Ingenol Mebutate Field-Directed Treatment of UVB-Damaged Skin Reduces Lesion Formation and Removes Mutant p53 Patches

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Skin cancer is the most prevalent cancer worldwide and is primarily caused by chronic UV exposure. Here, we describe the topical field-directed treatment of SKH1/hr mice with UVB-damaged skin with ingenol mebutate, a new topical drug shown to be effective for the treatment of actinic keratosis (AK). Application of 0.05% ingenol mebutate gel to photo-damaged skin resulted in a ≈70% reduction in the number of skin lesions that subsequently emerged compared with placebo treatment. Ingenol mebutate treatment also reduced the number of mutant p53 keratinocyte patches by ≈70%. The treatment resulted in epidermal cell death, acute inflammation, recruitment of neutrophils, hemorrhage, and eschar formation, all of which resolved over several weeks. Ingenol mebutate field-directed treatment might thus find utility in the removal of subclinical precancerous cells from UV-damaged skin. Field-directed treatment may be particularly suitable for patients who have AKs surrounded by UV-damaged skin.

Journal of Investigative Dermatology (2012) **132,** 1263–1271; doi:10.1038/jid.2011.418; published online 22 December 2011

INTRODUCTION

Non-melanoma skin cancers (NMSCs) are the most common cancers worldwide, with the incidence increasing by 3–8% annually (Madan *et al.*, 2010; Rogers *et al.*, 2010). The direct cost of treating NMSC in the USA in 2004 was estimated to be US$ 1.4 billion (The Lewin Group, 2005) with over 2 million patients treated in 2006 (American Cancer Society, 2010). UV radiation, usually from sunlight, is the most important risk factor for skin cancer; however, despite increased public awareness of the dangers of sun exposure, the incidence continues to rise (Madan *et al.*, 2010; Rogers *et al.*, 2010). The reasons for this increase likely include better detection of the disease, the aging population (Madan *et al.*, 2010), and increasing UV radiation due to ozone depletion (Norval *et al.*, 2007; Chang *et al.*, 2010). Sunlight, particularly UVB, is able to act both as a tumor initiator and a tumor promoter, with UVB able to damage DNA directly (Ziegler *et al.*, 1994; Rass and Reichrath, 2008; Madan *et al.*, 2010).

Mutations of the p53 tumor suppressor gene are particularly common in skin cancers, and are considered to be an important early event in skin cancer oncogenesis (Rebel *et al.*, 2005; Benjamin *et al.*, 2008; de Gruijl and Rebel, 2008). Chronic UV exposure results in the accumulation in the skin of histologically detectable ''p53 patches'', which are clonal outgrowths of keratinocytes with elevated nuclear expression of mutated p53 (Berg *et al.*, 1996; Rebel *et al.*, 2001, 2005). A small number of these mutant p53 patches may progress to actinic keratosis (AK; Einspahr *et al.*, 1999) and ultimately NMSC, with further mutations, UV-induced immunosuppression, and/or human papilloma virus infection contributing to cancer formation (Hart *et al.*, 2001; Rebel *et al.*, 2005; Byrne *et al.*, 2008; Murphy, 2009; Madan *et al.*, 2010). Although a number of factors can influence the propensity of these mutated keratinocytes to develop into NMSC (Madan *et al.*, 2010), these UV-mutated cells appear to be a prerequisite for the development of most NMSC (Ananthaswamy *et al.*, 1998; Benjamin *et al.*, 2008; Rass and Reichrath, 2008). Consistent with this view is that prophylactic treatments, such as sunscreen application, that reduce the number of p53 patches, also reduce the number of skin cancers that subsequently develop (Ananthaswamy *et al.*, 2002; Conney *et al.*, 2008).

Ingenol mebutate is currently being developed as a new topical antineoplastic drug, which has been shown to be effective and well tolerated in the treatment of AKs and NMSC (Anderson *et al.*, 2009; Siller *et al.*, 2009, 2010).

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Abbreviations: AK, actinic keratose; NMSC, non-melanoma skin cancer

Received 24 May 2011; revised 29 September 2011; accepted 20 October 2011; published online 22 December 2011

Preclinical studies have shown that the mechanism of action involves the induction of primary necrosis in tumor cells, disruption of tumor vasculature, transient inflammation, and recruitment of neutrophils, with treatment resulting in a favorable cosmetic outcome (Challacombe *et al.*, 2006; Ogbourne *et al.*, 2007; Li *et al.*, 2010). Here we show, using the hairless SKH-1/hr mouse model, that field-directed treatment (Berman *et al.*, 2009; Jorizzo *et al.*, 2010; Feldman and Fleischer, 2011) of precancerous chronically UVB-irradiated skin with ingenol mebutate significantly reduced the number of skin lesions that subsequently developed. Ingenol mebutate treatment also significantly reduced the number of mutant p53 patches present in the skin. This study thus illustrates that ingenol mebutate may find utility for the field-directed treatment of UV-damaged skin to reduce the emergence of AKs and NMSC. Such treatment may be particularly suitable for patients with multiple or recurring AKs (Berman *et al.*, 2009; Jorizzo *et al.*, 2010; Feldman and Fleischer, 2011) and/or patients who are at high risk of developing NMSC (Marcil and Stern, 2000).

RESULTS

Field-directed treatment of UVB-damaged skin with 0.05% ingenol mebutate gel reduced the emergence of UVB-induced skin lesions

SKH1/hr mice were exposed to 1.25 minimal erythemal dose UVB three times per week over 10–11 weeks for a total of 30 doses. The day after the final irradiation dose, the dorsum of each mice was tattooed with rectangles to demarcate the treatment areas. Mice were randomly assigned into three groups. The first group was treated topically, daily for 2 days with ~100 µl of 0.05% ingenol mebutate gel within the tattooed areas (Figure 1a, Ing. meb.). The second group was treated with placebo gel within the tattooed areas (Figure 1a, placebo). The third group remained untreated (Figure 1a, control). The mice were examined weekly for 21 weeks for the development of UV-induced skin lesions. Ingenol mebutate gel field–directed treatment resulted in a significant 60–70% reduction in the number of skin lesions per cm^2 within the treatment areas that emerged over time compared with placebo treatment and the controls (Figure 1a). At all time points after 12 weeks, the ingenol mebutate group showed significantly fewer lesions than the other two groups ($P<0.05$, Mann–Whitney *U*-test). As the treatment areas encompassed much of the UVB-damaged skin of the mouse, this also resulted in a ≈70% reduction in the total number of lesions per mouse (Figure 1b). The slight increase in the number of lesions seen with placebo gel–treated group compared with controls (Figure 1a and b) may be because of mice scratching the treatment site, with skin wounding or abrasion known to promote epidermal carcinogenesis (Argyris, 1985).

The number of skin lesions outside the treatment areas was not significantly affected (Figure 1c), suggesting that the ingenol mebutate treatment did not induce effective systemic immunity against keratinocytes overexpressing mutated p53 (Le *et al.*, 2009). This is consistent with the view that mutant p53 patches are not under immune control (Remenyik *et al.*, 2003; de Graaf *et al.*, 2008).

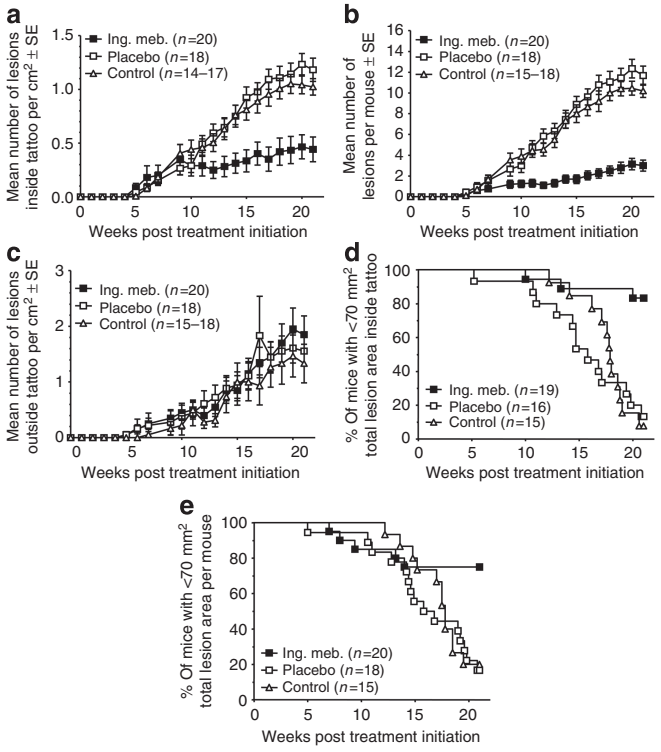

Figure 1. Development of UVB-induced lesions after treatment of UVB-irradiated skin with ingenol mebutate. After 30 doses of UVB, SKH1/hr mice were treated daily for 2 days with 0.05% ingenol mebutate gel (Ing. meb.) or placebo gel (Placebo) or were left untreated (Control). (**a**) The mean number of dorsal lesions per mouse per cm^2 within the treatment area over time; *n* = the number of mice. (**b**) The mean number of dorsal lesions per mouse over time. (**c**) The mean number of dorsal lesions outside the tattooed areas per mouse. (**d**) Kaplan–Meier curves showing the percentage of mice with a total lesion area within the treatment area of $<70\,mm^2$. (**e**) Kaplan–Meier curves showing the percentage of mice with a total lesion area of $<70\,mm^2$; (see Supplemental material online for mouse number details).

To analyze the effect of 0.05% ingenol mebutate gel treatment on tumor burden, Kaplan–Meier curves were generated by analyzing the total lesion area within the treatment area (Figure 1d) and the total dorsal lesion area per mouse over time (Figure 1e). For both analyses, an event was assigned when the total lesion area reached $70\,mm^2$. Both analyses showed that 0.05% ingenol mebutate gel treatment led to a significant reduction in the number of mice that had a total lesion area of $70\,mm^2$ when compared with placebo gel–treated or control mice (Figure 1d and e; ingenol mebutate vs placebo; log rank statistic; $P<0.001$ and $P<0.002$, respectively). Forty-five percent of mice treated with ingenol mebutate actually had no lesions within the treatment areas, whereas placebo and control groups all had at least five lesions at week 21 (data not shown). Thus, both the number of lesions and the lesion burden (as determined by total lesion area) were significantly reduced by field-directed treatment with 0.05% ingenol mebutate gel.

Growth rates, size distribution, and histology of lesions emerging after ingenol mebutate treatment were no different from those in control animals

To illustrate that ingenol mebutate treatment did not select for the emergence of more aggressive lesions, the growth rates of individual lesions within the treatment areas were examined for each group. The area of each individual lesion was plotted over time with week 0 assigned as the week before an individual lesion was identified (irrespective of the time of emergence of the lesion relative to the time of treatment; Figure 2a). No lesion in the ingenol mebutate–treated group grew faster than any lesion in the placebo group (Figure 2a). Five placebo mice with the large tumors (Figure 2a) were euthanized before week 21 because of an excessive overall tumor burden. The two larger tumors in the ingenol mebutate group (Figure 2a) grew larger only because an excessive overall tumor burden was not reached before week 21.

Analysis of the mean lesion growth rates also showed that there was no significant difference between control, placebo, and ingenol mebutate gel–treated mice (Figure 2b; the error bars for the mean area of lesions in the ingenol mebutate treatment group are larger as the number of lesions was lower).

The size distribution of lesions within the treatment area was analyzed to determine whether the lesions that remained 21 weeks after ingenol mebutate treatment showed any changes in size profile. Although more lesions were present in control and placebo gel–treated mice compared with ingenol mebutate–treated mice, a similar lesion size distribution profile was present in all three groups (Figure 2c).

Histological analysis of lesions from all three groups showed that lesions were of epidermal origin and resembled the classical picture described previously for lesions in this model (Kligman and Kligman, 1981). No differences in the histology were observed for lesions that emerged after ingenol mebutate treatment and those in the placebo or control groups (data not shown).

These data illustrate that treatment with ingenol mebutate gel did not result in, or select for, the emergence of more aggressive, or larger, or histologically distinct tumors.

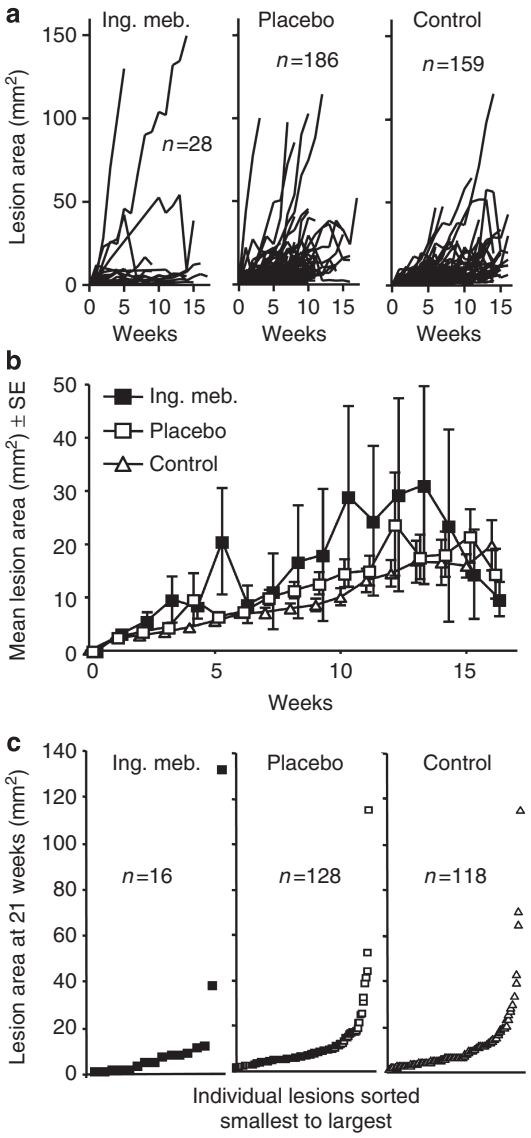

Figure 2. Growth and size profiles of UVB-induced skin lesions after ingenol mebutate (Ing. meb.) treatment. (**a**) Growth curves of individual lesions arising in ingenol mebutate- and placebo-treated mice, and control mice. The size of individual lesions within the tattoo areas from the mice described in Figure 1 were measured over time, with week 0 designated as the week before an individual lesion became apparent. (**b**) The mean size of the lesions shown in **a** over time. (**c**) The size profile of the lesions within the treatment areas in ingenol mebutate- and placebo-treated mice, and control mice at 21 weeks post treatment initiation. Lesions were sorted from the smallest to the largest area, with each lesion represented by one data point. The large tumor in **a** (Ing meb.) was not included, as at week 21 it had coalesced with another tumor, making size determination difficult.

Skin appearance after ingenol mebutate treatment of UVB-damaged and normal skin

Treatment with 0.05% ingenol mebutate gel of chronically UVB-irradiated skin caused reddening of the skin, visible within 1 hour of treatment, with erythema increasing with time (Figure 3a). By 48 hours (24 hours after the second dose), eschar formation had begun and by days 5–9 it had encompassed the treatment area. The eschar resolved after 3–4 weeks, leaving a pink treatment area (Figure 3a), which lightened over subsequent weeks.

Treatment of normal skin (with no UV exposure) with 0.05% ingenol mebutate ($n=5$ mice) appeared to result in less reddening of the skin at 24–48 hours, although eschar formation and skin contraction appeared to be similar (Figure 3b).

At 21 weeks after ingenol mebutate treatment of UVB-damaged skin, the number of lesions within the treatment areas was clearly lower, when compared with placebo and control mice (Figure 3c). The mean skin area within the ingenol mebutate treatment area (demarcated by tattoo) had also reduced by ≈3-fold (Figure 3c); this was also apparent at day 29 (Figure 3a and b). This contraction resulted in skin surrounding the treatment site being pulled toward the treatment site (data not shown). At week 21, after ingenol mebutate treatment, the treated skin felt and appeared largely

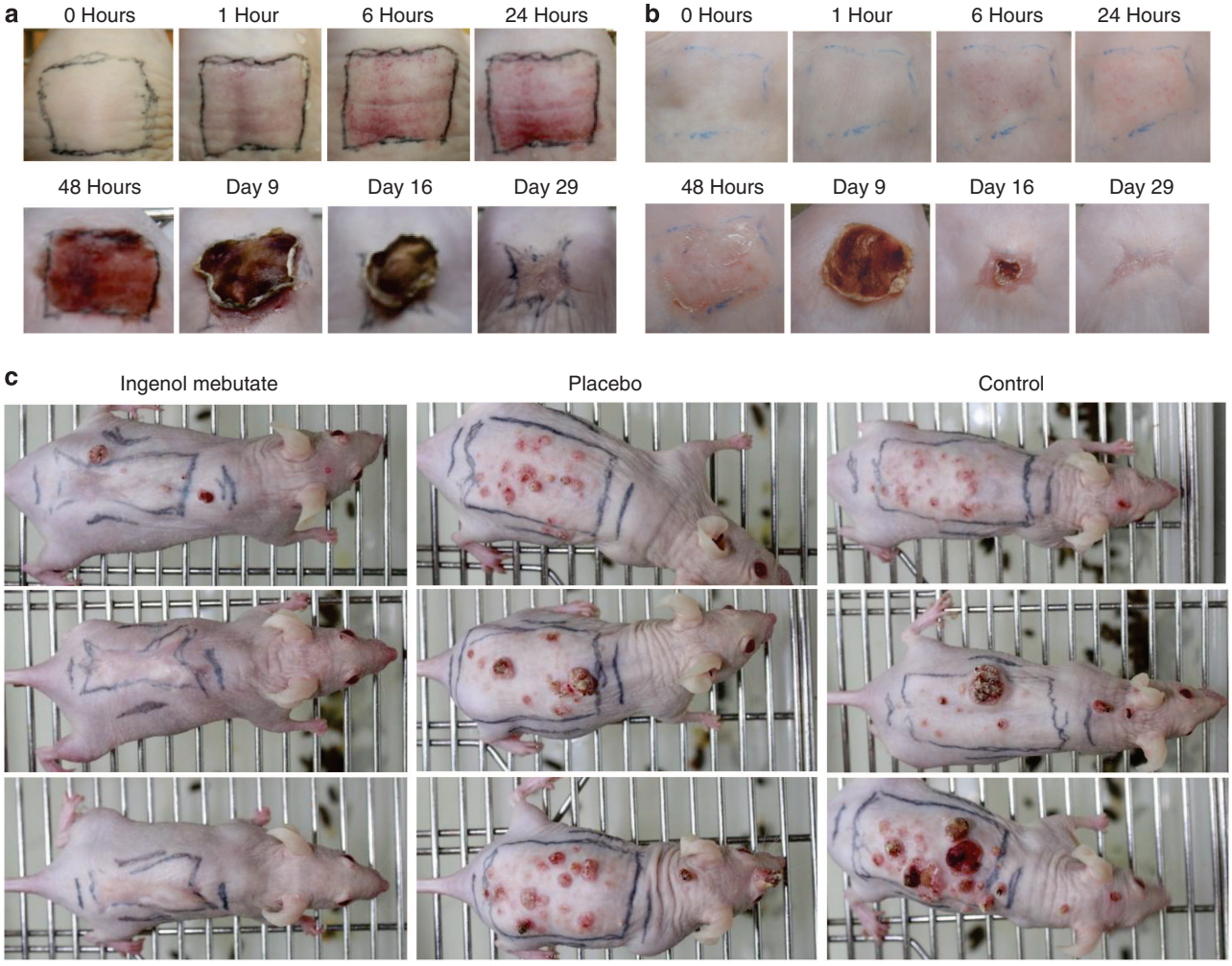

Figure 3. Skin appearance after ingenol mebutate treatment. (**a**) Photographs of UVB-irradiated skin within the tattooed areas at the indicated times after treatment initiation with 0.05% ingenol mebutate gel (48 hours represents 24 hours after the second treatment). (**b**) As in **a**, except that normal skin was treated. (**c**) Photographs of representative mice from ingenol mebutate and placebo treatment, and control groups taken 21 weeks post treatment initiation.

normal, although slightly darker pink irregular markings remained within the treatment areas (Figure 3c, ingenol mebutate).

Skin histology after ingenol mebutate treatment of UVB-damaged skin

Histology of placebo-treated skin showed the expected epidermal thickening that arises after chronic UVB irradiation (Chaquour *et al.*, 1995) when comparing normal skin (Figure 4a) with placebo (Figure 4b). Six hours after the first treatment with 0.05% ingenol mebutate gel, disruption of the basal epidermis was evident when comparing placebo (Figure 4b) with ingenol mebutate (Figure 4c). Higher magnifications showed ballooning degeneration of basal keratinocytes when comparing placebo (Figure 4f) with ingenol mebutate (Figure 4g). By 24 hours post ingenol mebutate treatment, extensive cellular infiltrates (predominant neutrophils) could be seen in the dermis, and extensive areas of epidermal keratinocytes showed hyper eosinophilic staining (Figure 4d). (This loss of blue basophilic hematoxylin staining in the cytoplasm is usually associated with the loss of ribosomal RNA with a consolidation of cytoplasmic components as the cell collapses.) Higher magnification of the epidermis showed keratinocytes with consistent pyknotic nuclei and widespread coagulative necrosis (Figure 4h). These features are consistent with acute primary necrosis of keratinocytes (Haschek *et al.*, 2009), and illustrated that ingenol mebutate treatment had killed most of the epidermal keratinocytes. At 48 hours (24 hours after second treatment), the epidermis was absent in many places and was replaced by an eschar (Figure 4e). Areas containing extensive cellular infiltrates were evident throughout the dermis (Figure 4e), and at higher magnifications these areas showed pyodermatitis, predominantly composed of red blood cells and degenerating neutrophils (Figure 4i), consistent with previous observations (Challacombe *et al.*, 2006). Healthy (hematoxylin staining) keratinocytes were clearly present in hair follicles and appeared, in several places, to be reconstituting the epidermis (Figure 4e and j). Strong Ki67 staining was apparent in the hair follicles and the epidermis at this time (Figure 4k), illustrating

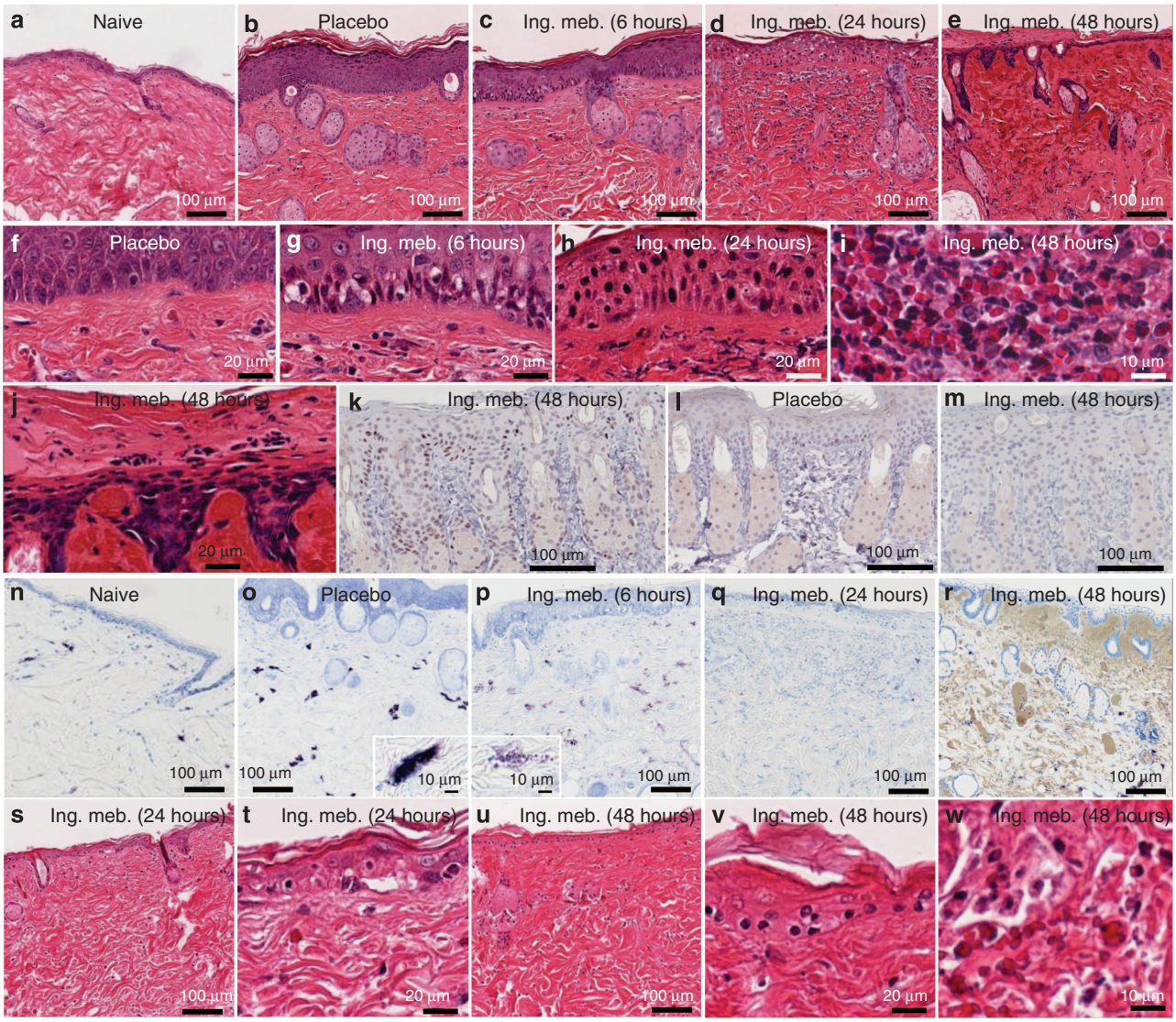

Figure 4. Histology of UVB-irradiated skin treated with 0.05% ingenol mebutate (Ing. meb.) gel. (**a–i**) Hematoxylin and eosin staining. (**a**) The skin of naive mice. (**b**) The skin of mice irradiated with UVB as described above and treated with placebo gel. (**c**) UVB-irradiated skin 6 hours after treatment with 0.05% ingenol mebutate gel. (**d**) UVB-irradiated skin 24 hours after treatment with 0.05% Ing. meb gel. (**e**) UVB-irradiated skin 48 hours after the first treatment with 0.05% ingenol mebutate gel. (**f**) High magnification of skin treated as in **b**. (**g**) High magnification of skin treated as in **c**. (**h**) High magnification of skin treated as in **d**. (**i**, **j**) High magnifications of dermis and epidermis of mice treated as in **e**. (**k**) Ki67 staining of skin treated as in **e**. (**l**) Ki67 staining of skin treated as in **b**. (**m**) Control antibody staining of skin treated as in **e**. (**n–r**) Toluidine blue staining of skin treated as in **a–e**; insets for **o** and **p** show high magnifications of individual toluidine blue–staining cells. (**s–w**) Treatment of normal skin with 0.05% ingenol mebutate; (**s**, **t**) 24 hours after treatment, (**u–w**) 48 h after treatment.

keratinocyte replication and suggesting keratinocyte emergence from hair follicles leading to re-epithelialization of the skin (Lau *et al.*, 2009). Ki67 staining was less apparent in placebo gel–treated skin (Figure 4l) and control staining of ingenol mebutate–treated skin was negative (Figure 4m). At 21 weeks post treatment, the lesion-free epidermis in ingenol mebutate–treated skin was indistinguishable by hematoxylin and eosin staining from normal skin (data not shown).

Toluidine blue staining of skin from naive (Figure 4n) and placebo gel–treated (Figure 4o) mice showed the increased number of dermal mast cells usually found in chronically UV-irradiated skin in mice and humans (Kligman and Murphy, 1996; Gonzalez *et al.*, 1999; Bosset *et al.*, 2003). Six hours post ingenol mebutate treatment, the mast cell granules, which are stained pink–purple by toluidine blue, appeared more dispersed, suggesting that the mast cells were either dying and/or degranulating (Figure 4p). At 24 hours, few toluidine blue–staining cells could be seen (Figure 4q). At 48 hours after treatment initiation, toluidine blue–staining cells could again be seen; however, the dominant feature was the extensive red/brown pigment throughout the dermis, indicating widespread hemorrhage (Figure 4r). At 21 weeks post treatment, the number of dermal toluidine blue–staining cells was similar in naive, placebo, and ingenol mebutate–treated groups, and no signs of hemorrhage were evident (data not shown).

Skin histology after ingenol mebutate treatment of normal skin
Treatment of normal skin with 0.05% ingenol mebutate resulted in similar, although delayed, changes to the epidermis compared with treatment of UV-irradiated skin. Ballooning degeneration of keratinocytes was observed at 24 hours (Figure 4s and t), and extensive coagulative necrosis and hyper eosinophilic staining were not seen until 48 hours after treatment initiation (Figure 4u and v). In addition, the extent of hemorrhage and neutrophil infiltration at 48 h (Figure 4w) was less than that seen for UV-irradiated skin. These histological findings are consistent with the reduced reaction seen on normal skin (Figure 3b). However, the similar eschar formation (Figure 3b) is consistent with the observation that in both UV-irradiated (Figure 4h) and normal skin (Figure 4v) epidermal keratinocytes were killed by the ingenol mebutate treatment.

Field-directed treatment of UVB-irradiated skin with ingenol mebutate reduced the number of mutant p53 patches
Chronic UVB irradiation results in the accumulation of mutant p53 epidermal patches, which are believed to be prerequisite precursors of UVB-induced skin lesions (Berg *et al.*, 1996; Rebel *et al.*, 2001, 2005). To ascertain whether post-UVB ingenol mebutate field-directed treatment affects p53 patch numbers, SKH1/hr mice were exposed to 1.25 minimal erythemal dose three times a week for 11–12 weeks. Mice were then divided into three groups, one was treated on the dorsum with 0.05% ingenol mebutate gel, one was treated with placebo gel, with the third group left untreated (control). After the healing process was complete in the ingenol mebutate treatment group, the mice from all the groups were euthanized and the epidermis within the treatment areas were analyzed for the presence of p53 patches using immunohistochemistry. An example of a typical mutant p53 patch at low and high magnifications is shown in Figure 5a.

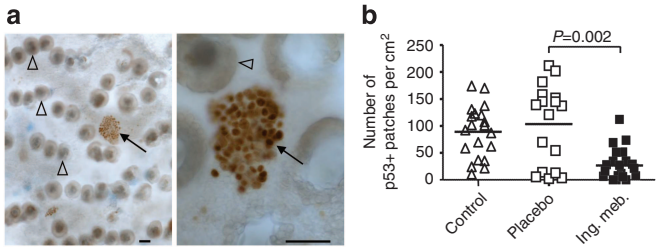

Figure 5. Ingenol mebutate (Ing. meb.) field-directed treatment reduced the number of mutant p53 patches. After 30 doses of UVB, SKH1/hr mice were left untreated (Control), or were treated with placebo or 0.05% ingenol mebutate (days 0 and 1). Four or five weeks after treatment, the epidermis was analyzed for p53 patches by immunohistochemistry. (**a**) Representative photographs of a p53 patch in a control animal (arrows). The epidermis was mounted basal side up and viewed from the top. White arrowheads show hair follicles. Bars = 100 μm. (**b**) The number of p53 patches per cm^2 in control UVB-irradiated untreated mice ($n = 20$), and UVB-irradiated mice treated with placebo gel (Placebo; $n = 17$) or Ing. meb. ($n = 22$). The data shown were generated in two independent experiments; each individual experiment showed significant differences (data not shown). Bars represent the mean (statistical analysis by Mann–Whitney *U*-test).

Enumeration of the number of p53 patches showed that chronically irradiated skin in the absence of treatment had 89 ± 10.8 (SE) p53 patches per cm^2 (Figure 5b, control), whereas unirradiated skin from the underbelly of the same mice showed no detectable p53 patches (data not shown). Placebo gel treatment had no significant effect on p53 patch density (Figure 5b, placebo) with 103.3 ± 18.1 (SE) p53 patches per cm^2 (Figure 5b, placebo). In contrast, treatment with 0.05% ingenol mebutate gel resulted in a significant ≈70% reduction ($P = 0.002$) in the number of p53 patches per cm^2 (26.8 ± 6.3 SE) within the treatment areas (Figure 5b, Ing. meb.). A parallel experiment using a 0.01% ingenol mebutate had no significant effect on the number of p53 patches (data not shown).

DISCUSSION

Herein, we show that field-directed treatment of chronically UVB-irradiated skin with 0.05% ingenol mebutate gel resulted in a ≈70% reduction in the number of skin lesions that emerged over time. Ingenol mebutate field-directed treatment caused loss of the epidermis, which appeared to be followed by rapid re-epithelization from hair follicles. The treatment also caused a significant reduction in the number of mutant p53 patches in the newly formed epidermis, suggesting removal of replication-competent mutant p53–expressing keratinocytes that give rise to these patches. These experiments highlight the potential for the use of ingenol mebutate as a field-directed treatment for chronically UVB-damaged skin to prevent the development of AKs and NMSC.

Ingenol mebutate field-directed treatment may be particularly suitable for individuals who have a history of UV exposure and are at high risk of developing further NMSC (Jonason *et al.*, 1996; Marcil and Stern, 2000; Bailey *et al.*, 2010). In humans, chronic UV exposure results in the accumulation of a substantial burden of premalignant keratinocytes (detectable as p53 patches) from which AKs (Einspahr *et al.*, 1999) and NMSC are able to develop (Jonason *et al.*, 1996; Feldman and Fleischer, 2011). An individual's risk of developing NMSC depends on a number of environmental and genetic factors (Madan *et al.*, 2010). However, meta-analysis has shown that patients who develop one NMSC have at least 10-fold higher probability of developing another NMSC within 3 years when compared with the general population (Marcil and Stern, 2000). Such patients currently have limited treatment options to reduce their NMSC risk (Bailey *et al.*, 2010) and currently rely on continued monitoring for, and treatment of, newly emerging AKs and NMSC (Cohen, 2010). A recent development is the notion of field-directed treatment, which seeks to remove both AKs and any surrounding subclinical lesions (Feldman and Fleischer, 2011). A phase III human trial of 0.05% ingenol mebutate treatment of 25 cm^2 areas containing AKs was recently completed and showed a 69% reduction in AK lesion count (Swanson, 2011). The current paper provides the first evidence that such field-directed treatments may also reduce the subsequent emergence of AKs and squamous cell carcinomas from the treatment area by removing keratinocytes with malignant potential.

Field-directed treatments of 25 cm^2 (Swanson, 2011) and up to 100 cm^2 areas (Schmieder, 2010) with ingenol mebutate in human trials were generally well tolerated. Importantly, skin contractions, like those seen in the mice (Figure 3), were not observed, suggesting that dermal hemorrhage and disruption is less pronounced in humans. Mouse and human skin differ in a number of aspects, and thus responses to ingenol mebutate are likely to differ. Whether the pronounced hemorrhage, erythema, and eschar formation seen in the mouse are required for the removal of mutant p53 patches in humans is unclear. However, clinical trial experience (Anderson *et al.*, 2009; Siller *et al.*, 2009; Swanson, 2011) suggests that effective removal of abnormal epidermal keratinocytes (in the form of AKs) can be achieved without such robust responses.

The treatment of normal skin (that had received no UVB irradiation) with 0.05% ingenol mebutate resulted in less erythema at 24–48 hours. The reason for this is unclear. Normal skin may be less permeable to ingenol mebutate than UVB-damaged skin (Wang *et al.*, 2010). Expression of the epidermal multidrug transporter (Li *et al.*, 2010) may be higher in UV-irradiated skin (Uchiumi *et al.*, 1993). Degranulation of mast cells, which are elevated in chronically UVB-irradiated skin, may also be involved (Chai *et al.*, 2011).

Mast cells are believed to have important roles in UV-induced immunosuppression and the development of NMSC (Andreu *et al.*, 2010; Chacon-Salinas *et al.*, 2011). Whether the ingenol mebutate–induced degranulation or the removal of mast cells contributed to the reduction in skin lesion development (Figure 1) remains unclear. However, the effect appeared to be transient with mast cell granule staining returning at 48 hours post treatment initiation (Figure 4r). Even if ingenol mebutate–induced degranulation did transiently reduce mast cell–mediated immunosuppression (de Vries *et al.*, 2009), the effect on subsequent lesion emergence in the current model would likely be minimal as precancerous keratinocytes are believed not to be controlled by the immune system (Remenyik *et al.*, 2003; de Graaf *et al.*, 2008).

Recently, chemical peels were also shown to reduce p53 patch numbers and lesion formation in chronically UVB-irradiated mice (Abdel-Daim *et al.*, 2010). However, this involved 10 treatments (1 treatment every 2 weeks), whereas a similar effect was achieved herein with only two ingenol mebutate treatments (once daily for 2 days). In addition, chemical peel treatments continued while lesions and tumors were developing (Abdel-Daim *et al.*, 2010), a scenario unlikely to be countenanced in humans. The chemical peel strategy did, however, result in reductions in lesions developing outside the treatment areas (Abdel-Daim *et al.*, 2010), something that was not observed for ingenol mebutate field-directed treatment (Figure 1c). Treatment of lesions with chemical peels may result in the production of systemic antitumor immunity that can regresses distant lesions. We have shown, for instance, that treatment of tumors with ingenol mebutate results in the induction of anticancer CD8 T-cell immunity, which was capable of regressing distant tumors (Le *et al.*, 2009). Herein, ingenol mebutate field-directed treatment was applied several weeks before skin lesions were visible, therefore significant induction of antitumor immunity is unlikely to have occurred. The ingenol mebutate field-directed treatment described herein may expose the immune system to antigens present in p53 patches. However, several groups have shown that p53 patches are not regressed by immune-based mechanisms (Remenyik *et al.*, 2003; de Graaf *et al.*, 2008), therefore such immunity, even if induced, would have limited effect on p53 patches and subsequent lesion development.

Imiquimod has also been used to reduce p53 expression in UV-damaged skin in humans, although the drug was applied three times a week for 4 weeks and the implications for the subsequent development of NMSC and AKs development was not reported (Smith *et al.*, 2007). Oral retinoids have also been reported as being effective in reducing NMSC in high-risk patients. However, treatment may need to be lifelong and retinoids have the potential to produce adverse effects (Hardin and Mydlarski, 2010; Marquez *et al.*, 2010). Herein, we show that only two treatments over 2 days with ingenol mebutate were sufficient to reduce mutant p53 patch density and lesion formation.

MATERIALS AND METHODS

The SKH1/hr model

Outbred SKH1/hr mice were obtained from Charles River Laboratories (Wilmington, NC) and a breeding colony for outbred SKH1/hr mice was established at Queensland Institute of Medical Research (QIMR). Male SKH1 mice were used for this study. To prevent any fighting and injury (which promotes tumors), two mice were kept per cage, separated by a physical barrier. All animal experiments were approved by the QIMR Animal Ethics Committee.

UVB irradiation

Outbred SKH1/hr mice were irradiated three times per week for 10–11 weeks with 1.25 times the minimal erythemal dose of UVB at each exposure (Rebel *et al.*, 2001). For details see Supplementary material online.

Ingenol mebutate. Isopropanol-based gel containing ingenol mebutate (>95% pure) was supplied by Peplin Ltd (Brisbane, Queensland, Australia).

Immunohistochemistry and histology

One to three days after discontinuation of UVB irradiation, a ≈4 cm^2 dorsal area was treated topically daily for 2 days with 40 μl of 0.05% (w/v) ingenol mebutate gel or placebo gel. The treatment area was demarcated by a tattoo. An untreated group served as an additional control. Once the treated area had healed (4–5 weeks), the mice were euthanized for analysis of mutant p53 patches. The treated skin areas were surgically excised and the epidermis was stained with a mutant-specific anti-p53 antibody. For details see Supplemental material online.

For Ki67 staining, paraffin sections were treated with Target Retrieval Solution of pH6 (Dako Australia, Noble Park, Victoria, Australia) and Ki67 stained with rat anti-mouse Ki67 (TEC-3; Dako). Detection was by horseradish peroxidase as above.

For histological studies, samples were fixed in 10% formalin and processed for paraffin embedding at the indicated times after ingenol mebutate treatment. Paraffin sections were stained with hematoxylin and eosin or toluidine blue using standard protocols.

Ingenol mebutate treatment and lesion monitoring

One to three days after discontinuation of UVB irradiation, $\sim 10\,cm^2$ rectangular areas (one per mouse) located centrally on the dorsa and previously demarcated by tattoos were treated topically, daily for 2 days with 100 µl of 0.05% ingenol mebutate gel or 100 µl of placebo gel. An untreated group served as an additional control. The mice were monitored weekly for the development of lesions both within and outside the treatment areas. Lesions arising on the tattooed lines were considered outside the treatment area. The number and size of lesions were monitored over time. All visible lesions were counted. Lesions <2 mm were mostly AKs, whereas lesions >3 mm were mostly squamous cell carcinomas (de Gruijl, 2008). The mice were euthanized at the end of the study or earlier in cases where total lesion area was excessive or other welfare issues required euthanasia.

Data analysis

Statistical analysis was carried out using Graphpad Prism version 5.03. (Graphpad Software, San Diego, CA, http://www.graphpad.com) or SPSS for Windows (version 15.0, 2007; SPSS, Chicago, IL).

CONFLICT OF INTEREST

S-J Cozzi is now an employee of LEO Pharma Pty Ltd, Australia. LEO Pharma acquired Peplin Ltd in 2009. S Ogbourne was an employee of Peplin Ltd. A Suhrbier was a paid consultant for Peplin Ltd and is currently a paid consultant for LEO Pharma.

ACKNOWLEDGMENTS

S-J C was funded by an NHMRC Industry Fellowship from the National Health and Medical Research Council (NHMRC), Australia. AS is a Principle Research Fellow with the NHMRC. The research was conducted at Queensland Institute of Medical Research with consumables and research assistants funded by Peplin Ltd.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Abdel-Daim M, Funasaka Y, Kamo T *et al.* (2010) Preventive effect of chemical peeling on ultraviolet induced skin tumor formation. *J Dermatol Sci* 60:21–8

American Cancer Society (2010) *Cancer Facts and Figures 2010*. American Cancer Society, Atlanta, USA

Ananthaswamy HN, Loughlin SM, Ullrich SE *et al.* (1998) Inhibition of UV-induced p53 mutations by sunscreens: implications for skin cancer prevention. *J Investig Dermatol Symp Proc* 3:52–6

Ananthaswamy HN, Ullrich SE, Kripke ML (2002) Inhibition of UV-induced p53 mutations and skin cancers by sunscreens: implication for skin cancer prevention. *Exp Dermatol* 11(Suppl 1):40–3

Anderson L, Schmieder GJ, Werschler WP *et al.* (2009) Randomized, double-blind, double-dummy, vehicle-controlled study of ingenol mebutate gel 0.025% and 0.05% for actinic keratosis. *J Am Acad Dermatol* 60:934–43

Andreu P, Johansson M, Affara NI *et al.* (2010) FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* 17: 121–34

Argyris TS (1985) Regeneration and the mechanism of epidermal tumor promotion. *Crit Rev Toxicol* 14:211–58

Bailey HH, Kim K, Verma AK *et al.* (2010) A randomized, double-blind, placebo-controlled phase 3 skin. *Cancer Prev Res (Phila)* 3:35–47

Benjamin CL, Melnikova VO, Ananthaswamy HN (2008) P53 protein and pathogenesis of melanoma and nonmelanoma skin cancer. *Adv Exp Med Biol* 624:265–82

Berg RJ, van Kranen HJ, Rebel HG *et al.* (1996) Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. *Proc Natl Acad Sci USA* 93:274–8

Berman B, Amini S, Valins W *et al.* (2009) Pharmacotherapy of actinic keratosis. *Expert Opin Pharmacother* 10:3015–31

Bosset S, Bonnet-Duquennoy M, Barre P *et al.* (2003) Photoageing shows histological features of chronic skin inflammation without clinical and molecular abnormalities. *Br J Dermatol* 149:826–35

Byrne SN, Limon-Flores AY, Ullrich SE (2008) Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression. *J Immunol* 180:4648–55

Chacon-Salinas R, Limon-Flores AY, Chavez-Blanco AD *et al.* (2011) Mast cell-derived IL-10 suppresses germinal center formation by affecting T follicular helper cell function. *J Immunol* 186:25–31

Chai OH, Han EH, Choi YH *et al.* (2011) The role of mast cells in atrial natriuretic peptide-induced cutaneous inflammation. *Regul Pept* 167:79–85

Challacombe JM, Suhrbier A, Parsons PG *et al.* (2006) Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. *J Immunol* 177:8123–32

Chang NB, Feng R, Gao Z *et al.* (2010) Skin cancer incidence is highly associated with ultraviolet-B radiation history. *Int J Hyg Environ Health* 213:359–68

Chaquour B, Seite S, Coutant K *et al.* (1995) Chronic UVB- and all-trans retinoic-acid-induced qualitative and quantitative changes in hairless mouse skin. *J Photochem Photobiol B* 28:125–35

Cohen JL (2010) Actinic keratosis treatment as a key component of preventive strategies for nonmelanoma skin cancer. *J Clin Aesthet Dermatol* 3:39–44

Conney AH, Kramata P, Lou YR *et al.* (2008) Effect of caffeine on UVB-induced carcinogenesis, apoptosis, and the elimination of UVB-induced patches of p53 mutant epidermal cells in SKH-1 mice. *Photochem Photobiol* 84:330–8

de Graaf YG, Rebel H, Elghalbzouri A *et al.* (2008) More epidermal p53 patches adjacent to skin carcinomas in renal transplant recipients than in immunocompetent patients: the role of azathioprine. *Exp Dermatol* 17:349–55

de Gruijl FR (2008) UV-induced immunosuppression in the balance. *Photochem Photobiol* 84:2–9

de Gruijl FR, Rebel H (2008) Early events in UV carcinogenesis–DNA damage, target cells and mutant p53 foci. *Photochem Photobiol* 84:382–7

de Vries VC, Wasiuk A, Bennett KA *et al.* (2009) Mast cell degranulation breaks peripheral tolerance. *Am J Transplant* 9:2270–80

Einspahr JG, Alberts DS, Warneke JA *et al.* (1999) Relationship of p53 mutations to epidermal cell proliferation and apoptosis in human UV-induced skin carcinogenesis. *Neoplasia* 1:468–75

Feldman SR, Fleischer AB Jr (2011) Progression of actinic keratosis to squamous cell carcinoma revisited: clinical and treatment implications. *Cutis* 87:201–7

Gonzalez S, Moran M, Kochevar IE (1999) Chronic photodamage in skin of mast cell-deficient mice. *Photochem Photobiol* 70:248–53

Hardin J, Mydlarski PR (2010) Systemic retinoids: chemoprevention of skin cancer in transplant recipients. *Skin Therapy Lett* 15:1–4

Hart PH, Grimbaldeston MA, Finlay-Jones JJ (2001) Sunlight, immunosuppression and skin cancer: role of histamine and mast cells. *Clin Exp Pharmacol Physiol* 28:1–8

Haschek WM, Rousseaux CG, Wallig MA (eds) (2009) Epidermal Lesions. In: *Fundamentals of Toxicologic Pathology*. Academic Press: San Diego, CA

Jonason AS, Kunala S, Price GJ *et al.* (1996) Frequent clones of p53-mutated keratinocytes in normal human skin. *Proc Natl Acad Sci USA* 93:14025–9

Jorizzo JL, Markowitz O, Lebwohl MG *et al.* (2010) A randomized, double-blinded, placebo-controlled, multicenter, efficacy and safety study of 3.75% imiquimod cream following cryosurgery for the treatment of actinic keratoses. *J Drugs Dermatol* 9:1101–8

Kligman LH, Kligman AM (1981) Histogenesis and progression in ultraviolet light-induced tumors in hairless mice. *J Natl Cancer Inst* 67:1289–93

Kligman LH, Murphy GF (1996) Ultraviolet B radiation increases hairless mouse mast cells in a dose-dependent manner and alters distribution of UV-induced mast cell growth factor. *Photochem Photobiol* 63:123–7

Lau K, Paus R, Tiede S *et al.* (2009) Exploring the role of stem cells in cutaneous wound healing. *Exp Dermatol* 18:921–33

Le TT, Gardner J, Hoang-Le D *et al.* (2009) Immunostimulatory cancer chemotherapy using local ingenol-3-angelate and synergy with immunotherapies. *Vaccine* 27:3053–62

Li L, Shukla S, Lee A *et al.* (2010) The skin cancer chemotherapeutic agent ingenol-3-angelate (PEP005) is a substrate for the epidermal multidrug transporter (ABCB1) and targets tumor vasculature. *Cancer Res* 70:4509–19

Madan V, Lear JT, Szeimies RM (2010) Non-melanoma skin cancer. *Lancet* 375:673–85

Marcil I, Stern RS (2000) Risk of developing a subsequent nonmelanoma skin cancer in patients with a history of nonmelanoma skin cancer: a critical review of the literature and meta-analysis. *Arch Dermatol* 136:1524–30

Marquez C, Bair SM, Smithberger E *et al.* (2010) Systemic retinoids for chemoprevention of non-melanoma skin cancer in high-risk patients. *J Drugs Dermatol* 9:753–8

Murphy GM (2009) Ultraviolet radiation and immunosuppression. *Br J Dermatol* 161(Suppl 3):90–5

Norval M, Cullen AP, de Gruijl FR *et al.* (2007) The effects on human health from stratospheric ozone depletion and its interactions with climate change. *Photochem Photobiol Sci* 6:232–51

Ogbourne SM, Hampson P, Lord JM *et al.* (2007) Proceedings of the First International Conference on PEP005. *Anticancer Drugs* 18:357–62

Rass K, Reichrath J (2008) UV damage and DNA repair in malignant melanoma and nonmelanoma skin cancer. *Adv Exp Med Biol* 624:162–78

Rebel H, Kram N, Westerman A *et al.* (2005) Relationship between UV-induced mutant p53 patches and skin tumours, analysed by mutation spectra and by induction kinetics in various DNA-repair-deficient mice. *Carcinogenesis* 26:2123–30

Rebel H, Mosnier LO, Berg RJ *et al.* (2001) Early p53-positive foci as indicators of tumor risk in ultraviolet-exposed hairless mice: kinetics of induction, effects of DNA repair deficiency, and p53 heterozygosity. *Cancer Res* 61:977–83

Remenyik E, Wikonkal NM, Zhang W *et al.* (2003) Antigen-specific immunity does not mediate acute regression of UVB-induced p53-mutant clones. *Oncogene* 22:6369–76

Rogers HW, Weinstock MA, Harris AR *et al.* (2010) Incidence estimate of nonmelanoma skin cancer in the United States, 2006. *Arch Dermatol* 146:283–7

Schmieder G (2010) Multicenter, open-label, dose-area escalation, cohort study to evaluate the safety and tolerability of PEP005 (ingenol mebutate) Gel, 0.05% applied for 2 consecutive days to treatment area(s) of up to a total of 100 cm2 in patients with actinic keratoses (AK) on the extensor (dorsal aspect) forearm(s). *American Academy of Dermatology Annual Meeting, Poster Presentation*

Siller G, Gebauer K, Welburn P *et al.* (2009) PEP005 (ingenol mebutate) gel, a novel agent for the treatment of actinic keratosis: results of a randomized, double-blind, vehicle-controlled, multicentre, phase IIa study. *Australas J Dermatol* 50:16–22

Siller G, Rosen R, Freeman M *et al.* (2010) PEP005 (ingenol mebutate) gel for the topical treatment of superficial basal cell carcinoma: results of a randomized phase IIa trial. *Australas J Dermatol* 51:99–105

Smith K, Hamza S, Germain M *et al.* (2007) Does imiquimod histologically rejuvenate ultraviolet radiation-damaged skin? *Dermatol Surg* 33: 1419–28

Swanson N (2011) A multicenter, randomized, parallel-group, double-blind, vehicle-controlled study to evaluate the efficacy and safety of PEP005 (ingenol mebutate) gel, 0.05% in patients with actinic keratoses on non-head locations. [abstract P2182] *22nd World Congress of Dermatology, Seoul Poster*

The Lewin Group, Inc. (2005) *The Burden of Skin Diseases*. Prepared for: The Society for Investigative Dermatology and The American Academy of Dermatology Association

Uchiumi T, Kohno K, Tanimura H *et al.* (1993) Enhanced expression of the human multidrug resistance 1 gene in response to UV light irradiation. *Cell Growth Differ* 4:147–57

Wang YN, Fang H, Wang HM *et al.* (2010) [Effect of chronic exposure to ultraviolet on skin barrier function]. *Zhejiang Da Xue Xue Bao Yi Xue Ban* 39:517–22

Ziegler A, Jonason AS, Leffell DJ *et al.* (1994) Sunburn and p53 in the onset of skin cancer. *Nature* 372:773–6