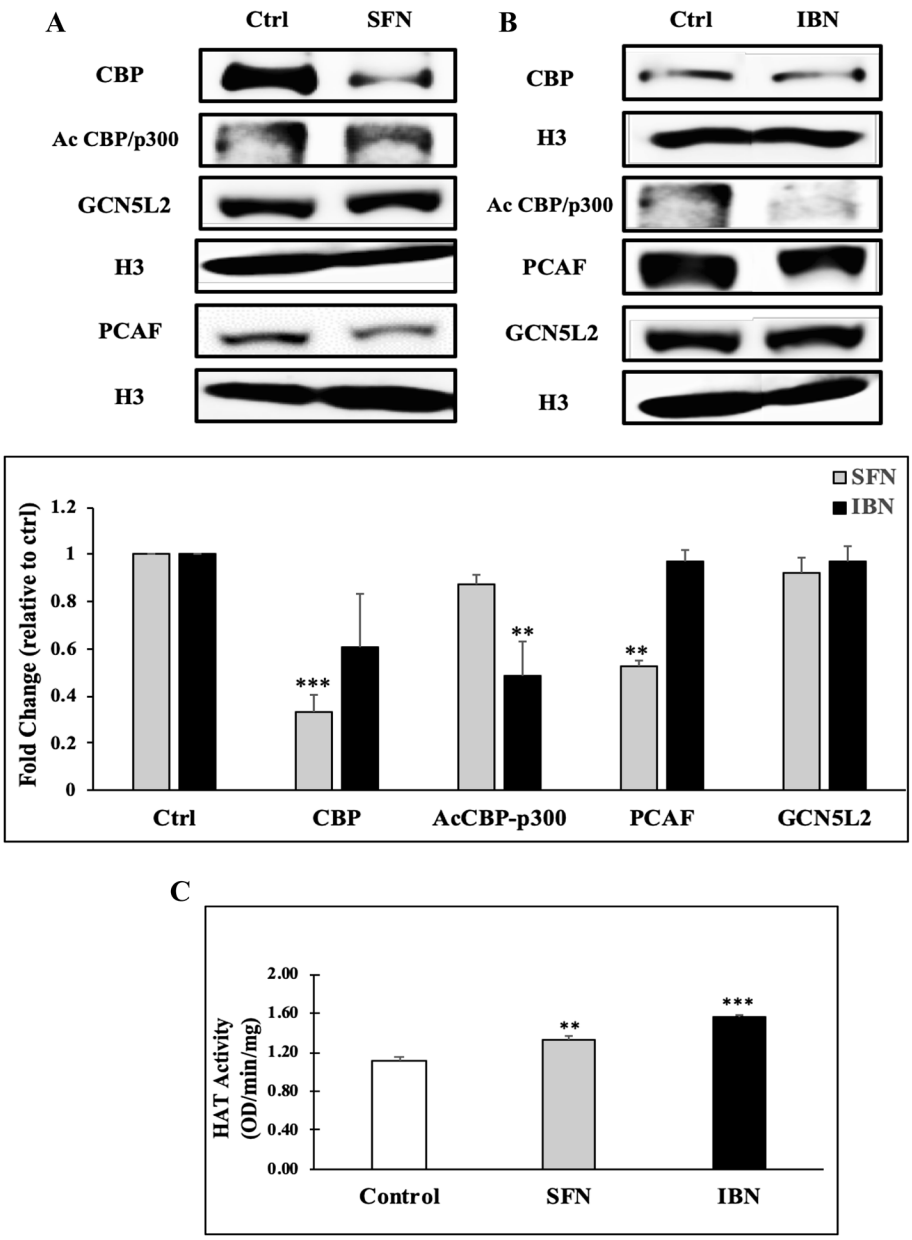


Fig. 4 The effect of SFN and IBN on expression and activity levels of HATs. A375 cells were treated with 10 μM of either SFN or IBN for 48 h. Western immunoblotting was used, in nuclear extracts, to determine the expression levels of CBP, Acetyl-CBP/p300, PCAF and GCN5L2 after exposure to **a** SFN and **b** IBN. Western blots are representative of three independent experiments. Protein expression levels were quantified by densitometry relative to H3. Statistical significance was set at *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ relative to corresponding control; **c** total HAT activity was assessed using the Epigenase HDAC Activity/Inhibition Direct assay kit. Western blots and HDAC activity assay are representative of three independent experiments

residues on the tails of both H3 and H4. To this end, total histone extracts of A375 cells exposed to 10 μM of either SFN or IBN were utilised for the determination of the acetylation profile of H4 on lysines (K) 5, 8 and 12 and of H3 on K9, K27, K14 and K18 (Fig. 5). Significantly reduced protein expression levels of AcH4K8 and AcH4K12 were observed with SFN and IBN (Fig. 5a) whilst SFN significantly reduced the expression of AcH3K9, AcH3K14 and AcH3K27. Similarly, treatment of A375 cells with IBN although decreased the expression levels of AcH3K27 only, this was not statistically significant when compared to untreated cells (Fig. 5b).

SFN and IBN differentially affect the expression levels of various HMTs as well as specific histone H3 lysine methylation marks in human malignant melanoma (A375) cells

The effect of SFN and IBN on various HMTs (e.g., ESET, RBBP5, ASH2L, SUV39H1, SET 8 and SET 7–9) was assessed; however, only the expression levels of SET 8 and SET7-9 were affected in A375 cells. More specifically, our data showed that the expression levels of SET8 were significantly reduced, in A375 cells, upon treatment with IBN only when compared to untreated control cells. On the other hand,

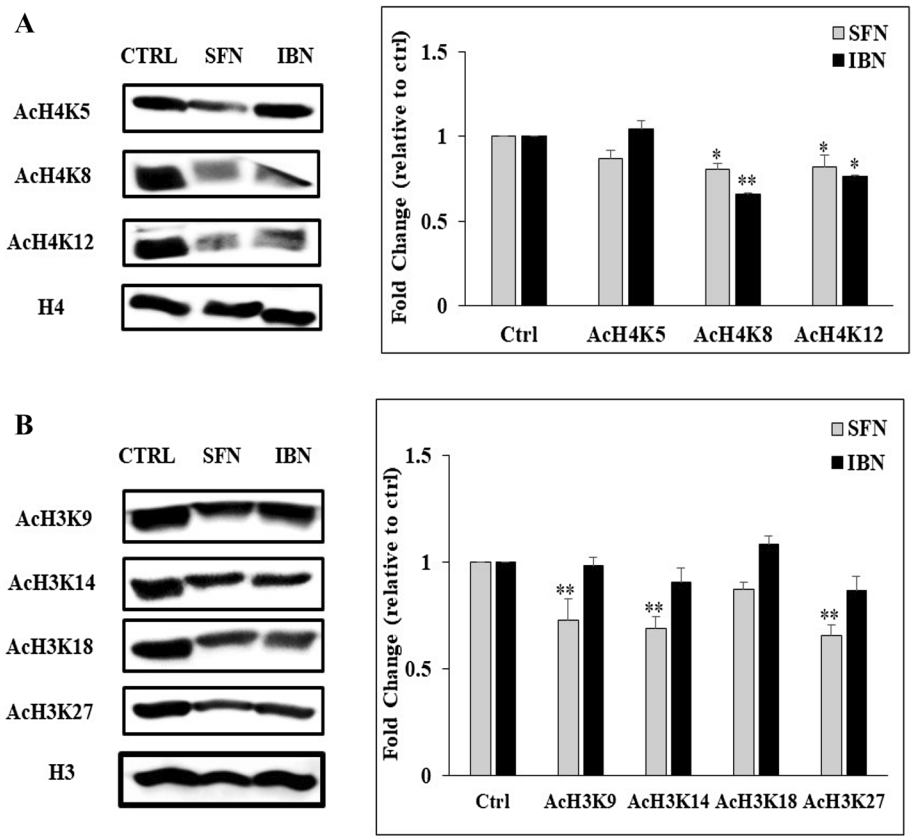


Fig. 5 The effect of SFN and IBN on specific H4 and H3 lysine acetylation marks. A375 cells were exposed to 10 μM of either ITC for 48 h. Western immunoblotting was used, in total histone extracts, to assess the expression levels of **a** the acetylation status of H4K5, H4K8 and H4K12 as well as **b** the acetylation status of H3K9, H3K14, H3K18 and H3K27. Western blots are representative of three independent experiments. Protein expression levels were quantified by densitometry relative to H3 and H4, respectively. Statistical significance was set at *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ relative to corresponding controls

protein levels of SET7-9 were shown to be significantly over-expressed under treatment with both ITCs (Fig. 6a). Next, we determined the effect of both ITCs on the di- and tri-methylation levels of K4, K9, K27, K36 and K79, on H3, but only those levels of K9, K36 and K79 were found to be significantly affected (Fig. 6b–d). More specifically, it was shown that upon exposure to both ITCs, the tri-methylation levels of K9, 36 and 79 were overexpressed (Fig. 6b) whilst the di-methylation levels of K9 and 79 only were shown to be overexpressed upon exposure to IBN only (Fig. 6c, d).

Discussion

A number of studies have investigated the potential of ITCs to inhibit melanogenesis through induction of apoptosis and cell cycle arrest whilst blocking invasion and metastasis both in vitro [29–36] and in vivo [37–39]. The anticancer potential of diverse ITCs has been associated with variations in their structures as well as differences in length or other functional groups on their side chains (e.g., double bond, benzene ring, sulphur or hydroxyl moiety, etc.) [40–42]. In this study, we have shown that two structurally similar ITCs significantly inhibited melanoma cancer cell growth, in vitro, in diverse malignant melanoma cell lines including those of human-based A375 and Hs 294T and murine-based B16F-10 in a concentration- and time-dependent manner. These results are in line with previous observations, from our lab, where exposure to allyl isothiocyanate (AITC) also resulted in a significant decrease on the viability levels of A375, Hs 294T and B16F-10 cells which was shown to be accompanied with modulations in specific lysine acetylation and methylation marks, in A375 cells, together with alterations in apoptotic gene expression [29]. In our current study, we have reported a differential targeting of melanoma cells compared to normal ones as well as a differential degree of sensitivity amongst cancer cell lines upon ITC treatment. A number of studies have reported that ITCs, and other phytochemicals, are able to differentially induce cytotoxicity amongst various cancer cells as well as between cancer and normal cells [43–45]. Such differences in cell susceptibility have been shown to be complex and the actual mechanism may involve multiple cellular pathways. Possible explanation(s) may involve differences in (1) magnitude of ROS production as increased ROS levels make cells highly vulnerable to further oxidative damage [46, 47], (2) glutathione (GSH) and glutathione S-transferases (GSTs) metabolism rates as changes in intracellular GSH levels play an important role in ITC cytotoxicity [46, 48], (3) basal activity and transcriptional activation of genes, involved in cell survival such as *NRF2*, *NFκB* and *Bax* [46, 49], and (4) mutational status of *BRAF*. The latter

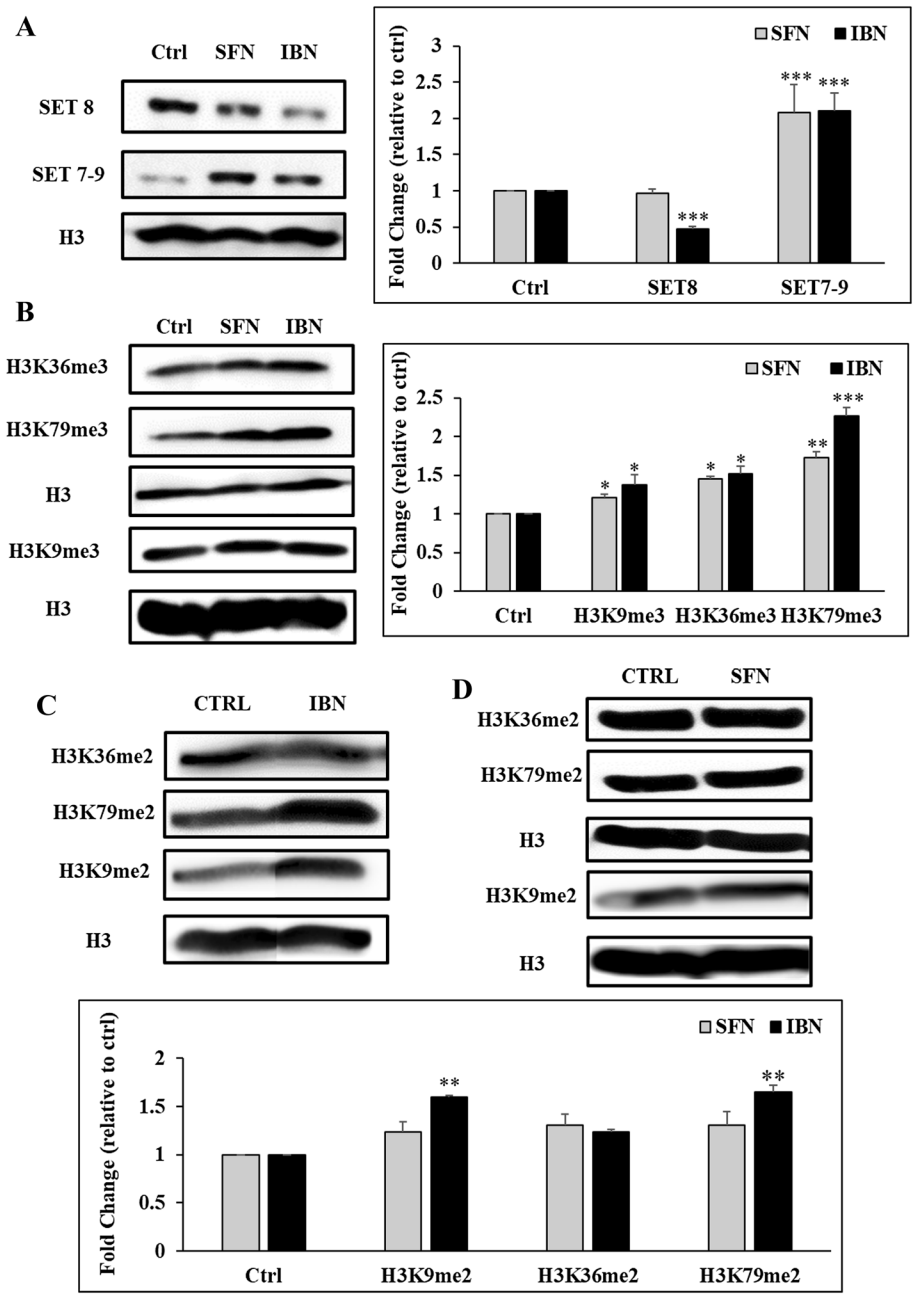


Fig. 6 The effect of SFN and IBN on protein expression levels of specific HMTs as well as tri- and di-methylation status of specific lysine residues on H3. A375 cells were exposed to 10 μM of SFN and IBN for 48 h. Western immunoblotting was used in nuclear **a** as well as in total histone **b**–**d** extracts to determine the expression levels of SET8, SET7-9 as well as the tri-methylation and di-methylation status of lysines (K) 9, 36 and 79 on histone H3 upon treatment with SFN (**b**, **d**) and IBN (**b**, **c**), respectively. Western blots are representative of three independent experiments. Protein expression levels were quantified by densitometry relative to H3. Statistical significance was set at *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ relative to corresponding control

is of particular importance as, for instance, VMM1 cells are wild type for $BRAF^{V600E}$ mutation in contrast to A375 and Hs 294T cells.

A number of aberrant epigenetic modifications have been shown to play a major role in the development and progression of melanoma including overexpression of class I and II HDACs which have been associated with melanoma progression, survival and drug resistance [50–53]. As a result, a range of HDAC inhibitors (HDACi) have been documented to promote apoptosis and cell cycle growth arrest [54], overcome resistance against various clinically used drugs [55–57] and also enhance immunogenicity of melanoma cells [58, 59]. To this end, ITCs have been shown to act as potent epigenetic modulators by inhibiting HDAC activity and consequently disrupting HAT/HDAC ratio thus inhibiting cancer cell growth through modulations on the expression of key tumour suppressor genes [60–62]. In line to these observations, we have recently reported that AITC reduced the expression levels of HDACs 4 and 6 (in addition to the significantly reduced expression levels of HDACs 1 and 2) in A375 melanoma cells [29], a finding similar to what was observed in this study as well. On the other hand, we have reported diminished expression levels of specific HATs, in A375 cells, namely those of CBP, Acetyl CBP/p300 and

PCAF. These enzymes have long been implicated in proliferation and cell cycle progression whereas their inhibition has been linked to increased apoptosis in various cancer cells [63, 64]. In melanoma, downregulation of these HATs has also been shown to trigger senescence and suppression of cell growth as well [65–67]. In this context, AITC, Phenethyl (PEITC) and Benzyl (BITC) ITCs have all been described as promising HDAC and HAT inhibitors capable of suppressing cancer cell growth [29, 68, 69]. Although the majority of studies support the importance of targeting these enzymes as potential therapeutic mediators, yet others indicate their tumour suppressor capacity through p53 acetylation [70, 71].

Next, we investigated the acetylation status of specific lysine residues on both H3 and H4 N-terminus tails targeted by HATs. To this end, H4K8, H4K5, H3K14, H3K18 and H3K27 are all known to be target sites for the action of CBP/p300 [72]. Overall, we have observed that both SFN and IBN induce hypo-acetylation of H4K8, H4K12 and H3K27 whereas SFN alone can further hypo-acetylate H3K9, H3K14 and H3K18. Decreased acetylation of these lysine residues has been previously reported in various tumours thereby causing decreased cancer cell growth and metastatic potential [29, 64].

Histone methylation is also important in the context of gene regulation and so we have aimed to examine the expression status of specific HMTs and methylation marks. To this end, we have observed a dramatic decrease on the protein levels of SET8 after exposure to IBN whilst both ITCs significantly increased the expression of SET7/9. Both of these HMTs are mono-methylases capable of regulating the methylation of H4K20 and H3K4 respectively, thereby playing an important role in DNA damage responses and cell cycle progression. In addition, these HMTs also regulate the methylation status of non-histone proteins like p53 [73]. The role of SET7/9 and SET8 in carcinogenesis is rather controversial as they can exert a tumour suppressor as well as an oncogenic function depending on the cancer cell type [74–77]. For instance, in acute myeloid leukaemia, breast and gastric cancers, SET7/9 has been reported to induce apoptosis thus preventing cell proliferation and migration [78–80] whilst in non-small cell lung cancer, it had the opposite effect by inhibiting apoptosis [78]. On the other hand, increased levels of SET8 have been associated with (1) decreased survival in gastric and hepatocellular carcinomas [81], (2) increased prostate cancer proliferation through interaction with the androgen receptor (AR) [82] and (3) enhanced metastatic potential and invasiveness in breast cancer [83]. In addition, reduced levels of H4K20 mono-methylation (the main target of SET8) have been observed in melanoma tissues associated with increased proliferation rates [84]. Finally, we assessed the methylation status of specific lysine residues of H3. Our data showed an elevation on the di- and tri-methylation levels of H3K9, H3K36 and H3K79 under treatment with both ITCs, but IBN appeared to have a more potent effect on levels of H3K9me2, H3K79me2 and HeK79me3. To these ends, studies have shown that increased methylation levels of H3K36 are linked to defective proliferation as well as induction of senescence, via re-activation of $p15^{Ink4}$ cell cycle inhibitor, in primary mouse embryonic fibroblasts [85]. In general, actively transcribed genes are characterised by methylation(s) on H3K4, H3K36 and H3K79 whilst methylation(s) of H3K9 and H3K27 are characteristic(s) of decreased gene expression. Furthermore, the degree and position of the methylation marks also affect the transcriptional status of target genes. For instance, tri-methylation of H3K9 and H3K27 is associated with gene silencing whilst mono- and di-methylation of H3K9 usually results in transcriptional activation [86, 87]. The diverse patterns of these epigenetic marks, in cancer, can provide useful prognostic, diagnostic and therapeutic value. For example, low levels of H3K4me2 are considered a poor prognostic factor in breast cancer [88] and when alongside with low levels of H3K9me2 and AcH3K18, they were shown to be associated with an overall decreased survival in patients with pancreatic adenocarcinoma [89].

To conclude, we have investigated on the effect of two structurally similar ITCs, SFN and IBN, in modulating acetylation and methylation of specific lysines on histone proteins 3 and 4 through alterations on the protein expression profile of various HDACs, HATs and HMTs (Fig. 7). Overall, we have demonstrated that SFN and IBN act as potent epigenetic regulators via their ability to modulate cancer methylation and acetylation marks (Fig. 7). To our knowledge, this is the first report to document a detailed

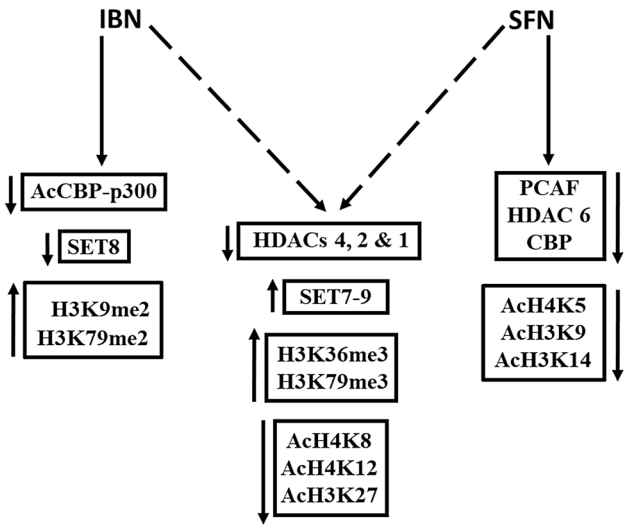


Fig. 7 General scheme of the SFN- and IBN-induced epigenetic responses in human malignant melanoma (A375) cells. Broken and solid lines represent common and individual epigenetic responses respectively under treatment with SFN and IBN

characterization of the interaction of SFN and IBN with the epigenome thereby supporting the potential use of ITCs as potentially promising therapeutic agents in malignant melanoma.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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