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Combined Effects of Capsaicin and HA14-1 in Inducing Apoptosis in Melanoma Cells

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ABSTRACT

Abnormal regulation of apoptosis is an important aspect of tumour development. Capsaicin, an extract of red chilli peppers, has been shown to inhibit growth of melanoma and other malignant cell lines and HA14-1 is an organic compound that directly induces apoptosis by binding to Bcl-2 protein. The aim of this work was to investigate whether combination therapy with capsaicin and HA14-1 might hold any promise for the treatment of melanoma. Three melanoma cell lines of a range of aggressive potential, melanocytes and fibroblasts were examined, looking at the effects of both drugs singly and in combination on cell viability and induction of apoptosis. This comparative study showed that melanoma cells and melanocytes have a similar sensitivity to capsaicin while fibroblasts are more resistant to it. HA14-1, as expected, induced apoptosis in all cells at relatively low concentrations. A combination of the two agents produced the expected results of an additive effect for 2 (HBL and A375SM) out of 3 melanoma cell lines in inducing apoptosis, but encouragingly for the most metastatically aggressive cancer cell line (C8161), a combination of the two showed a synergistic induction of apoptosis.

Keywords: Capsaicin; HA14-1; Bcl-2 Inhibitors; Melanoma; Apoptosis

1. Introduction

Many studies have suggested a strong association between inflammation and cancer [1-6]. A recent review shows a significant decline in cancer risk with increasing intake of non-steroidal anti-inflammatory drugs (NSAIDs) (primarily aspirin and ibuprofen) for at least four major types of cancer: colon, breast, lung and prostate [7]. Preclinical investigations also provide consistent evidence that both selective and non-selective NSAIDs effectively inhibit chemically-induced carcinogenesis of epithelial tumours [8].

In 1998, capsaicin, a common component which produces the burning hot sensation of chilli-laced spicy foods (8-methyl N-vanillyl 6-nonamide), was used as an anti-inflammatory/anti-cancer medication [9] as a treatment for human bladder cancer. In this study, 20 patients had repeated instillations of intravesical capsaicin (at 1 - 2 mmol/l) over 5 years. Throughout the following 5 years with capsaicin treatment, there was no further progression of the cancer in these patients.

With respect to melanoma Morré *et al*. [10] reported that NADH oxidase activity was inhibited preferentially in A-375 melanoma cells but not in primary melanocytes, by capsaicin. They speculated that the inhibition of cell surface NADH oxidase activity may be correlated to the effects of capsaicin in inhibiting growth and inducing apoptosis.

Brar *et al.* [11] reported that catalase, N-acetylcysteine, ebselen, dicumarol and capsaicin also inhibited growth of melanoma and other malignant cell lines. These results raise the possibility that ROS (Reactive Oxygen Species) produced endogenously by mechanisms involving the NAD (P) H: quinone oxidoredutase (NQO) can constitutively activate NF-kappaB in an autocrine fashion. These authors suggested that there was potential for new antioxidant strategies for interruption of oxidant signalling of melanoma cell growth.

Treatments for melanoma remain challenging. While the outcome for superficial melanomas remains good, the treatment for tumours which have invaded and spread to distant lymph nodes or other sites in the body surgery is not a very useful option. Ongoing clinical trials continue to evaluate chemotherapy approaches to the treatment of

metastatic melanoma. Chemotherapy, with Dacarbazine (DTIC) and temozolomide (Temodar) is currently used, however, there has been no improvement over 5-year survival for patients with advanced melanoma [12]. Recently Oblimersen, an antisense oligonucleotide that stops the translation of Bcl-2 mRNA to protein, was found to significantly improve progression-free survival when administered in combination with dacarbazine in patients with normal lactate dehydrogenase [13,14]. Additionally more recently there are now 2 new treatments for melanoma-ipilimumab, a CTLA-4 targeting agent [15] and vemurafenib, a BRAF inhibitor, [16] both of which have improved on dacarbazine and temozolomide. It has been suggested by some authors that another promising candidate might be HA14-1 as an inducer of apoptosis in drug resistant cancer either as a monotherapy or in combination with current cancer therapies [17]. HA14-1 binds to Bcl-2 protein and induces apoptosis of tumour cells [18]. The regulation of apoptosis may be a central part of tumour development. Bcl-2 over-expression has been linked to tumour development and is associated with inhibition of apoptosis and also with chemotherapy resistance [19].

Accordingly the aim of this study was to investigate the effects of capsaicin and HA14-1 used individually and combined, in reducing melanoma cell viability and in inducing apoptosis in three melanoma cell lines to assess whether any useful synergy could be obtained by a combination of these agents.

2. Methods

2.1. Culture of Melanoma Cells

The human C8161 melanoma line was established from an abdominal wall metastasis of a menopausal woman with recurrent melanoma (kindly donated by Professor F. Meyskens, University of California, Irvine, USA, via Professor M. Edwards, University of Glasgow, UK). Cells were cultured in Eagle's modified essential medium (EMEM) supplemented with 10% (v/v) FCS, 2 mM L-Glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulphate, 1.2 mg/ml amphotericin B, 1.5% (v/v) (of 100 × stock solution) vitamin concentrate, 1 mM sodium pyruvate, 1% (v/v) non essential amino acids (NEA) and 0.187% (w/v) sodium hydrogen carbonate (Sigma, Poole, Dorset, UK). Cells were incubated at 37˚C in a humidified 5% carbon dioxide/95% air environment under standard conditions.

The melanoma cell line A375-SM was a generous gift from Professor I. J. Fidler (USA) via Professor M. J. Humphries (University of Manchester, UK). This cell line was established in culture from a lymph node metastasis. These cells are heterogeneous in nature and a highly metastatic variant (A375-SM) was established in culture from lung metastasis produced by parental A375 cells growing subcutaneously in nude mice [20]. These cells were cultured in Eagle's modified essential medium as described for C8161 cells. The human cutaneous cell line HBL used was originally established and described by Professor Ghanem Ghanem (Laboratory of Oncology and Experimental Surgery, Free University of Brussels, Belgium) from a lymph node metastasis of a modular malignant melanoma [21]. Cells were cultured in Ham's F10 medium (Gibco; Paisley, Scotland) supplemented with 5% (v/v) foetal calf serum (FCS), 5% (v/v) newborn calf serum (NBCS; Advanced Protein Products, UK), 2 mM L-Glutamine, 100 units/ml penicillin, 100 μg/ml Streptomycin, 250 μg/ml amphotericin.

Each cell line was subcultured when 80% - 90% confluent using 0.02% (w/v) EDTA for five minutes. Cells were used between passages 20 and 50. Some melanoma cells were incubated prior to use with TNF-α (300 units/ml to 1000 units/ml) Sigma; Poole, Dorset, UK.

2.2. Culture of Melanocytes

Fibroblasts and melanocytes were established from skin donations from patients undergoing elective abdominoplasties and breast reductions who gave written informed consent for excised skin to be used for research purposes on an anonymous basis. The Kroto Research Laboratory holds a Human Tissue Authority Research Tissue Bank License for anonymous tissue donations for research purposes.

Melanocytes were isolated from harvested split-thickness skin grafts (STSGs) obtained from routine plastic surgery of breast reduction and abdominoplasties as previously described [22]. The STSGs were harvested and placed in sterile saline and stored at 4˚C until processing (within 24 h).

Samples of this skin were cut into 0.5 cm^2 pieces using a scalpel blade and were incubated overnight (12 - 24 h) at 4˚C in 0.1% w/v trypsin. FCS was added to neutralize the trypsin and the epidermal and dermal layers were carefully separated using a pair of forceps with fine points. A scalpel blade was used for gently scraping basal keratinocytes and melanocytes from the under surface of the epidermis and the papillary surface of the dermis.

The cells were collected into a mixture of FCS and PBS in sterile 25 ml universal containers. The cell suspension was then centrifuged at 200 g (1000 rpm) for 5 min. Once isolated, cell suspensions were seeded at 4 × 10^6 cells/T25 flask in a low calcium MCDB 153 basal media supplemented with 2% chelated FCS, 25 μg/ml bovine pituitary extract (BPE), 0.6 ng/ml basic fibroblasts growth factor (bFGF), 10 μg/ml insulin, 10 μg/ml transferrin, 2.8 μg/ml hydrocortisone, 2 mM/l L-glutamine, 100 IU/ml; 100 μg/ml penicillin/streptomycin, 10 U/ml nystatin, 1 μg/ml α-tocopherol, 100 ng/ml cholera

toxin and 10 nM/l phorbol 12-myristate 13-acetate (PMA). Geneticin (100 μg/ml) was added to the media over the first few days to prevent fibroblast contamination. Melanocyte cultures were fed twice weekly with this Melanocyte Growth Medium. For co-culture, melanocytes were trypsinised using 2 ml of a 1:1 mixture of 0.1% w/v trypsin and 0.02% w/v EDTA. Once detached 1:2 trypsin inhibitor (TI) was used to block the effects of trypsin and melanocyte growth medium was added to the cells prior centrifuging. Cells were re-suspended in this medium and seeded at the desired density. Melanocytes were not used for experiments after passage 4.

2.3. Culture of Fibroblasts

STSGs were trypsinized as described previously for the isolation of keratinocytes. The epidermal and dermal layers were separated and the dermal parts of the specimens were collected. These dermal samples were washed several times in sterile PBS and then finely minced with a scalpel blade. The dermal mince was incubated at 37˚C overnight in 10 ml of a 0.5% collagenase A solution. The following day, the collagenase digest was spun down in a centrifuge at 2000 g for 10 min, the supernatant was discarded and the pellet of cells was re-suspended in Fibroblasts Culture Medium. Cells were passaged when fibroblasts reached 80% - 90% confluence using 2 ml of a 1:1 mixture of 0.1% w/v trypsin and 0.02 5 w/v EDTA per flask. Fibroblasts were used between passages 4 and 9.

2.4. Preparation of Capsaicin (8-Methyl-N-Vanillyl-Trans-6-Nonenamide) and HA14-1 (2-Amino-6-Bromo-*α*-Cyano-3-(Ethoxycarbonyl)-4H-1-Benzopyran-4-Acetic Acid Ethyl Ester)

A stock solution of 0.33 M capsaicin (Sigma-Aldrich™) was made by dissolving 50 mg in 0.5 ml of ethanol. Capsaicin stock was then dissolved in cell culture medium. A stock solution of 0.02 M HA14-1 (Tocris™) was made by dissolving 10 mg in 1 ml of ethanol. This HA14-1 stock was then dissolved in cell culture medium. Camptothecin (Sigma-Aldrich™) was used as a positive control to induce apoptosis.

2.5. MTT (3-(4,5-Dimethyl Thiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

MTT (Sigma-Aldrich™) acts as an artificial hydrogen acceptor substrate producing a coloured formazan product which is then eluted from the cells using acidified isopropanol. This cytobiochemical assay provides a direct indication of the viability of cells and can be used to provide an indirect reflection of cell number. MTT assays were carried out to assess the impact of drugs on the viability of melanoma cells and fibroblasts. Cells were seeded in a 24 well plate at concentrations of 5×10^4 cells/ml per well and incubated for 72 h. Media was then removed and either ibuprofen, capsaicin, HA14-1 or a combination of capsaicin and HA14-2 were added to the cells and incubated for a further 24 h period prior to assessment of cell viability. Cells were then washed in PBS. MTT solution 1 ml of 0.5 mg/ml in PBS was added to each well and incubated at 37˚C for 40 minutes. This was then removed and 300 μl acidified isopropanol was added to each well to elute the stain. The optical density was measured using a plate reader set at 540 nm wavelength with a reference wavelength of 630 nm.

2.6. Assessment of Apoptosis by Annexin-V

The Guava Nexin™ assay exploits the use of Annexin V which has a strong affinity and specificity in the presence of calcium for phosphatidylserine (PS). The externalisation of PS to the cell surface is one of the early apoptotic events. The kit includes Annexin V conjugated phycoerythrin (PE; λex = 480 nm; λem = 578 nm) and 7-aminoactinomycin D (7-AAD; λex = 555 nm; λem = 655 nm), a viability stain. 7-AAD is excluded from live cells and binds to GC rich regions of DNA within the cell allowing identification and distinguishing between apoptotic cells (Annexin V positive) in early (7-AAD negative) and late (7-AAD positive) stages. Annexin V is able to access internal PS as a result of increased permeability so simultaneous staining with 7-AAD is essential.

Melanoma cells were plated at a density of 5×10^4 cells/ml per well in 24 well plates (Costar) containing EMEM or HAMS plus 10% (v/v) FCS and incubated for 72 hours. After this period media was removed and 1 ml of capsaicin or HA14-1 or both at desired concentrations were added to the cells and incubated for 24 hours. Control samples were incubated with medium alone. Camptothecin (20 μM) was used as a positive control.

Cells were prepared for flow cytometry by removing the culture medium, washing with PBS (×2) and detached using 0.02% (w/v) EDTA (Sigma). Aspirated culture medium and PBS washes (potentially containing late apoptotic and dead cells) were combined together with detached cells and centrifuged at 1000 rpm for 5 minutes. Cell pellets were re-suspended in 150 μl cold PBS buffer and divided into three parallel samples. Samples were then used to measure cellular viability or investigate phosphatidylserine (PS) externalisation.

Cells (~1×10^5) suspended in 100 μl cold PBS buffer were washed once with 1 ml ice cold 1 × Nexin® buffer (Guava Technologies) and centrifuged at 1000 rpm for 5 minutes. Cells were re-suspended in 40 μl ice-cold 1 × Nexin® buffer and incubated on ice in the dark with 5 μl annexin-V PE and 5 μl 7-AAD for 20 minutes. 450 μl 1 × Nexin® buffer was added to each tube, gently agitated

and measurement of 1000 single cell PE versus 7-AAD fluorescence events were made via flow cytometry (Guava PCA). Acquired data was analysed using Cytosoft (v 2.0) software, with different stages in apoptosis determined by relative Annexin V versus 7-AAD fluorescence.

2.7. Assessment of Apoptosis by DNA Damage

Double stranded DNA breaks were measured as a marker of apoptosis using a fluorescent DNA Damage Assay (Active Motif, Carlsbad, CA). Cells (1×10^5) in a 96 well plate were cultured in the presence of various concentrations of capsaicin for 24 hours. Control cells were incubated with medium alone (negative control) or camptothecin 20 μM (positive control). Cells were fixed with ice-cold methanol for 10 min, washed, incubated with 5% BSA in PBS for 1 hour at RT and washed again. Cells were incubated at 4˚C overnight with an anti-DNA break antibody, washed and then incubated with a fluorescently labelled secondary antibody for 1 hour at RT. Washed cells were incubated with propidium iodide for 30 minutes to stain all fixed cells. The plate was then scanned using a fluorescent plate reader and the ratio of DNA-damaged cells to total cells was calculated.

2.8. Statistics

Non-parametric unpaired t-tests were used throughout for assessment of effects of drugs on cell viability and apoptosis. Differences between means were taken as $p < 0.05$ (significant); $p < 0.01$ (very significant); and, $p < 0.001$ (extremely significant) level.

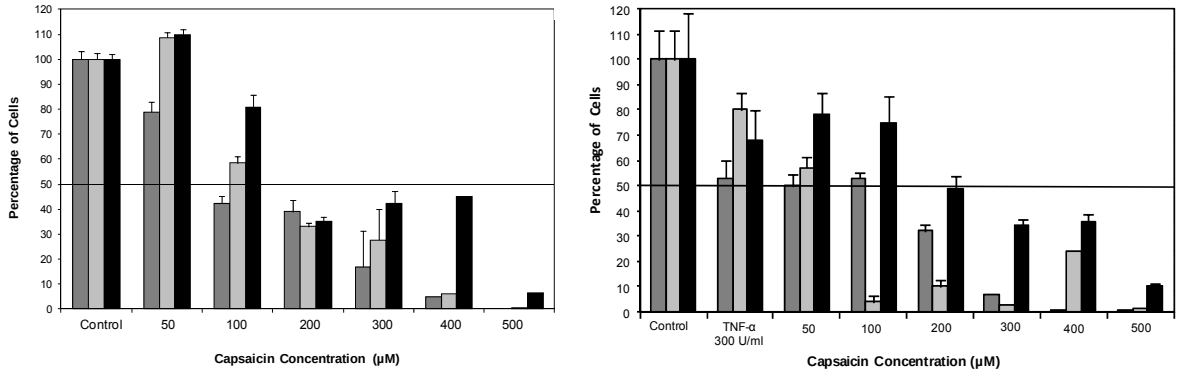
3. Results

Initial studies looked at the effects of capsaicin on melanoma cell viability in the absence and presence of TNF-*α*. For these cells the presence of the pro-inflammatory cytokine increased the cytotoxic effect of capsaicin fourfold for the HBL cells and approximately threefold for the A375SM cells. This did not apply to C8161 cells where the addition of TNF-*α* did not make the cells more susceptible to the inhibitory effects of capsaicin (see **Figures 1(a)** and **(b)**). The IC50 values show the cytotoxic effect of capsaicin concentrations and the reduced cell viability by 50% (see **Figure 1**, annexed table). All subsequent studies were conducted in the absence of TNF-*α*.

The effect of capsaicin in inducing cell apoptosis was then examined both in the presence and absence of a stimulator of apoptosis to examine whether there was any benefit in combining capsaicin with a pro-apoptotic agent.

3.1. Effects of Capsaicin and HA14-1 on Melanoma Cells and Skin Cell Viability

Initially, a dose-response curve for each drug and the combination of both was tested using MTT assays to assess cell viability and to determine appropriate concentrations for investigation of the effects of both drugs in inducing apoptosis in these cells.



(a) (b)

IC5O values (μM) for cell viability		
Melanoma Cells	Unstimulated	TNF-*α* stimulated
HBL	200	50
A375SM	200	75
C8161	200	300

Figure 1. Effects of Capsaicin in melanoma cells viability (MTT assays), (TNF-*α* stimulated and non-stimulated cells). (a) = Capsaicin on melanoma cells; N = 6, in triplicates. (b) = TNF-*α* pre-stimulated melanoma cells and capsaicin, N = 2, in triplicates. ▢ = HBL, ▢ = A375SM, ■ = C8161. Values expressed as percentage of viable cells and SD. Horizontal black line = IC50 values. Table: IC50 values of the effect of capsaicin on melanoma cell viability under basal and TNF-*α* stimulated conditions.

Figures 2(a), **(b)** and **(c)** show the effects of capsaicin and **Figures 2(d)**, **(e)** and **(f)** show the effects of HA14-1 on melanoma cells, melanocytes and fibroblast viability respectively. Overall, capsaicin reduced melanoma cell viability. For HBLs the IC50 value was 100 μM. (Capsaicin significantly reduced HBL cell viability to 16.5% at 300 μM ($p < 0.001$), and to 5% at 400 μM ($p < 0.001$). No viable cells were found at 500 μM capsaicin).

For A375SM cell the IC50 value was 133 μM capsaicin. (Capsaicin significantly reduced cell viability at concentrations of 200 μM up to 400 μM and no viable cells were found by 500 μM).

For C8161 cells the IC50 value was 166 μM capsaicin. (Cell viability was significantly reduced to 35% at 200 μM ($p < 0.05$) and to 6.5% at 500 μM capsaicin ($p < 0.01$))

For melanocytes the IC50 value was 150 μM capsaicin ($p < 0.001$) and concentrations of 50 - 200 μM significantly induced inhibition of cell viability. For fibroblasts the IC50 value was 350 μM capsaicin and cell viability was significantly reduced by 400 μM and 500 μM capsaicin ($p < 0.001$).

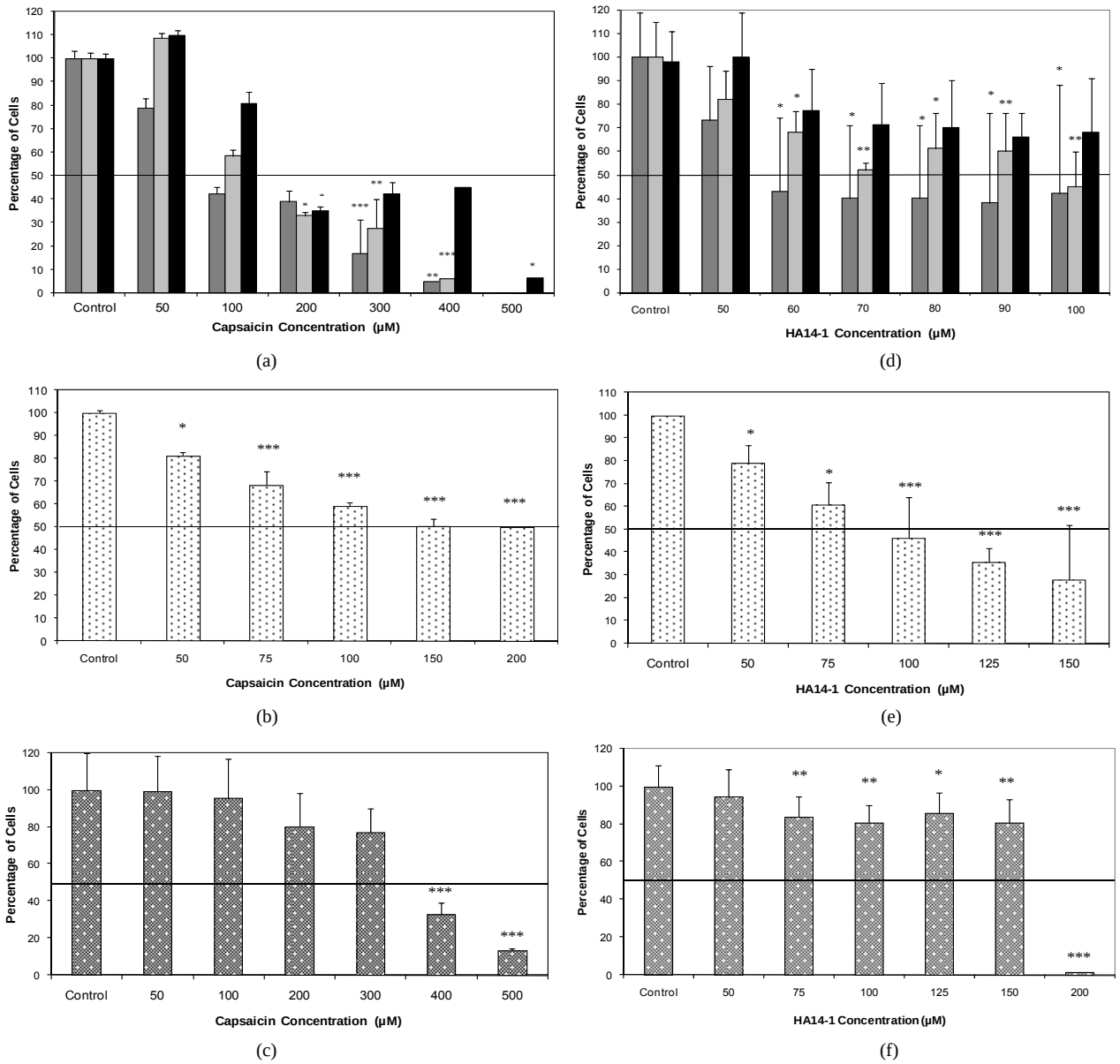


Figure 2. Effects of Capsaicin or HA14-1 in melanoma cells, melanocytes and fibroblasts viability (MTT assays). (a) = Capsaicin on melanoma cells; N = 6, in triplicates. (b) = Capsaicin on melanocytes, N = 2, in triplicates. (c) = Capsaicin on fibroblasts, N = 3, in triplicates. (d) = HA14-1 on melanoma cells, N = 2, in triplictaes. (e) = HA14-1 on melanocytes, N = 2, in triplicates. (f) = HA14-1 on fibroblasts, N = 3, in triplicates. ■ = HBL, ■ = A375SM, ■ = C8161, ⁘ = Melanocytes, ▨ = Fibroblasts Values expressed as Mean + SD. Horizontal black line = IC50 values. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

In summary, the three melanoma cells and melanocytes showed a similar sensitivity to capsaicin with capsaicin concentrations above 200 μM becoming cytotoxic. However, fibroblasts tolerated capsaicin up to 300 μM without any effect with an IC50 of around 350 μM, showing these cells were more resistant to the metabolic inhibitory effects of capsaicin.

HA14-1 also reduced melanoma cell viability. The IC50 value for HA14-1 in HBL cells was around 60 μM ($p < 0.05$). For A375SM cells the IC50 value was around 100 μM HA14-1. For C8161 cells the IC50 value was greater than 100 μM HA14-1. For melanocytes the IC50 value was around 100 μM HA14-1 (see **Figure 2(e)**). Fibroblasts coped relatively well with HA14-1 up to concentrations of 150 μM but 200 μM reduced viability to only 1.3% (see **Figure 2(f)**).

In summary, the IC50 values for melanoma cells and normal skin cells were: HBL (55 μM) followed by melanocytes (80 μM), A375SM (95 μM), C8161 (higher than 100 μM) and fibroblasts (180 μM).

In conclusion, these results suggest that melanoma cells and melanocytes had similar sensitivities to HA14-1 whilst fibroblasts were more resistant to HA14-1 (by a factor of two-fold).

3.2. Effects of Combined HA14-1 and Capsaicin on Melanoma and Skin Cell Viability

Based on their IC50 values a combination of both drugs was then used to investigate their combined effect on melanoma cells, melanocytes and fibroblasts viability. The predicted values from an additive effect of using both agents were also calculated.

Figure 3 illustrates the effects of HA14-1 and capsaicin alone and in combination on melanoma cells (**Figures 3(a)**, **(b)** and **(c)**), melanocytes (**Figure 3(d)**) and fibroblasts (**Figure 3(e)**).

For HBL cells both agents significantly reduced cell viability to 50% ($p < 0.05$). This was not significantly different to the predicted additive effects of these two agents which predicted a reduction in HBL viability to 30%.

For A375SM cell the combined drugs reduced viability to 57% ($p < 0.001$). This was not significantly different to the predicted additive value of reduction in A375SM cell viability to 47%.

For C8161 cells the two drugs combined reduced viability to 56% ($p < 0.001$) which was exactly the predicted value for an additive effect (57%) of these agents.

For melanocytes the combined agents reduced viability to 19% ($p < 0.05$) which was exactly as predicted for an additive effect of these agents.

For fibroblasts the combination of the two agents reduced viability to only 0.4% ($p < 0.001$) which showed a strong synergistic effect as the predicted effect was 35% ($p < 0.001$). However, it should be noted that higher concentrations of HA14-1 and capsaicin were deliberately used for these cells as they had been shown to be more resistant to both of these agents compared to the other melanoma cells and melanocytes.

3.3. Effects of Capsaicin and HA14-1 Alone and in Combination in Inducing Apoptosis in Melanoma Cells and Skin Cells

Figure 4 shows the effects of increasing capsaicin in inducing apoptosis in melanoma cells and in fibroblasts using the Annexin-V assay for apoptosis.

For HBL cells (**Figure 4(a)**), a significant increase in late apoptosis with reduced cell viability started at capsaicin concentration of 300 μM. For A375SM cells (**Figure 4(b)**) capsaicin significantly increased late apoptotic cells and reduced cell viability at 400 μM. For C8161 cells (**Figure 4(c)**) capsaicin increased nuclear debris and reduced cell viability at around 400 μM capsaicin. Overall, fibroblasts coped well with capsaicin at the highest concentration tested, 500 μM, see **Figure 4(d)**.

The concentration of capsaicin which reduced cell viability to 50% was calculated to be 220 μM for HBL cells, 350 μM for A375SM cells and 320 μM for C8161 cells. Capsaicin had no effect on fibroblasts at concentrations up to 500 μM.

20 μM camptothecin was used as a positive control and significantly induced apoptosis in 2 out of 3 melanoma cell lines and fibroblasts. This was not significant for HBL cells due to sample variation.

3.4. Effects of Capsaicin in Inducing Apoptosis in Melanoma Cells Assessed by DNA Damage Assessment

The effects of capsaicin in inducing apoptosis in melanoma cells were also investigated using a DNA Damage assay.

For HBL cells (see **Figure 5**) capsaicin concentrations of 300 μM, 400 μM, and 500 μM significantly reduced HBL cells viability to 57.8%, 57.1% and 48% ($p < 0.001$ in all cases). The IC50 value was around 480 μM capsaicin.

A375SM cell viability was significantly reduced to 68.7% at 300 μM capsaicin concentrations ($p < 0.01$) and the IC50 value was 440 μM. The IC50 value for C8161 cells was 500 μM capsaicin ($p < 0.05$).

Fibroblasts were not affected by the effects of capsaicin up to 500 μM ($p > 0.05$).

20 μM camptothecin, which was used as a positive control, significantly reduced viability of HBL and A375-SM cells ($p < 0.001$) whilst not significantly reducing C8161 cell viability ($p > 0.05$). 20 μM camptothecin had

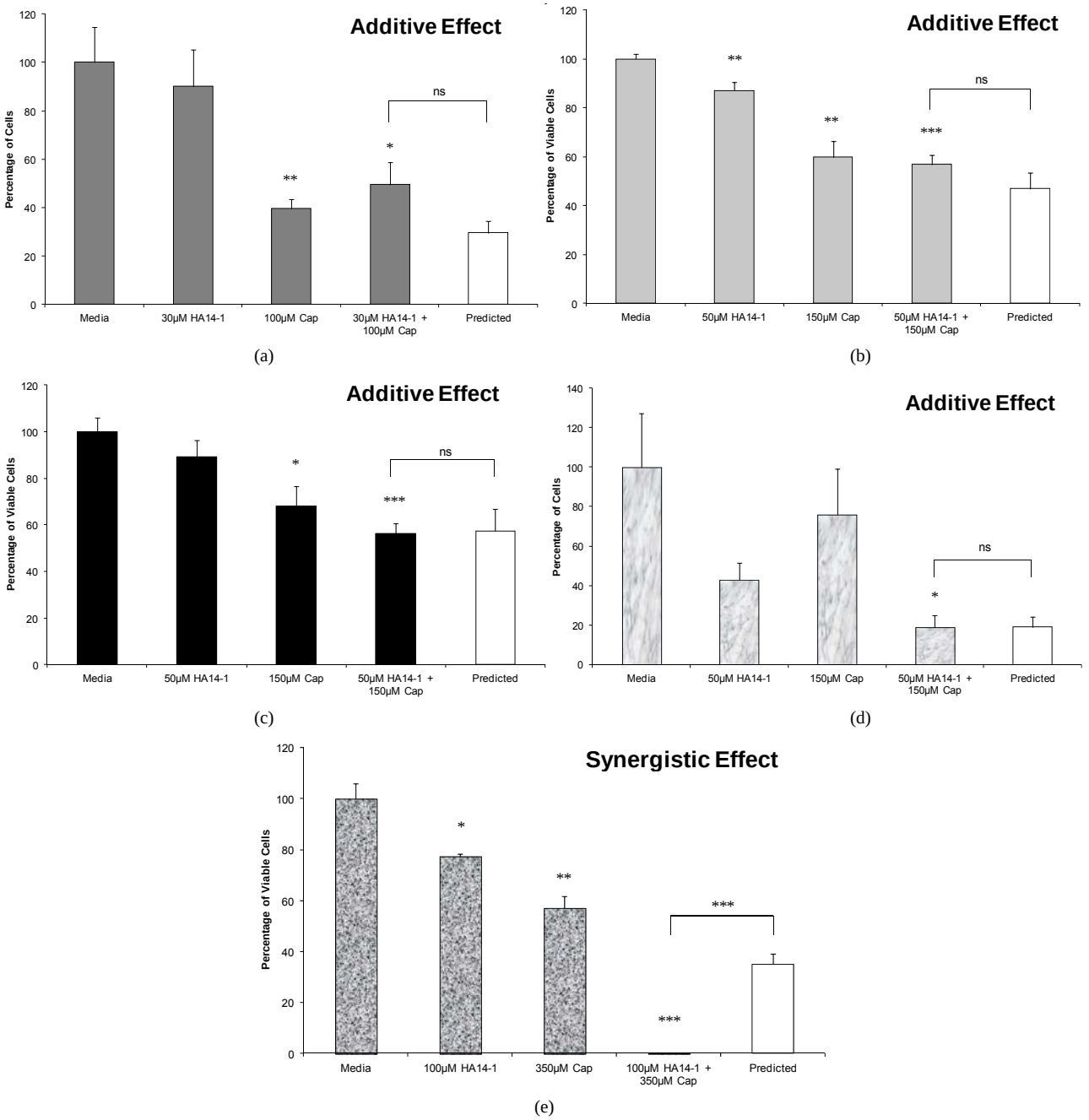


Figure 3. The non-additive, synergistic and additive effects of combined HA14-1 and Capsaicin on (a) HBL, (b) A375SM, (c) C8161 melanoma cells, (d) melanocytes and (e) fibroblast viability. □ = predicted values for each cell line. HA14-1 (μM), Cap = Capsaicin (μM). Combined agents were maintained at the same concentrations as used isolated. Predicted values are shown by the grey coloured columns. Values are expressed as percentage of viable cells. For each melanoma cells N = 2 in triplicates; Melanocytes N = 2 in triplicates; Fibroblasts N = 1 in triplicates. * = p > 0.05; ** = p < 0.01; *** = p < 0.001.

no effect on fibroblast viability.

3.5. Effects of HA14-1 in Inducing Apoptosis in Melanoma Cells and Fibroblasts

Figure 6 shows the effects of increasing HA14-1concentrations in inducing apoptosis in melanoma cells and fibroblasts. For HA14-1 the concentration which reduced cell viability to 50% was 102 μM for fibroblasts. This is higher than the concentration required to demonstrate the same effects on HBL cells which was 48 μM and 85 μM for A375SM and C8161 cells respectively. As before this shows that fibroblasts were more resistant to higher HA14-1 concentrations compared to melanoma cells.

Figure 7 shows the apoptotic effects of HA14-1 and

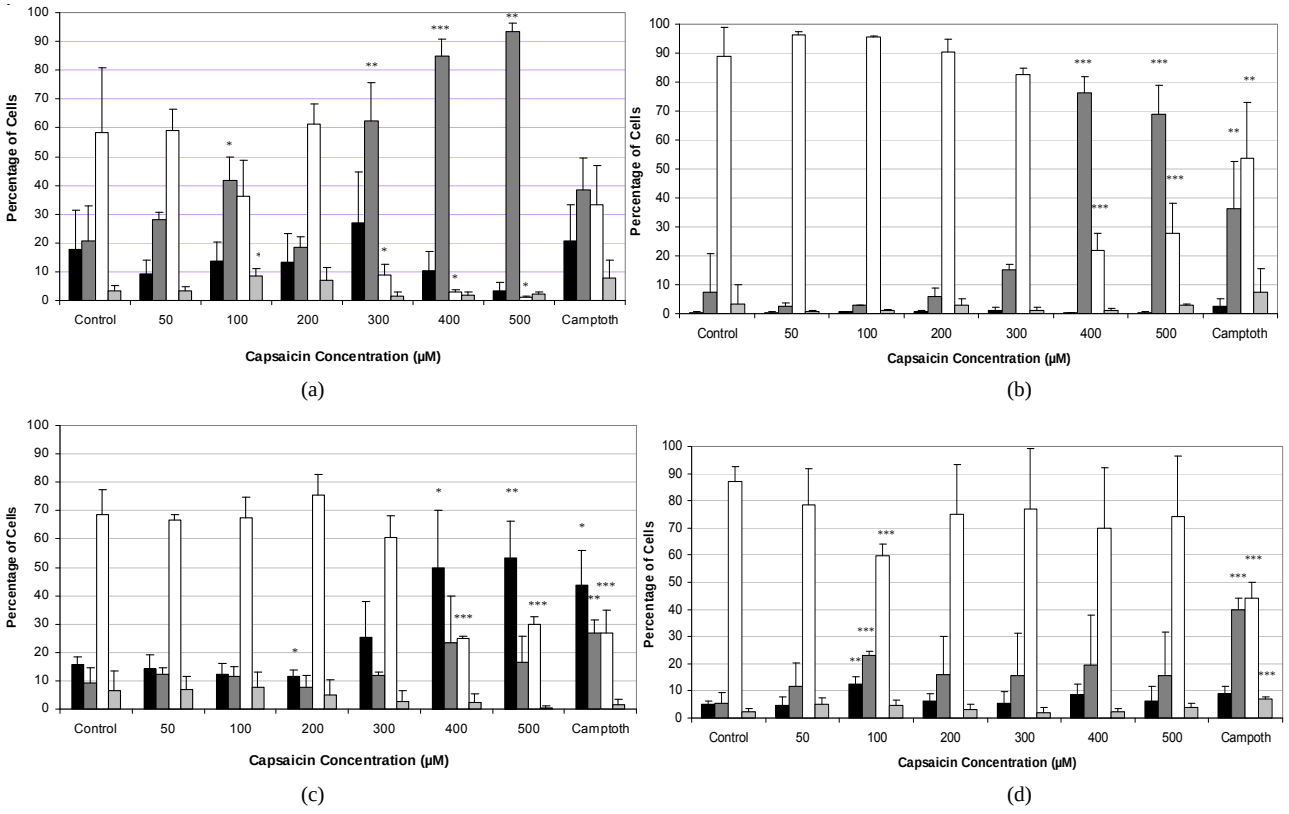


Figure 4. Effects of capsaicin in inducing apoptosis on (a) HBL, (b) A375SM, (c) C8161 melanoma cells and (d) fibroblasts. ■ = Nuclear debris, ▒ = Late apoptotic, □ = Live viable, ▒ = Early apoptotic. Values are expressed as Mean + SD. N = 2 in duplicates. * indicates $p < 0.05$. Camptoth = 20 μM camptothecin.

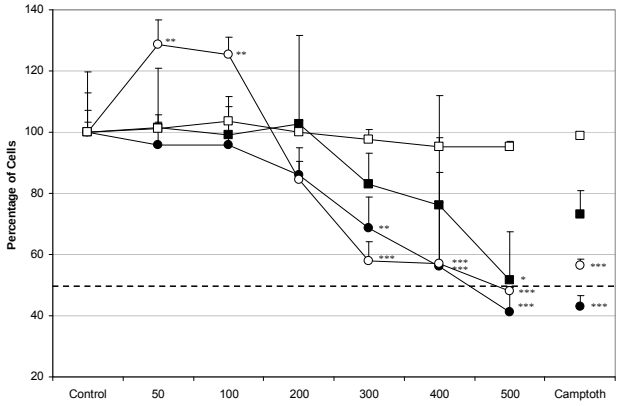


Figure 5. Effects of capsaicin (μM) in inducing apoptosis on melanoma cells and fibroblasts—DNA damage assay. ○ = HBL cells, ● = A375SM cells, ■ = C8161 cells, □ = Fibroblasts. Values express percentage of cells. N = 2 in duplicates. IC50 values are indicated by a red line. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Campth = 20 μM camptothecin.

capsaicin alone and combined compared to the predicted values for the additive effects of these agents in inducing apoptosis.

Figure 7(a) shows that the individual treatments with 30 μM HA14-1 and 200 μM capsaicin had little effect on live and apoptotic cells ($p > 0.05$). Combined HA14-1 and capsaicin showed an additive effect on HBL cells which significantly reduced live HBL cells from 76% of control to 40% ($p > 0.05$) and increased late apoptotic cells from 18% in control cells to 52% ($p < 0.05$). These results were as predicted for an additive effect.

For A375SM cells (**Figure 7(b)**), 75 μM HA14-1 on its own had little effect. 300 μM capsaicin on its own reduced the live cells to 44% and increased the apoptotic cells to 55%, but this was not significant ($p > 0.05$). However, the combined agents significantly reduced live A375SM cells to 18% ($p < 0.001$) and significantly increased late apoptotic cells to 80% ($p < 0.01$), showing an additive effect of these combined agents as predicted.

For C8161 cells, HA14-1 and capsaicin individually had no effect. However the combined agents significantly reduced live C8161 cells from 84% in control cultures to 50% ($p < 0.05$) and significantly increased late apoptotic cells from 12% in control cells to 44% ($p < 0.01$). This outcome clearly shows a synergistic effect ($p < 0.05$) of these combined agents compared to the predicted additive values (see **Figure 7(c)**).

For fibroblasts (see **Figure 7(d)**), 75 μM HA14-1 and 200 μM capsaicin individually had no effect on cell viability, neither did a combination of the two.

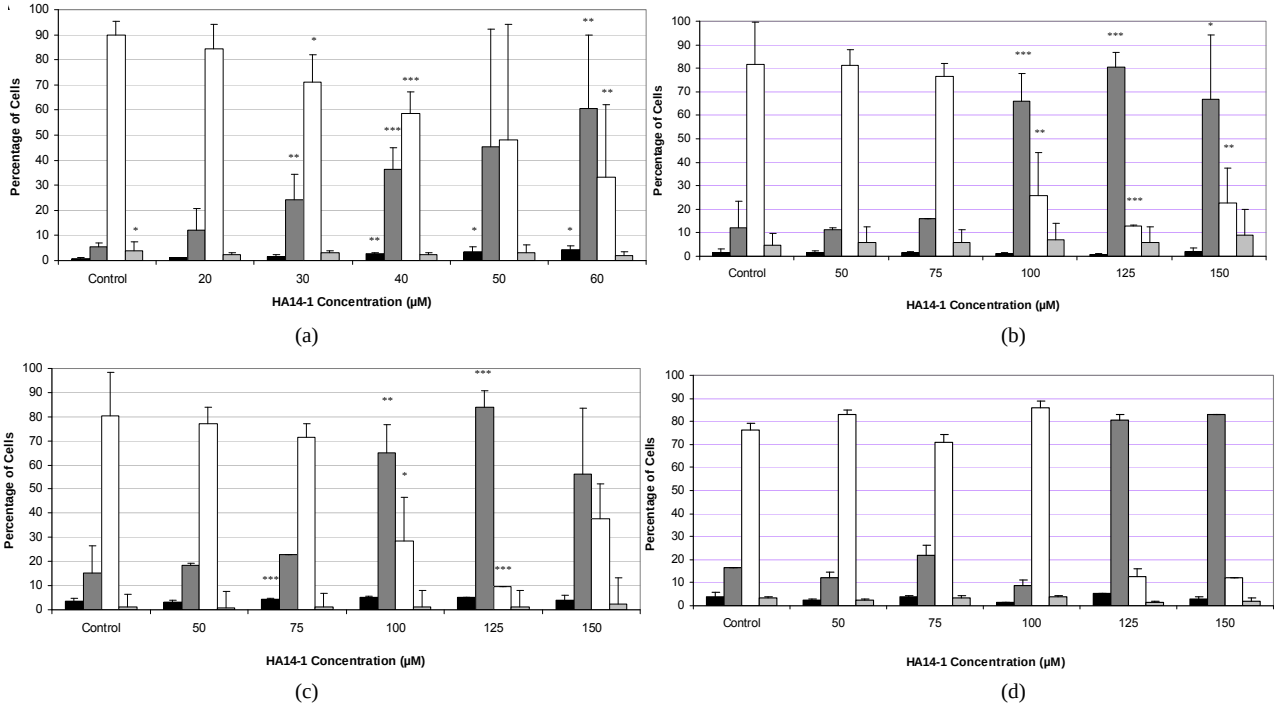


Figure 6. Effects of HA14-1 on (a) HBL, (b) A375SM, (c) C8161 melanoma cells and (d) fibroblasts. Values are expressed as Mean + SD. ■ = Nuclear debris, ▒ = Late apoptotic, □ = Live viable, ▒ = Early apoptotic. Values are expressed as Mean + SD. N = 2 in duplicates. Fibroblasts N = 1 in duplicates. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

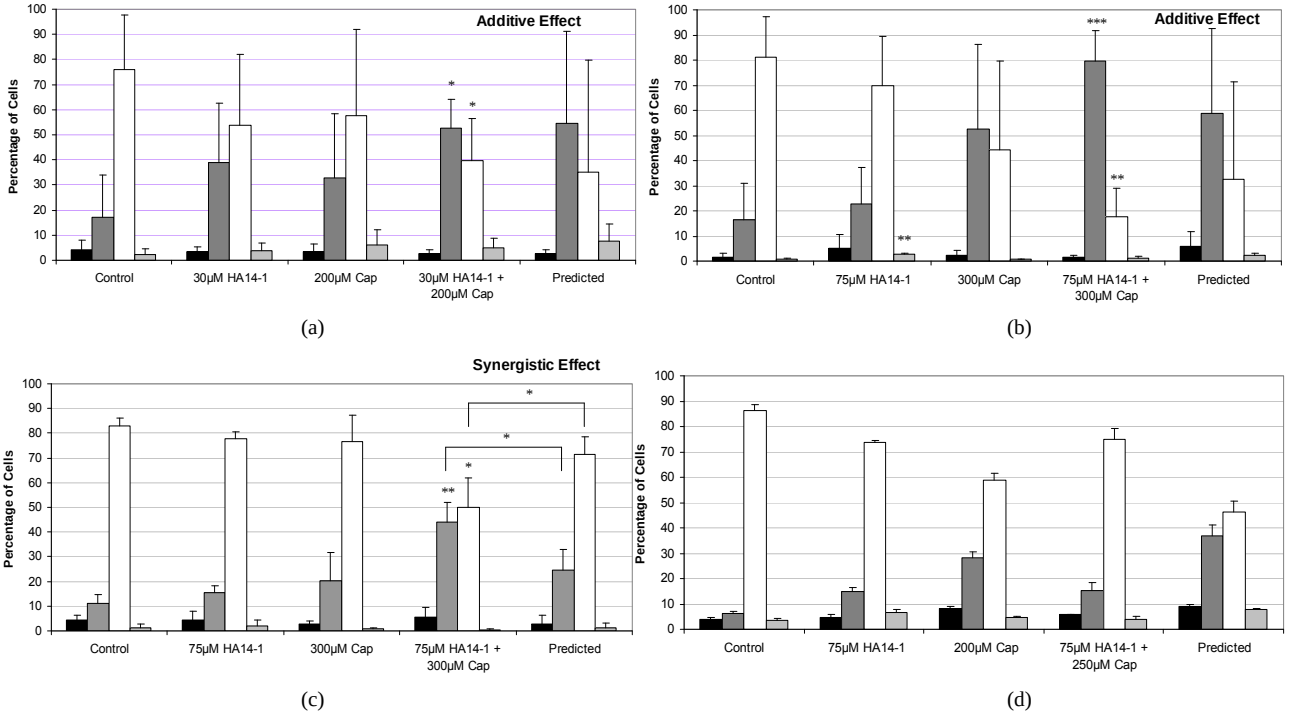


Figure 7. Effects of HA14-1 and Capsaicin on (a) HBL, (b) A375SM, (c) C8161 melanoma cells and (d) fibroblasts. ■ = Nuclear debris, ▒ = Late apoptotic, □ = Live viable, ▒ = Early apoptotic. Values are expressed as Mean + SD. N = 2 in duplicates. For fibroblasts N = 1 in duplicates. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Table 1 summarises the effects of capsaicin and HA-14-1 on melanoma cells, melanocytes and fibroblasts in reducing cell viability and inducing apoptosis comparing the three different methods used.

Table 1. Summary of the effects of capsaicin and HA14-1 viability and apoptosis in melanoma cells and normal skin cells. (IC50 values).

	Capsaicin (μM)			HA14-1 (μM)		Combined	
	MTT	Annexin V	DNA Damage	MTT	Annexin V	MTT	Annexin V
HBL	100	220	480	60	48	100 μM Capsaicin 30 μM HA14-1 ADDITIVE	200 μM Capsaicin 30 μM HA14-1 ADDITIVE
A375SM	150	350	440	70	85	150 μM Capsaicin 50 μM HA14-1 ADDITIVE	300 μM Capsaicin 75 μM HA14-1 ADDITIVE
C8161	150	320	500	> 100	85	150 μM Capsaicin 50 μM HA14-1 ADDITIVE	300 μM Capsaicin 75 μM HA14-1 SYNERGISTIC
Melanocytes	150	Not done	Not done	100	Not done	150 μM Capsaicin 50 μM HA14-1 ADDITIVE	Not done
Fibroblasts	350	> 500	> 500	175	112	350 μM Capsaicin 100 μM HA14-1 SYNERGISTIC	250 μM Capsaicin 75 μM HA14-1 NO EFFECT

4. Discussion

The aim of this study was to examine whether a combination of capsaicin and HA14-1 would have a useful pro-apoptotic effect on melanoma cells. To do this, the effects of both drugs were examined on melanoma cells, melanocytes and fibroblasts looking at their effect on cell viability and apoptosis. Viability was assessed using a simple metabolic assay (MTT test) and two methods for examining apoptosis were used, Annexin V binding to PS on the cell membrane and DNA damage.

Firstly in this study, a major pro-inflammatory cytokine TNF-α was used to activate NF-kB in melanoma cells. NF-kB is a genetic intracellular transcription factor which regulates the pre-synthesis of genes involved in the inflammatory and immune response.

With one exception (for the more aggressive melanoma cell line), experiments on the effects of capsaicin in TNF-α pre-stimulated cells suggested that melanoma cell viability was reduced in pre-stimulated cells compared to unstimulated ones. This may be explained by the effect of capsaicin in preventing protein expression of TNF-α and NF-kB in melanoma cells and the additional apoptotic effect of capsaicin on reducing melanoma cell viability in inflamed cells.

Although 300 U/ml TNF-α only slightly increased activation of NF-kB, capsaicin attenuated melanoma cell viability. An inflamed microenvironment is known to up-regulate integrins, ICAM-1 and ICAM-3 [23] expression which facilitates melanoma cell attachment and invasion [24,25]. Some studies have reported that TNF-α can mediate either apoptotic or anti-apoptotic effects [26-28]. However, a 2006 clinical study already suggests TNF-α blockade as an intervention to improve chemotherapy in cancer patients and for eradication of the primary causes of cancer and NF-kB inhibition to halt tumour progression [29].

The main findings of this study were that capsaicin at around 150 μM reduces the total metabolic activity of the three melanoma cell lines and melanocytes by around 50% (HBL cells were slightly more sensitive than A375SM and C8161) while fibroblasts were able to cope with approximately twice as much capsaicin before any significant loss of viability. Capsaicin induces apoptosis (assessed by the Annexin-V assay) with an IC50 range from 220 μM to 320 μM for the melanoma cells but greater than 500 μM for fibroblasts. Higher concentrations were required to induce DNA damage: 440 μM to 500 μM for the melanoma cells but greater than 500 μM for the fibroblasts.

HA14-1 induced a 50% loss of viability at concentrations ranging from 60 μM to greater than 100 μM for melanoma cells and melanocytes. For fibroblasts the IC50 was 175 μM. Similar concentrations of HA14-1 induced apoptosis in these cells.

Capsaicin and HA14-1 in combination showed additive inhibitory effects on melanoma and melanocyte viability. Synergistic effects were observed on fibroblasts but this was at much higher concentrations of both drugs than were tested on melanoma cells or melanocytes.

Capsaicin and HA14-1 in combination also showed additive effects on inducing HBL and A375SM apoptosis and, surprisingly, a synergistic effect on C8161 cells. These combined agents (at similar concentrations) showed no effect in inducing fibroblast apoptosis. Fibroblasts were also sensitive to the combined effects of capsaicin and HA14-1 when used at concentrations that were sufficient to reduce viability by 50% (350 μM) and induce 50% apoptosis (100 μM), at which point a synergistic effect was seen.

Capsaicin is a well known inducer of apoptosis in cancer cells [30-32] and in melanoma cells [33-35]. In this study, experiments using a DNA damage profile demonstrated that capsaicin induced apoptosis in HBL and A375SM cells at 300 μM. C8161 cells were affected only at a higher capsaicin concentration of 500 μM. At 500 μM capsaicin all three melanoma cells had more

than a 50% reduction in cell viability. Fibroblasts were not affected by capsaicin at up to 500 μM. These results suggest that capsaicin may cause DNA breakage more readily in melanoma cells than in normal skin cells such as fibroblasts. The selective apoptosis inducing effect of capsaicin has been previously reported [36].

There are many reports suggesting mechanisms of action of capsaicin inducing apoptosis. For example, extra production of ROS by mitochondrial NADH oxidase [31,37], *ras* activation inducing apoptosis in transformed cells [35], Bcl-2 down-regulation and caspase 3 activation [38,39] and inhibition of interleukin-6-induced STAT3 activation [40]. In this study, the effects of capsaicin on inducing DNA damage are very clear suggesting that this is one mechanism for explaining apoptosis.

What are the molecular mechanisms by which capsaicin selectively induces apoptosis in transformed cells without affecting normal skin cells? One report from Bodó [41] demonstrated that functional VR1 (Vanilloid Receptor-1) which capsaicin interacts with, was present on human epidermal cells, specifically keratinocytes and Langerhans cells, but not on melanocytes; it was also present in dermal cells, mast cells, sweat gland epithetlium, sebocytes, endothelial cells and smooth muscle cells but not, interestingly, in connective tissue fibroblasts. The lack of response of fibroblasts to capsaicin may be based on their lack of receptors but on the other hand, Kim *et al*. [42] reported the existence of VR1 receptors on fibroblasts. Clearly this is an area that requires further investigation.

As summarised in **Table 1** capsaicin and HA14-1 each reduced cell viability and induced apoptosis in the melanoma cells, melanocytes and fibroblasts but at different concentrations. With respect to metabolic activity of the cells (which is what the MTT assay measures) this was reduced by capsaicin with the melanoma cells and melanocytes showing a similar sensitivity. Fibroblast viability was also reduced by capsaicin (arguing for the presence of VR1 receptors on these cells) but it required approximately twice the concentration of capsaicin compared to that which reduced viability in melanocytes and melanoma cells. For all cells higher concentrations of capsaicin (approximately twice as high) were required to show induction of apoptosis as evidenced by using the Annexin-V assay and still higher concentrations as measured by the assay for DNA damage.

The differences found using these different assays are much as expected. The MTT assay detects a reduction in cell viability and as such is quite sensitive. Not all cells with a reduced viability will necessarily go on to become apoptotic or die. Many cells may recover.

With the apoptotic assessment (Annexin-V and DNA damage assays), it was found that the cells had the same sensitivity pattern, in that HBL were more sensitive than A375SM which were in turn more sensitive than C8161 cells and fibroblasts but higher capsaicin concentrations were required to demonstrate apoptosis.

Annexin-V binds specifically to phospholipids at an early stage of the apoptotic process (a disruption of membrane phospholipids asymmetry exposes PS on the outer cytoplasmatic membrane). In contrast the DNA damage assay is based on breaks in doubled-stranded DNA resulting in the phosphorylation of the histone variant H2AX at serine 139. This measures late apoptosis (by which time physical breaks in the DNA have occurred) so it is entirely as expected that the IC50 values for capsaicin assessed using this assay were higher than when using Annexin-V.

With respect to HA14-1, its mechanism of action was identified by Wang [43]. These authors verified that HA14-1 interacts with Bcl-2 *in vitro*. This protein induces apoptosis in a variety of tumour cell lines and cooperates with other drugs [44,45].

Melanoma cells and melanocytes had a roughly similar sensitivity to HA14-1 as assessed by a loss of cell viability with IC50 values ranging from 60 μM to over 100 μM. For fibroblasts the IC50 was 175 μM, again suggesting that these cells are more resistant than melanocytes and melanoma cells.

With respect to induction of apoptosis slightly lower concentrations of HA14-1 were required to produce apoptosis (compared to reducing cell viability) as assessed by Annexin-V (DNA damage was not undertaken in these experiments). The IC50 values were 48 μM - 85 μM for the three melanoma cells and 112 μM for fibroblasts. This again is much as expected as this drug is known to interact directly with Bcl-2 to induce apoptosis directly.

The two agents were then tested together to see if agents thought to act by different mechanisms would show any useful additivity or synergy in their actions on melanoma cells. Combination therapies have been reported by others [46,47].

For both HBL and A375SM cells the effects of combining both were additive rather than synergistic, but interestingly for C8161 cells there was evidence of some synergy when Annexin-V was measured as an early indicator of apoptosis. This was encouraging as C8161 melanoma cells are particularly aggressive with respect to metastases. Concerning loss of viability the effects of combining the two were additive rather than synergistic.

For melanocytes combining the two gave a loss of viability that was additive (effects on apoptosis were not studied in these cells). For fibroblasts the results appear contradictory at first glance (**Table 1**) in that there is apparent synergy with respect to loss of viability but no effect when these two agents were combined on apoptosis. However this is explained by the concentrations used.

The concentrations of capsaicin and HA14-1 that were used in measuring early stage apoptosis in fibroblasts were similar to those used for the melanoma cells. However, in studying viability, as the fibroblasts had proven relatively resistant to capsaicin and HA14-1, higher concentrations of both were studied and then a combination of the two was found to be synergistic. This argues strongly for fibroblasts having a lower concentration of the VR1 receptor rather than being entirely lacking in it.

Capsaicin and HA14-1 showed a synergistic effect on C8161 cells. This study suggests that these agents may have different mechanisms of action in inducing apoptosis in melanoma cells. As previously reported capsaicin induces oxidative stress [10,11,31], regulates activation of NF-*k*B and IL-8 [40] and hypoxia inducing factor-1-alpha in human melanoma [34], but also inhibits Bcl-2 anti-apoptotic activity [38,39], whilst HA14-1 blocks the anti-apoptotic Bcl-2 protein [17,18] inducing melanoma cell apoptosis. Thus there is some overlap in their activity in that both are reported to inhibit Bcl-2 anti-apoptotic protein.

Capsaicin has been used as an anti-inflammatory agent [47-49]. Topical capsaicin has been used for peripheral neuropathies [50] and patients with soft tissue pain and chronic back pain [51] and for treating burning mouth syndrome [52]. Capsaicin instillation has been used for postoperative pain following knee arthroplasty [53]. It has been also reported that capsaicin can be topically used as an add-on therapy in systematic pain medication [54]. Finally the topic of this study, systemic administration of capsaicin has been demonstrated to induce tumour cell apoptosis [55].

It has been suggested that HA14-1 can be used in therapeutic combination with other anticancer agents [17] as it has no effect in normal cells. It has been reported to inhibit the expression of anti-apoptotic Bcl-2 proteins which are associated with chemotherapy resistance in various human cancers. Preclinical studies have shown that agents targeting antiapoptotic Bcl-2 family members can be used as a single agent and in combination with other anticancer agents. Its mechanism of action is related to the intrinsic apoptotic pathway which can be initiated by many signals such as cellular damage or cytokine deprivation [57]. Another molecular antagonist of the Bcl-2 family members known as deoxyglucose-ABT-263/737 has been demonstrated to be a potent apoptotic inducer by releasing cytochrome c from the mitochondria activating caspases which are required to complete the apoptosis process and has also been suggested as a combined therapy for cancer treatment [58]. There are now some experimental and pre-clinical trials in which capsaicin has been used as a treatment for pain in advanced cancer [59].

5. Conclusion

In conclusion this comparative study has shown that melanoma cells and melanocytes have a similar sensitivity to capsaicin while fibroblasts are more resistant to this agent. HA14-1 induces apoptosis at relatively low concentrations and a combination of the two agents produces a useful additive affect for 2 out of 3 of the melanoma cancer lines and studies and encouragingly for the most metastatically aggressive cancer cell line (C8161), a combination of the two showed some evidence of synergy.

The results suggest that a natural agent (which is perhaps common in one's diet), such as capsaicin, can be used in combination with another organic compound, HA14-1, as a pro-apoptotic agent. The advantages of a combined therapy include using the two drugs at lower concentrations which reduces toxicity and side effects for the patient while promoting improved cell apoptosis. This may be a promising alternative therapy for those patients with malignent melanoma, as there are few effective chemotherapy approaches at present. The newer more promising agents of ipilimumab and vemurafenib could also be studied in combination with capsaicin in future studies.

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