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Development and characteristics of a human cell assay for screening agents for melanoma prevention

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This paper describes the development and initial evaluation of a human cell assay to identify potentially efficacious agents for preventing melanoma. Four human cell lines were used: normal melanocytes, a radial growth-phase-like melanoma cell line (WM3211), a vertical growth-phase-like melanoma cell line (Lu1205), and 83-2c, a cell strain cloned from metastatic melanoma. Four endpoints were evaluated in ultraviolet B-treated cells: annexin V, human leukocyte antigen-DR; E-cadherin, and N-cadherin. Annexin V was induced by nimesulide, 4-hydroxyphenylretinamide, and difluoromethylornithine in ultraviolet-B-treated radial growth-phase-like melanoma cells. None of the agents inhibited human leukocyte antigen-DR expression in ultraviolet-B-treated radial growth-phase-like melanoma cells, the only cells that strongly expressed human leukocyte antigen-DR. E-cadherin was overexpressed only in radial growth-phase-like melanoma cells relative to melanocytes, and ultraviolet B exposure dramatically reduced this expression. E-cadherin was only induced by difluoromethylornithine in ultraviolet-B-treated radial growth-phase-like melanoma cells. N-cadherin was overexpressed in all melanoma cell lines relative to melanocytes. In this study, all candidate preventive agents inhibited N-cadherin in ultraviolet B-treated radial growth-phase-like melanoma cells. Four agents inhibited N-cadherin in ultraviolet B-treated vertical growth-phase-like melanoma cells. The mean ratios of N-cadherin to E-cadherin levels and specific endpoint responses for both the radial growth-phase-like melanoma and vertical growth-phase-like melanoma cells were used to rank the agents. Agents were evaluated at clinically relevant concentrations. The rankings were difluoromethylornithine > 4-hydroxyphenylretinamide > nimesulide > 9-*cis*-retinoic acid > polyphenon E. Diphenylhydramine, D-mannitol, and nordihydroguaiaretic acid were inactive. The results of these initial studies suggest that ultraviolet-B-treated radial growth-phase-like melanoma cells are the most responsive to chemopreventive agents, and may be the cell line of choice for screening melanoma prevention agents. *Melanoma Res* 17:42–50 © 2007 Lippincott Williams & Wilkins.

Introduction

The frequency of malignant melanoma has been increasing in the United States, Australia, Northern Europe, and Canada over the past 60 years and has shown a dramatic rise in the past 10 years with more than 59 000 new cases projected for 2005 in the US alone (http://seer.cancer.gov) [1,2]. Of the skin cancers, malignant melanoma is the most refractory to treatment and, unless managed in an early stage of development, has the worst prognosis. Epidemiological studies have implicated sunlight exposure as a major factor in melanoma development [3]. Furthermore, Caucasians have an increased risk of melanoma in sun-exposed areas relative to Blacks [4]. A hereditary component seems to exist in 5–10% of the individuals with melanoma [5].

Although advances in the early detection and surgical treatment of melanoma have resulted in increased lifespan and improved quality of life, an effective systemic therapy for metastatic disease has not been forthcoming. It would therefore be beneficial to identify agents with potential efficacy for preventing melanoma. The prevention of melanoma could occur by preventing the initial events, inhibiting or reversing the development of early premalignant lesions, arresting the growth of existing early-stage melanoma, or killing the cells via apoptosis or other mechanisms. This paper describes the development of an in-vitro screening assay that uses human melanocytes and melanoma cell lines at different stages of progression and biomarkers known to be important in melanoma progression to identify agents with potential cancer prevention efficacy. In planning the development of such an assay, the following approach was used:

- Perform initial studies in human cell lines that reflect the progression of melanoma from normal melanocytes

to metastatic melanoma. Cultures included: primary cultures of normal melanocytes; a radial growth melanoma (RGM) cell line, WM3211; a vertical growth melanoma (VGM) cell line, Lu-1205; and a metastatic melanoma cell strain, 83-2c.

- As melanoma has been strongly associated with sun exposure, ultraviolet light (UVB) treatment was made a part of the assay protocol. Studies with RGM cell lines have demonstrated that UVB irradiation can induce genomic instability [6].
- As it is important to establish agent effectiveness in relation to potential toxicity, the assay should be capable of examining multiple concentrations of an agent at the same time.
- Several biomarkers associated with melanoma development and their responses to candidate agents were investigated. The markers included the induction of E-cadherin, the inhibition of N-cadherin, the induction of annexin V, and the inhibition of human leukocyte antigen (HLA)-DR . As a control for toxicity, propidium iodide staining was carried out for each agent concentration.

Melanocyte growth and differentiation is, to a great extent, regulated by the homeostatic control, which is mediated through E-cadherin and N-cadherin expression [7]. E-cadherin, which facilitates cell-to-cell interaction between keratinocytes and melanocytes [7,8], is expressed in normal melanocytes and RGM cells at higher levels than in metastatic melanoma and can be regarded as a marker of the more benign phenotype. E-cadherin expression in melanocytes facilitates the binding to keratinocytes and allows them to control the homeostatic expression of melanocytes [7,9]. N-cadherin is strongly expressed in more malignant cells and appears to facilitate interactions between the melanoma cells, fibroblasts, and endothelial cells, and the switch from E-cadherin to N-cadherin during the development of melanoma may be indicative of malignant progression [8–10].

The regulation of histocompatibility antigens by melanoma plays a key role in allowing the melanoma cells to escape immune surveillance. Melanoma cells show reduced HLA class I antigens and increased expression of HLA class II antigens as they progress [11]. As HLA expression is thought to transition from class I to II during melanoma development, we chose the inhibition of HLA-DR (a class II antigen) expression as an endpoint.

Although melanocytes *in vivo* appear to be resistant to UV-induced apoptosis [2], treatment of some melanoma cells with preventive agents has induced apoptosis [12]. As cancer formation is also associated with a loss of apoptosis, this endpoint was also explored, using annexin V staining (an early biomarker of apoptosis).

The goal of these initial studies was to evaluate various biomarkers related to melanoma and cell lines at different stages of progression to determine: (i) an optimal cell line(s) and (ii) suitable biomarkers for use in screening preventive agents. In this paper, data for five candidate preventive agents are presented: nimesulide, difluoro-methylornithine (DFMO), 4-hydroxyphenyl retinamide (4HPR), polyphenon E, and 9-*cis*-retinoic acid (9-*cis*-RA). These agents are in clinical trials for prevention of cancer of the skin and/or other organs. In addition, diphenyl-hydramine, D-mannitol, and nordihydroguaiaretic acid (NDGA), which have not demonstrated cancer preventive activity in multiple animal bioassays, were also tested.

Materials and methods
Cell culture conditions
Melanocytes were isolated from normal human foreskin tissues obtained from the University of California Irvine Medical Center under a protocol approved by the University of California Irvine Institutional Review Board. The method used to isolate normal melanocytes was a combination of the procedures developed by Eisinger and Marko [13] and Halaban and Alfano [14] and modified by Jambrosic and co-workers [15]. One to two human Caucasian neonatal foreskins (not more than 48 h after excision) were placed in 0.25% trypsin at 4°C overnight. The epidermal and dermal layers were separated and the melanocytes removed with gentle scraping. The cells were pooled and cultured in MCDB 153 medium (Sigma-Aldrich, St. Louis, Missouri, USA) containing 2% fetal bovine serum (FBS), 0.3% bovine pituitary extract (BioWhittaker, Inc., Walkersville, Maryland, USA), 10 ng/ml phorbol myristate-13-acetate, 2.0 mmol/l calcium chloride, 5 g/ml insulin, 0.1 mmol/l 3-isobutyl-1-methyl-xanthine (Sigma), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Fibroblast contamination was suppressed by adding 250 µg/ml Gentacin (Sigma) to the growth media for 2 days. Each normal melanocyte culture contained the total yield of cells from one or two Caucasian neonatal foreskins. Normal melanocytes were used through passage 5.

The RGM cell line (WM3211) and the VGM cell line (Lu1205) were kindly provided by Dr Meenhard Herlyn [16]. The RGM cells were grown in RPMI 1640 (Invitrogen, Carlsbad, California, USA) with L-glutamine supplemented with 5% FBS, 5% fetal calf serum, 10 µg/ml insulin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The VGM cells were maintained in MCDB 131/L15 (1 : 1 v/v) (Invitrogen) medium supplemented with 5% FBS, 5% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The human metastatic melanoma cell strain (83-2c), derived by Thomson and Meyskens [17], was cultured in F-10 (Invitrogen)

containing 5% FBS, 5% newborn calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Fisher Scientific).

All cell lines were grown in monolayer culture. Normal melanocytes were slow growing whereas the RGM, VGM, and 83-2c cells exhibited rapid growth rates. All cells were cryopreserved in the appropriate culture medium containing 10% dimethylsulfoxide (DMSO) and 10% FBS and stored in the vapor phase of a liquid nitrogen freezer. All cells were thawed at 37°C and centrifuged in 5 ml of the appropriate medium for 5 min at 800g, resuspended in 10 ml of complete media, and transferred into a 75-cm^2 flask. The cultures were incubated at 37°C in 5% CO_2 at approximately 95% relative humidity. Primary cells were maintained by changing the medium twice a week until ready for cryopreservation or testing.

To subculture, cells were washed twice with calcium/magnesium-free Hank's balanced salt solution, 0.25% trypsin-ethylenediaminetetraacetic acid (Invitrogen) added to the culture container, and the cells incubated for 1–3 min. The detached cell solution was neutralized with complete culture medium and centrifuged at 800g for 5 min. The cells were diluted and seeded at an appropriate plating density.

All culture materials and cell cultures were tested routinely for microbial contamination. If contamination was found, the media, media components, or cultures were not used in the experiments. Each agent was tested for its solubility in culture media. If the chemical was insoluble in media, or soluble only at very low levels, then DMSO was used as a solvent. After dissolving in DMSO, the compound was added to culture medium such that the concentration of DMSO did not exceed 0.2%. To ensure optimal compound stability, agents were stored as recommended by the supplier. Fresh chemical was diluted for each experimental treatment and always handled under yellow and/or reduced light to minimize any photodegradation.

The cells were exposed to UVB irradiation at a dose (25 mJ/cm^2) that clinically produces suberythema. The cells were irradiated using a UVP 2000 Ultraviolet Crosslinker (UVP, Inc., Upland, California, USA), which produces an emission maximum centered at 302 nm. Wavelengths of 280 nm or less were filtered out by irradiating the cultures through the plastic culture flasks. To correct for irradiation through plastic, the internal sensor in the instrument was covered with a plastic shield of the same type and thickness as the flasks containing the cells.

Preliminary assay
An initial study was carried out to determine the effects of UVB exposure on the expression of each biomarker in each cell line. In addition, the expression of these markers was also measured following exposure to UVB plus chemopreventive agents. UVB treatment was included as part of the standard assay for preventive agent efficacy, but a parallel drug treatment arm with no-UVB treatment was not. The reasons included the fact that patients taking preventive agents would probably have some exposure to UV during their treatments. Second, UVB treatment produced an inhibitory response for E-cadherin and annexin V expression in WM cells with only minimal effects on other cell types relative to no-UVB (Table 1).

To choose a nontoxic concentration range suitable for the assay, a preliminary viability assay was performed. This assay included normal melanocytes and 83-2c cells. Cells were seeded in triplicate into a 96-well plate at a density of 3300–3500 cells/well and allowed to attach overnight. Agents, in culture medium, were tested in half-log increments (five concentrations not exceeding 1 mmol/l or 1 mg/ml) for 72 h. To assess cellular viability following agent exposure, Aqueous One Solution (Promega Corp., Madison, Wisconsin, USA) was added (10 μl/well) for the last 2 h of the 72-h incubation. Absorbance of the Aqueous One formazan product, which is directly proportional to the number of viable cells, was quantified at 490 nm by a plate reader (Bio-Rad, Hercules, California, USA) [18,19]. The data were used to select the highest nontoxic concentration of the agent for the human cell assay. A comparison of the concentrations used in the assay and clinically achievable levels is shown in Table 2.

Table 1 The effects of UV exposure on the expression of biomarkers in all cell lines[a]

Cell type	Annexin V		E-cadherin		N-cadherin		HLA/DR	
	No UV	UV	No UV	UV	No UV	UV	No UV	UV
Melanocytes	259.9	263.9	64.9	67.6	102.9	110.6	10.2	10.8
RGM (WM3211)[b]	233.2	161.0	267.6	142.5	276.4	302.1	370.3	296.9
VGM (LU1205)	71.5	77.0	81.3	94.7	274.0	283.2	31.3	29.7
83-2c	256.0	346.0	72.5	81.7	264.7	265.6	30.2	31.6

HLA, human leukocyte antigen; RGM, radial growth phase-like melanoma; UV, ultraviolet; VGM, vertical growth phase-like melanoma.
[a]Values shown for expression are the mean peak fluorescence for each endpoint.
[b]The differences in expression observed for E-cadherin with no UV and UV are reproducible (three experiments).

Biomarker assays

The concentrations of test agents used in the assays consisted of the highest, nontoxic test agent concentration (HC) from the cytotoxicity assay above, plus four half-log dilutions of that concentration. Cells were seeded (2–2.25×10^5) into 25-cm^2 flasks and allowed to attach overnight. The test agents were added with fresh media and incubated for 24 h. At the end of the incubation, media were aspirated and cells were washed once with phosphate-buffered saline (PBS) and 1 ml of PBS added to cover the cells. Fresh treatment medium containing preventive agents was added and UVB-treated cells incubated for an additional 48 h. Cells were assayed for all endpoints by flow cytometry using a Becton-Dickinson FACScan with Cell Quest software (Becton-Dickinson, Inc, Franklin Lakes, New Jersey, USA). In preliminary experiments, the peak response for the different endpoints was determined to be 48 h after UV exposure.

Measurement of biomarkers

Annexin V, an early biomarker for apoptosis, was measured according to the manufacturer's protocol (Pharmingen, San Diego, California, USA). Briefly, cells were harvested, washed once in PBS and resuspended in binding buffer (10 mmol/l *N*-2-hydroxyl piperazine-*N'*-2-ethane sulfonic acid/NaOH, pH 7.4, 140 mmol/l NaCl, 2.5 mmol/l CaCl$_2$) and resuspended at a concentration of 1×10^6 cells/ml. A 100-μl sample of the cells was incubated with 5 l of annexin V (conjugated to fluorescein isothiocyanate) and 15 mol/l propidium iodide for 15 min at room temperature. The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for annexin V. Cells used for annexin V staining were also stained with propidium iodide to determine whether substantial cell toxicity was produced by mechanisms other than apoptosis.

The cells were analyzed for HLA-DR expression by direct immunofluorescence staining. The cells were harvested and washed once in washing buffer (PBS, 0.1% sodium azide) and resuspended in staining buffer (PBS, 0.1% sodium azide, 2% FBS) at a concentration of 1×10^6 cells/ml. The cells were incubated on ice for 30 min with the fluorescein isothiocyanate-labeled HLA-DR monoclonal antibody (2 g/ml) (Becton Dickinson). At the end of the incubation, 2 ml of wash buffer was added and the cells were centrifuged at 1200 r.p.m. for 5 min. The supernatant was discarded and the cells were resuspended in 0.5 ml of wash buffer and analyzed by flow cytometry.

Cells were analyzed for E-cadherin expression [20] by indirect immunofluorescence staining. Cells were harvested and washed once in washing buffer (PBS, 0.1% sodium azide) and resuspended in staining buffer (PBS, 0.1% sodium azide, 2% FBS) at a concentration of 1×10^6/ml. Cells were incubated on ice for 30 min with

Table 2 Comparison of in-vitro and clinical plasma concentrations[a]

	Effective concentrations	Clinical concentrations	
Agents	*In vitro*[b]	Plasma	C$_{max}$
DFMO	5.5–548.9	4.97–41.29 [22]	
4HPR	0.046–4.55	0.34–1.41 [23,24]	0.6–12.9 [25]
Nimesulide	0.001–0.1		2.81 [26]
Polyphenon E	0.53–53		0.16–0.82 [27]
9-*cis*-retinoic acid	0.067–6.67		0.82–1.95 [28]

9-*cis*-RA, 9-cis-retinoic acid; DFMO, difluoromethylornithine; 4HPR, 4-hydroxyphenylretinamide.
[a]Concentrations are in micromoles/liter.
[b]For all agents, the lowest effective concentration was the lowest concentration evaluated.

Table 3 Summary of the biomarker responses of all agents evaluated in RGM and VGM cells

	RGM cells (WM3211)			VGM cells (Lu1205)		
Agents	Annexin V	E-cadherin	N-cadherin	Inhibition of N-cadherin/ E-cadherin ratios (%)[a]	N-cadherin	Inhibition of N-cadherin/ E-cadherin ratios (%)
DFMO	+ (3)[b]	+ (3)	+ (4)	67.6	+ (5)	76.5
4HPR	+ (5)	NE	+ (5)	45.4		19.3
Nimesulide	+ (4)	NE	+ (3)	38.3	+ (3)	23.8
Polyphenon E	NE	NE	+ (4)	5.2	+ (5)	48.5
9-*cis*-RA	NE	NE	+ (3)	44.5	+ (5)	52.6
Diphenylhydramine	NE	NE	NE	13.9	NT	NT
Mannitol	NE	NE	NE	13.3	NT	NT
NDGA	NE	NE	NE	15.0	NT	NT

9-*cis*-RA, 9-*cis*-retinoic acid; DFMO, difluoromethylornithine; NDGA, nordihydroguaiaretic acid; 4HPR, 4-hydroxyphenylretinamide; NE, not effective; NT, not tested; RGM, radial growth phase-like melanoma; UV, ultraviolet; VGM, vertical growth phase-like melanoma.
[a]The percent inhibition ratios are calculated relative to the ratios obtained from the UV-only treated cultures. The maximum values observed with each agent are shown.
[b]For an agent to be considered as positive for an endpoint, it must produce a 40% induction (annexin V or E-cadherin) or 40% inhibition (N-cadherin) at two consecutive concentrations. The number in parenthesis is the number of positive concentrations.

mouse anti-human E-cadherin (1 g/ml) (Calbiochem, San Diego, California, USA). At the end of the incubation, 2 ml of wash buffer was added and cells centrifuged at 1200 r.p.m. for 5 min. The supernatant was discarded and cells resuspended in wash buffer with the fluorescein-conjugated goat anti-mouse IgG (Sigma, St Louis, Missouri, USA) for 30 min on ice. At the end of the incubation, 2 ml of wash buffer was added and the cells were centrifuged at 1200 r.p.m. for 5 min. The super-natant was discarded and cells were resuspended in 0.5 ml wash buffer analyzed by flow cytometry. A control, which omitted the primary antibody, was also analyzed.

Cells were analyzed for N-cadherin expression by indirect immunofluorescence staining. Cells were harvested and washed once in washing buffer (PBS, 0.1% sodium azide) and resuspended in staining buffer (PBS, 0.1% sodium azide, 2% FBS) at a concentration of 1×10^6/ml. Cells were incubated for 30 min with rabbit anti-human N-cadherin (1 g/ml) (Calbiochem). At the end of the incubation, 2 ml of wash buffer was added and cells centrifuged at 1200 r.p.m. for 5 min. The supernatant was discarded and cells were resuspended in wash buffer with the phycoerythrin-conjugated goat anti-rabbit IgG Calbiochem) for 30 min on ice. At the end of the incubation, 2 ml of wash buffer was added and the cells were centrifuged at 1200 r.p.m. for 5 min. Supernatant was discarded and the cells were resuspended in washing buffer and analyzed by flow cytometry. A control was also performed, omitting the primary antibody.

Responses of the various endpoints to the agents were based on their ability to induce expression (E-cadherin and annexin V) or inhibit expression (N-cadherin and HLA/DR) in UV-treated cells relative to the cells treated with UV alone. 'Tentative' endpoint response criteria were established. For an agent to be considered as positive for an endpoint, it must either induce annexin V or E-cadherin, or inhibit N-cadherin mean expression by 40% at two consecutive agent concentrations. These criteria were established on the basis of the data from the agents that have been evaluated. These criteria will allow us to distinguish between agents with known preventive activity from animal studies compared with agents for which no animal data are available to suggest activity. Once additional agents are tested and data collected, these criteria will be subject to further refinements.

Results and discussion

Before testing the effects of the chemopreventive agents on biomarker expression in UVB-treated cells, each agent was evaluated for cytotoxicity in normal melanocytes and the metastatic melanoma line, 83-2c. With the exception of 9-*cis*-RA at the highest concentration tested (6.7 μmol/l), none of the agents demonstrated any toxicity after a 3-day

exposure over a wide concentration range. Propidium iodide staining in the biomarker expression assays confirmed that the biomarker expression changes were not because of unassociated agent toxicity.

Although the assay was primarily aimed at examining the effect of chemopreventive agents on UVB-treated cells, additional information was obtained on the effect of UVB-alone exposure on the expression of the various biomarkers in each of the cell lines. The results are shown in Table 1. Interestingly, the effects of UVB were relatively minor, with the exception of the inhibition of E-cadherin expression and annexin V in the RGM cell line. Furthermore, the RGM cells were the only ones to overexpress E-cadherin relative to the melanocytes. All melanoma cell lines overexpressed N-cadherin; however, only the RGM cells showed high expression levels of HLA-DR. As normal melanocytes, VGM cells, and 83-2c cells lacked sufficient expression of HLA-DR, inhibition could not be measured in these cell lines.

As class II HLA expression is maintained in melanoma cells while the class I HLA antigens are reduced or lost, inhibition of HLA-DR by a compound in melanoma cells may indicate activity that could be beneficial. Only the RGM cell line overexpressed this antigen; however, none of the agents tested for this biomarker were able to inhibit expression.

Figure 1 shows examples of histograms that show the responses observed for annexin V in RGM cells. At 48 h, UVB-alone showed minor inhibition of annexin V compared with untreated cells (Fig. 1a). At the lowest active concentration, DFMO induced annexin V (Fig. 1b). Nimesulide, 4HPR, and DFMO also induced annexin V in RGM cells, whereas the remaining agents were inactive.

Figure 2 compares examples of histograms that show the responses observed for E-cadherin in RGM cells. Note the inhibition of E-cadherin expression with UVB expo-sure relative to the unexposed cells (Fig. 2a). Figure 2b shows that DFMO at the lowest active concentration was able to reverse the inhibition of E-cadherin produced by UVB exposure. Only DFMO was active in inducing the re-expression of E-cadherin in these UV-treated cells.

Figure 3 compares examples of histograms that show the responses observed for N-cadherin in RGM cells. UVB treatment resulted in only minor changes (Fig. 3a). Figure 3b shows the inhibition produced by DFMO at the lowest active concentration. Nimesulide, polyphenon E, 4HPR, and 9-*cis*-RA also inhibited N-cadherin expression in these UVB-treated cells, whereas D-mannitol, diphe-nylhydramine, and NDGA did not.
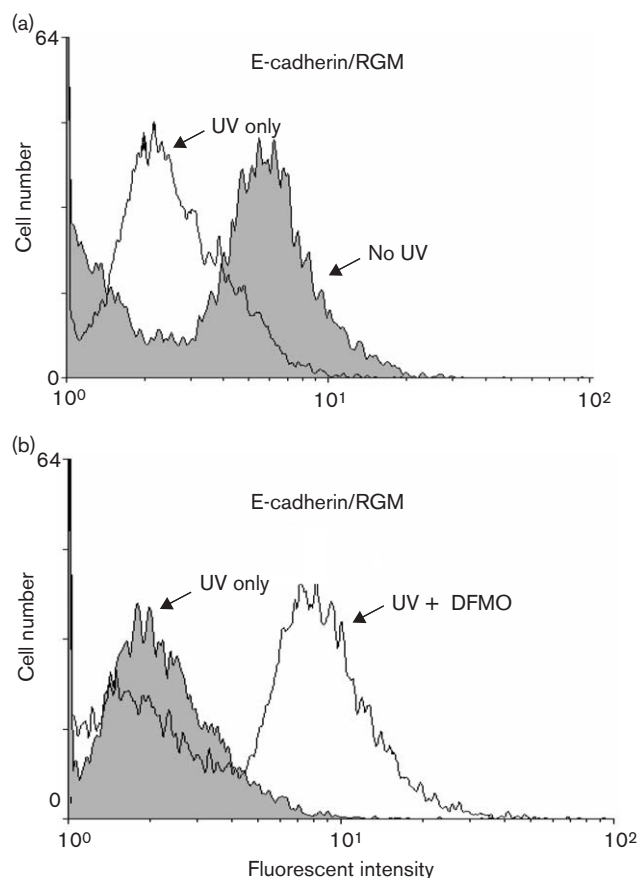
Fig. 1
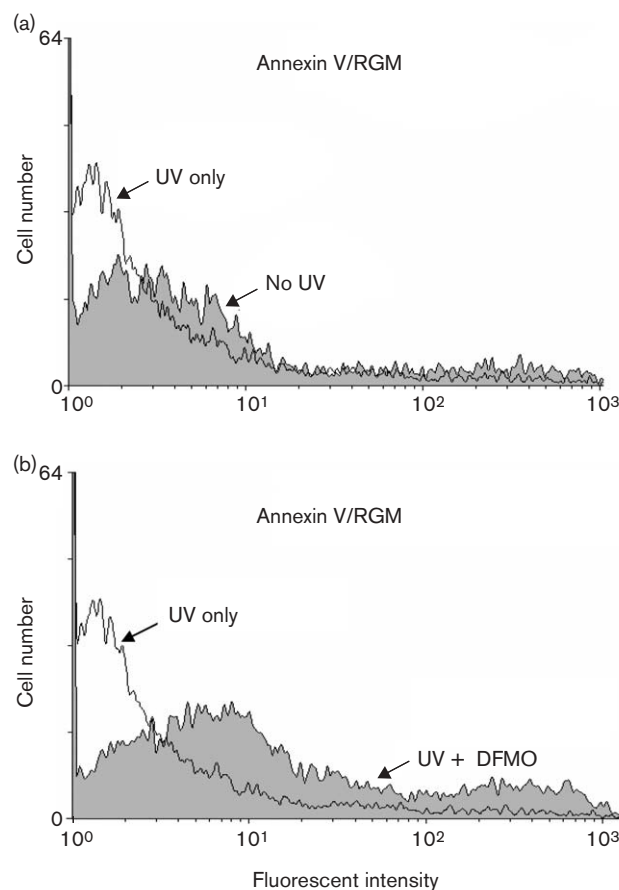
Examples of Annexin V expression in the WM2311 radial growth-phase-like melanoma (RGM) cell line. Comparison of flow cytometry histograms of annexin V expression: (a) control cultures [no ultraviolet (UV)] vs. UV-treated cell cultures; (b) UV-treated cell cultures vs. cultures treated with both UV and 5.5 µmol/l difluoromethylornithine (DFMO).
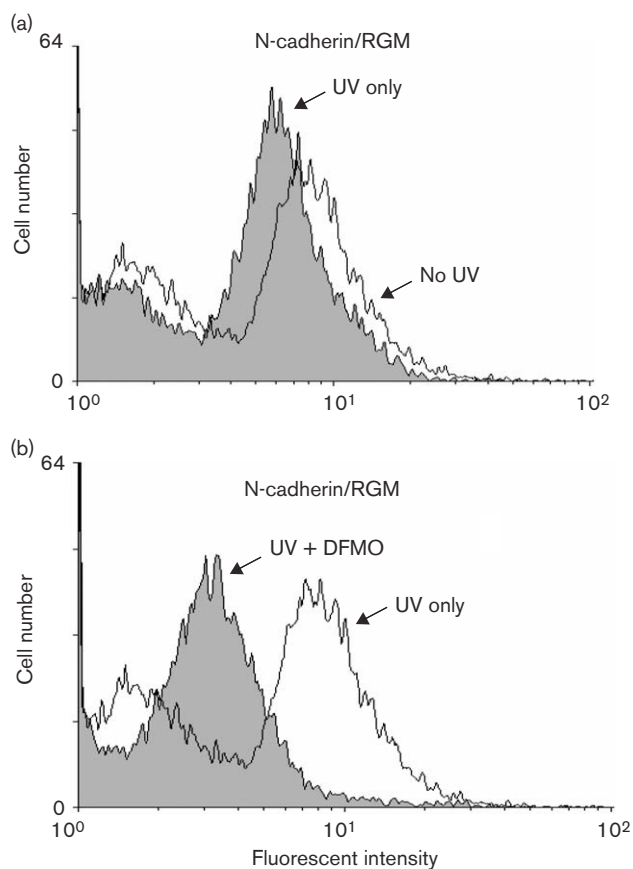
Fig. 2

Examples of E-cadherin expression in the WM2311 radial growth-phase-like melanoma (RGM) cell line. Comparison of flow cytometry histograms of E-cadherin expression: (a) control cultures [no ultraviolet (UV)] vs. UV-treated cell cultures; (b) UV-treated cell cultures vs. cultures treated with both UV and 16.5 µmol/l difluoromethylornithine (DFMO).

None of the agents evaluated induced E-cadherin in VGM cells. The lack of response in this cell line contrasts with the inhibition of E-cadherin in the RGM cell line.

Figure 4 compares examples of histograms that show the responses observed for N-cadherin in the VGM cells. UVB treatment resulted in only minor changes in N-cadherin expression (Fig. 4a). This response was similar to that observed with RGM cells (Fig. 2a). Figure 4b shows the inhibition of N-cadherin expression produced by DFMO at the lowest active concentration. Nimesulide, polyphenon E, 4HPR, and 9-*cis*-RA also inhibited N-cadherin expression.

Table 3 presents a summary of the data endpoints in which a positive response was observed in both RGM and VGM cells. Testing of the chemopreventive agents revealed that expression of annexin V showed some

response in RGM cells; however, HLA-DR showed little or no change in UVB-treated cells with or without the agent in any of the cell lines. On the other hand, significant changes were seen for E-cadherin and/or N-cadherin, in particular for the RGM and VGM cell lines. Table 3 also shows the percent inhibition of the N-cadherin/E-cadherin ratios for cells treated with UVB plus agent for both cell lines relative to the N-cadherin/E-cadherin ratios of the UVB only treated cultures.

For the RGM cells, all agents with known preventive activity in animals inhibited N-cadherin expression whereas agents with no known preventive activity *in vivo* did not. N-cadherin is preferentially expressed in melanoma whereas, with the exception of RGM cells, E-cadherin expression was reduced or lost. Therefore, N-cadherin expression in premetastatic melanoma cells could be considered an in-vitro biomarker for progression

Fig. 3

(a) N-cadherin/RGM — UV only, No UV

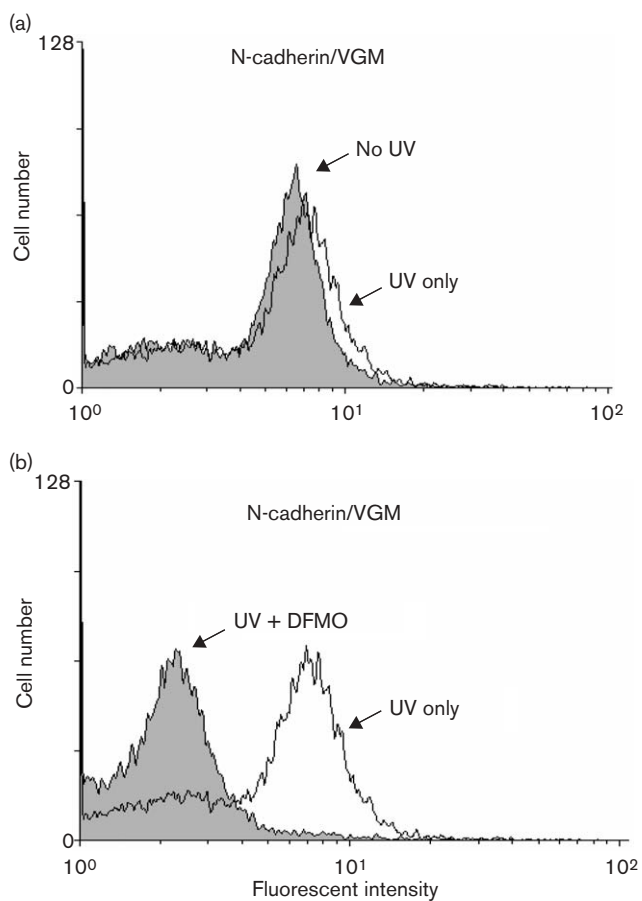(b) N-cadherin/RGM — UV + DFMO, UV only

Examples of N-cadherin expression in the WM2311 radial growth-phase-like melanoma (RGM) cell line. Comparison of flow cytometry histograms of N-cadherin expression: (a) control cultures [no ultraviolet (UV)] vs. UV-treated cell cultures; (b) UV-treated cell cultures vs. cultures treated with both UV and 5.5 µmol/l difluoromethylornithine (DFMO).

Fig. 4

(a) N-cadherin/VGM — No UV, UV only

(b) N-cadherin/VGM — UV + DFMO, UV only

Examples of N-cadherin expression in the Lu-1205 vertical growth-phase-like melanoma (VGM) cell line. Comparison of flow cytometry histograms of N-cadherin expression: (a) control cultures [no ultraviolet (UV)] vs. UV-treated cell cultures; and (b) UV-treated cell cultures vs. cultures treated with both UV and 5.5 µmol/l difluoromethylornithine (DFMO).

and its inhibition by a compound a favorable response. Furthermore, annexin V was induced by nimesulide, 4HPR, and DFMO in the RGM cells. The lack of induction of apoptosis at 48 h in melanocytes with induced apoptosis in RGM cells would suggest a differential effect on the RGM cells.

For the VGM cell line, polyphenon E, nimesulide, 9-*cis*-RA, and DFMO inhibited N-cadherin expression. In contrast to the RGM cells, annexin V was not induced by any agent in the VGM cells. None of the biomarkers were responsive to the tested compounds in the 83-2c melanoma cells.

As the expressions of both E-cadherin and N-cadherin have been shown to be involved in the control of differentiation and the progression to metastases, we compared the ratios of the mean immunofluorescence levels of N-cadherin/E-cadherin for different agent

concentrations. Figure 5 compares the normalized N-cadherin/E-cadherin ratios for all agents in RGM cells. The ratios are normalized relative to the N-cadherin/E-cadherin ratio of the UVB only treated RGM cells. The data suggest that DFMO was the most effective of the agents evaluated with a maximum inhibition of 67.6%. The agents, D-mannitol, diphenylhydramine, and NDGA, which have not shown preventive activity in animal studies, were all ineffective in lowering the N-cadherin/E-cadherin mean ratios. Although the lack of inhibition observed with the inactive agents was encouraging, we are uncertain as to the significance of the increases in N-cadherin/E-cadherin ratios observed with the other agents.

The data suggest that normal melanocytes, RGM cells, VGM cells, and 83-2c melanoma cells respond differently to the chemopreventive agents. The RGM cells were

Fig. 5
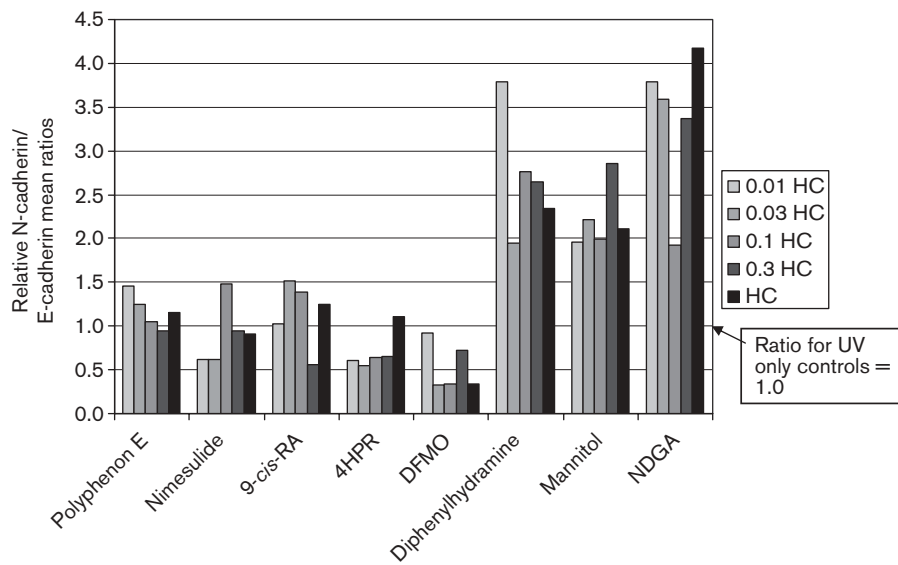
Comparative agent-induced N-cadherin/E-cadherin mean ratios of ultraviolet (UV) plus agent-treated cultures of radial growth-phase-like melanoma cells relative to the UV-alone treated cells. The highest concentration (HC) for each agent: polyphenon E – 100 µg/ml, nimesulide – 0.1 nmol/l, 9-*cis*-retinoic acid (9-*cis*-RA) – 6.66 µmol/l, 4-hydroxyphenylretinamide (4HPR) – 4.6 µmol/l, difluoromethylornithine (DFMO) – 550 µmol/l, diphenylhydramine – 0.034 µmol/l, mannitol – 549 µmol/l, and nordihydroguaiaretic acid (NDGA) – 0.41 µmol/l.

the most sensitive to chemopreventive agent-induced biomarker change. Both E-cadherin and N-cadherin were highly expressed in the RGM cell line. This confirms the finding of Hsu and co-workers [21] with this cell line. As E-cadherin plays an important role in the control of melanocyte growth in the skin via the interaction with keratinocytes [7,8], the observation that RGM cells overexpress E-cadherin suggests that keratinocytes play a key role in controlling their growth *in vivo*. The inhibition of E-cadherin expression following UV exposure is of potential significance to melanoma progression because loss of E-cadherin in the presence of a high N-cadherin expression level is associated with a more malignant phenotype. The induction of E-cadherin, if present *in vivo*, would increase the ability of the cells to maintain the normal controls between keratinocytes and melanocytes.

As there are no other in-vitro screening assays available for identifying a potential efficacy for melanoma prevention, it would be important to determine if this assay can predict which agents should have the better chance of preventing melanoma in humans. With this goal in mind, we have ranked the agents on the basis of the N-cadherin/E-cadherin ratios and their efficacy for specific endpoints. The rankings are: DFMO > 4HPR > nimesulide > 9-*cis*-RA > polyphenon E. Diphenylhydramine, D-mannitol, and NDGA were inactive.

Table 2 [22–28] compares the in-vitro agent concentrations that produced biomarker change and the clinical

plasma levels. Although the correlation of in-vitro efficacy and clinical efficacy remains to be shown, the finding that agent concentrations that showed efficacy in the assay are achievable *in vivo* is encouraging.

The results obtained indicated that RGM cells, and to some extent VGM cells, were responsive to treatment with known chemopreventive agents as ascertained by alteration of biomarker expression to levels associated with a less-malignant phenotype. Thus, this assay with the cadherin biomarkers can be considered as useful for identifying agents that may have efficacy against melanoma progression in these intermediate stages of melanoma. The biomarkers studied were not responsive in the more advanced melanoma cell strain. Additional studies will be needed to confirm the potential of the assay for identifying agents for preventing melanoma. The results of these initial studies suggest that UVB-treated RGM cells are the most responsive to chemopreventive agents. For future studies, we will initially use the WM3211 cell line for screening. In addition, we plan to evaluate other RGM melanoma cell lines to determine the universality of the response and to determine whether inclusion of other cell lines would add value to the assay.

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