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LAV694, a new antiproliferative agent showing improved skin tolerability vs. clinical standards for the treatment of actinic keratosis

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Abstract

The skin tolerability of the tubulin polymerisation inhibitor LAV694 was compared to that of 5% 5-fluorouracil (5-FU) and 0.5% podophyllotoxin *in vitro* using a human reconstructed epidermis (HRE), and *in vivo* using minipigs. Topical treatment of HRE for 1 or 3 days with a 0.2, 0.6 or 1% LAV694 cream or the placebo showed no signs of irritation in terms of morphology, cell viability (lactate dehydrogenase leakage) or interleukin-8 mRNA expression and release. 5-FU increased interleukin-8 production and induced morphological signs of irritation. The substances were also applied under occlusion to the back of two minipigs, twice daily, for 9 days to allow intraindividual comparison of skin effects and tolerability. Skin reactions were monitored by visual scoring, chromometry, pro-inflammatory activity, cell cycle and apoptosis by RT-PCR, laser scanning cytometry and histopathological examination of biopsies. Application of podophyllotoxin and 5-FU had to be stopped on days 4 and 8, respectively, due to severe skin lesions. LAV694 (1%) induced only moderate skin reddening after 9 days. 5-FU and podophyllotoxin, but not LAV694, increased mRNA expression of pro-inflammatory cytokines. LAV694 arrested keratinocytes in the M phase of the cell cycle and apoptosis was detected histologically in the basal layer. LAV694 increased the expression of pro-apoptotic genes in both experimental models. In conclusion, LAV694 selectively induced apoptosis, rather than necrosis, of growth-arrested keratinocytes, thus avoiding the occurrence of extensive inflammation. This resulted in an improved skin tolerability in comparison with 5-FU and podophyllotoxin.

Keywords: Apoptosis; Human reconstructed epidermis; Minipig; Pro-inflammatory cytokines; Irritation; Actinic keratosis

1. Introduction

Actinic keratosis (AK) is an epidermal cutaneous dysplasia. It is the most common epithelial pre-cancerous lesion among fair-skinned individuals, and frequently progresses to squamous cell carcinoma [1]. Patients have multiple lesions on sun-exposed skin areas, presenting as ill-defined macules or papules with a dry, adherent scale. On histological examination, changes in cell polarity, and nuclear atypia of epidermal layers are seen. The epidermis becomes hyperkeratotic or parakeratotic, with a sharp border between dysplastic and normal keratinocytes. Nuclei in the basal layer are often crowded closely together, with atypical keratinocytes occasionally forming buds. Degenerated keratinocytes and cellular fragments may be present. The dermis often shows basophilic actinic changes, with a mild inflammatory infiltrate of lymphocytes and edema of the upper layers [2].

Treatment of these lesions can include destructive methods such as cryotherapy, electrodesiccation and curettage, excisional therapy and photodynamic therapy. The risks

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Abbreviations: CF, control formulation; 5-FU, 5-fluorouracil; HRE, human reconstructed epidermis; LDH, lactate dehydrogenase; LSC, laser scanning cytometry; PBS, phosphate-buffered saline; SLS, sodium lauryl sulphate.

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associated with these methods include pain, blistering, scars and pigmentary changes. Alternatively, chemotherapy with topical 5-FU is commonly used, especially in the case of patients with multiple lesions. However, 5-FU therapy is usually associated with severe irritation, frequently preventing patient compliance. This fact, together with the increasing incidence of AK in our modern society, has given rise to a clear need for new, effective and well tolerated products for the treatment of multiple lesions [3].

It has been recently discovered that certain derivatives of lavendustin A possess antiproliferative activity [4] through binding to tubulin and inhibiting its polymerisation, thus precluding microtubule formation. This results in cell cycle arrest in dividing cells, with accumulation of metaphase figures. An example is SDZ 281-977 (or SDZ LAP 977), which inhibits keratinocyte proliferation in a potent and selective manner, both *in vivo* and *in vitro* [5]. This compound has been shown to have therapeutic efficacy in AK [6]. LAV694 is a novel lavendustin derivative with identical pharmacological mechanism of action, that has no cytotoxic effect on non-dividing cells. LAV694 has antiproliferative potency comparable to other antimitotic agents, such as 5-FU or podophyllotoxin both *in vitro* and *in vivo*, but it has an improved selectivity for keratinocytes. LAV694 has a better solubility than SDZ LAP 977, higher *in vitro* and *in vivo* potency, increased metabolic stability and improved skin penetration properties. In addition, LAV694 still shows the required properties for a topical ''soft drug'', i.e. very low systemic exposure after topical application and fast systemic metabolic inactivation [7].

Our goal was to assess the skin tolerability to LAV694 after topical application, both *in vitro* using a HRE and *in vivo* in minipigs, by analysing the morphological changes induced by LAV694, and the biochemical and molecular mechanisms involved in the response. The clinically used antiproliferative agents 5-FU and podophyllotoxin were used for comparison.

2. Materials and methods

2.1. Determination of in vitro skin irritation potential using a HRE

A HRE (SkinEthic® Laboratories) containing fully differentiated normal human keratinocytes showing morphological, biochemical and functional features similar to human epidermis [8] was used for *in vitro* skin irritation testing. Ten mg/cm^2 of a cream formulation containing 0.2, 0.6 or 1% LAV694, as well as the corresponding placebo, were applied topically on the surface of the HRE and incubated for 1 or 3 days under culture conditions, as described previously [9]. The placebo was formulated with 8% isopropyl myristate, 6% polysorbate 60, 2% sorbitan monostearate (Span 60), 2% cetyl palmitate, 4% cetyl alcohol, 4% stearyl alcohol, 1% benzyl alcohol and 73% water. For comparison, a cream containing 5% 5-fluorouracil (Efudix®, Roche) and an innocuous CF (composed of 1% polyacrylacid (Carbomer 974P), 15% propylenglycol-1, -2, 16% of a 0.25 M NaOH solution, and 68% water) were used. Additionally, a solution of the skin irritant 0.4% SLS in phosphate buffer was used as a positive control.

As a marker of cell membrane integrity and cell viability, the leakage of LDH [10] into the tissue subnatant was measured with a commercially available spectrophotometry-based kit (Sigma). The levels of IL-8 were determined in the subnatants by ELISA (Quantikine, R + D systems), with a detection limit of 10 pg/mL. In addition, IL-8 mRNA expression in the epidermal tissue was quantified by real-time RT-PCR. For this, each tissue was subjected to a total RNA extraction using the FastRNA kit-green (Bio 101 Vista) combined with the RNAzol B solution (Cinna/Biotex). The total RNA amount was measured spectrophotometrically and samples were diluted to 10 ng/μL. IL-8 mRNA was quantified using an ABI 7700 equipment (Perkin-Elmer). An equal amount of total RNA from each tissue (50 ng) was subjected to RT-PCR, using TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer recommendations. In brief, PCR was conducted in a 50 μL reaction volume containing 5 μL cDNA, 25 μL 2× TaqMan universal PCR master mix (Applied Biosystems), 2 × 4 μL primers (10 pmol/μL), 6.75 μL probe (1.1 pmol/μL) and 5.25 μL nuclease-free water. The thermal conditions were as follows: stage 1 = 10 min at 95°; stage 2 = 15 s at 95°; stage 3 = 1 min °; 40 cycles between stages 2 and 3 were performed. The following primers and labelled probes were used:

Human IL-8: forward, TGC TAG CCA GGA TCC ACA AGT; reverse, TGA GGT AAG ATG GTG GCT AAT ACT; probe, CTT GTT CCA CTG TGC CTT GGT TTC TCC TT.

Human GAPDH: forward, GAA GGT GAA GGT CGG AGT C; reverse, GAA GAT GGT GAT GGG ATT TC; probe, CAA GCT TCC CGT TCT CAG CC.

GAPDH mRNA, a housekeeping gene, was carried out in parallel in each sample for normalization.

Finally, histological analysis of formalin-fixed, paraffin-embedded HRE samples stained with hematoxylin and eosin was performed and evaluated in conjunction with the rest of the investigated parameters.

All experiments were performed in triplicate. The integrated evaluation of all tested parameters was used for the assessment of the skin irritation potential of the different LAV694 formulations, and for estimating the relative intensity of the effects against the reference compounds.

2.2. Expression of pro-apoptotic genes

The expression of Bax, p53, caspases 3 and 9 and granzyme B [11–14] was quantified by real-time RT-PCR as molecular markers of the induction of apoptosis. The following primers and labelled probes were used:

Human Bax: forward, CTG CAG AGG ATG ATT GCC G; reverse, TGC CAC TCG GAA AAA GAC CT; probe, CGT GGA CAC AGA CTC CCC CCG A.

Human p53: forward, TGC TTT CCA CGA CGG TGA C; reverse, AGT GAC CCG GAA GGC AGT C; probe, CGC TTC CCT GGA TTG GCA GCC.

Human caspase 3: forward, TGT TCC ATG AAG GCA GAG CC; reverse, TGC GTA TGG AGA AAT GGG C; probe, TGG ACC ACG CAG GAA GGG CCT A.

Human caspase 9: forward, CAT CGA CTG TGA GAA GTT GCG; reverse, CCC TTC ACC TCC ACC ATG AA; probe, CGT CGC TTC TCC TCG CCG CA.

Human granzyme B: forward, CTG GAG GCC CTC TTG TGT GT; reverse, GCA TGC CAT TGT TTC GTC C; probe, AGG TGG CCC AGG GCA TTG TCT CC.

2.3. Evaluation of the in vivo skin tolerability in minipigs

Göttingen minipigs are commonly used for testing skin tolerability because their skin resembles the features of human skin more closely than that of other species [15,16]. One male and one female Göttingen SPF minipigs (Ellegard Breeding Centre) of ca. 9 months of age were topically treated on the back with 200 mg of the 0.2, 0.6 or 1% LAV694 cream formulations, and the placebo formulation. The animals also received 200 mg of the 5% 5-FU cream, and 200 mg of a 0.5% podophyllotoxin liniment (Condyline®, Nycomed). This study was especially designed for intraindividual comparison of specific dermal effects of the tested compounds and their skin tolerability. Applications were performed with each preparation on the right and left side of the back of the animal under occlusive bandage (Finn chambers of 22 mm diameter), twice daily (8 and 14 hr of exposure with approximately 1 hr recovery after each application) for a maximum of 9 days. A sham control and an untreated site were also included in the evaluations.

Visual skin observations comprised scoring of skin reddening and swelling according to OECD Guideline No. 404 (July 17, 1992). Skin reflectance reading (colour changes recorded using a Minolta CR300 Chroma-Meter) was performed to quantify skin reddening. Skin biopsies of a diameter of 4 mm were taken from the application sites using a medical punch when the skin changes reached grade 3 (moderate to severe erythema and/or moderate edema) to 4 (severe erythema to eschar formation and/or severe edema), or, in the case of well tolerated compounds, at the end of the treatment period. Biopsies were taken after disinfection with 70% ethanol and local anaesthesia with 2% Lidocaine (Chassot AG). Histopathological examination of formalin-fixed, paraffin-embedded tissue stained with hematoxylin and eosin was performed. In order to investigate the onset of a local inflammatory process and the induction of apoptosis, the mRNA expression of IL-1α, TNFα, IL-8 and Bax in the biopsied skin was determined by real-time RT-PCR, using the following primers and labelled probes:

Porcine IL-1α: forward, GGG TCA TCA ACC ACC AGT GC; reverse, GAC CTG ACG GGT CTC GAA TG; probe, TCC TGA ATG ATG CCC GCA ATC AAA.

Porcine TNFα: forward, GGC CCC CAG AAG GAA GAG T; reverse, TGA GTC CTT GGG CCA GAG G; probe, CCA GCT GGC CCC TTG AGC ATC A.

Porcine IL-8: forward, CTC GTG TCA ACA TGA CTT CCA AA; reverse, CAC AGA GAG CTG CAG AAA GCA; probe, TGG CTG TTG CCT TCT TGG CAG TTT TC. IL-8 mRNA levels were only analysed in the biopsies obtained from control, 1% LAV694 and 5-FU treated sites.

Porcine bax has not been sequenced. Since bax has a high degree of homology between human and rat or mice (around 92%), it was reasonable to expect a high homology also with pigs. Therefore, the human bax primers and probes were used in the minipig samples.

2.4. Evaluation of cell cycle parameters and apoptosis in minipig skin by LSC

Cell cycle parameters, as well as the presence of apoptosis-related proteins, were assessed by LSC analysis: first, single-cell suspensions were obtained from whole skin samples by trypsin digestion, as follows: after incubation of each skin specimen in PBS (Hyclone) containing 0.25 mg/mL trypsin (Sigma) for 35 min at 37°, the dermis was separated with forceps in PBS. The remaining epidermis was then incubated in a fresh trypsin solution for 15 min and gently mixed on a shaker to separate the keratinocytes, resulting in a single-cell suspension. Cells were resuspended in PBS containing 20% foetal calf serum (clone III, Hyclone), fixed in ice-cold methanol and kept at −20° until analysis. The fixed cells were washed in PBS and cytocentrifuged on slides using a Shandon cytocentrifuge (Cytospin 2, Shandon Co.) at 50 *g* for 3 min.

For cell cycle studies, the slides were washed with PBS and incubated in a solution of 50 μg/mL propidium iodide (Sigma) containing 0.1 mg/mL RNAse A (Sigma) and 0.1% of Triton X-100 (Sigma) for 1 hr at room temperature. The slides were then covered and analysed with the LSC.

The fluorescence emission of the stained cells was measured on the LSC interfaced to a Compaq computer equipped with the WinCyte 2.1 software (Compucyte), as described in detail elsewhere [17]. The LSC was equipped with a 488 nm argon-ion laser and two standard photomultiplier tubes collecting in green (wavelength band centred at 530 nm), and red (wavelength band longer than 570 nm) wavelengths were used. The laser was imaged through a scan lens into an Olympus BX50 microscope. Slides were scanned using a 20× objective. The scan area was adjusted to the entire coverslip area and at least 5000 cells were analysed. Determination of cell cycle parameters was performed by gating cells based on contour area (reflecting nucleus size) and PI max pixel (highest

intensity of fluorescence within the threshold contour, representing chromatin condensation). Analysis of fluorescence peak (which represents the maximal fluorescence value among all the pixels over the measured cell) and fluorescence area (which represents the number of pixels over threshold) of the cells stained with DNA fluorochromes appeared to be a sensitive probe of chromatin structure, discerning cells differing in the degree of nuclear chromatin condensation. Thus, based on an analysis of the fluorescence parameters mentioned above, it was possible to easily distinguish mitotic cells (M phase) from interphase cells (G_2 phase).

The expression of Bax was evaluated as follows: the slides were incubated overnight at 4° with a rabbit anti-human Bax antibody (Ab-1, PC66, Oncogene), diluted 1:50 in PBS containing 0.5% bovine serum albumin and 0.01% Tween-20. After washing twice with PBS, the slides were incubated with the secondary antibody diluted 1:150 in PBS containing 0.5% bovine serum albumin and 0.01% Tween-20, for 1 hr at room temperature. As a secondary antibody, Alexa-488 goat anti-rabbit IgG (Molecular Probes) was used. The slides were washed again and counterstained with 10 μg/mL of PI and 0.1 mg/mL RNAse in PBS for 1 hr at room temperature before measurement. The cells were gated on green max pixel or integral (reflecting the local density of Bax) and integrated PI fluorescence. Overlapping nuclei were automatically excluded from the counting by special statistical filters.

The presence of fragmented nuclei (reflecting chromatin condensation) in minipig skin topically treated with LAV694 1% was analysed by confocal laser scanning microscopy. Images of paraffin sections stained for DNA using Draq5 (Alexis) dye were evaluated by confocal microscopy after stacking successively 20 XY images of variable thickness.

3. Results

3.1. Determination of in vitro skin irritation potential of LAV694 using a HRE

Fig. 1 shows that application of 0.2, 0.6, 1% LAV694 or the placebo to the HRE resulted in weak, non-significant increases in the expression and release of IL-8, in the absence of cytotoxicity (LDH release), when compared with the control formulation. 5-FU markedly enhanced the production of IL-8 protein and mRNA. The positive control, SLS, decreased cell viability, as shown by an increase in LDH leakage. In addition, a significant increase in IL-8 release was also observed upon SLS application, but no increase in IL-8 mRNA was detected after 24 or 72 hr incubation. This is due to the rapid loss of cell viability, which prevented the active process of IL-8 gene expression.

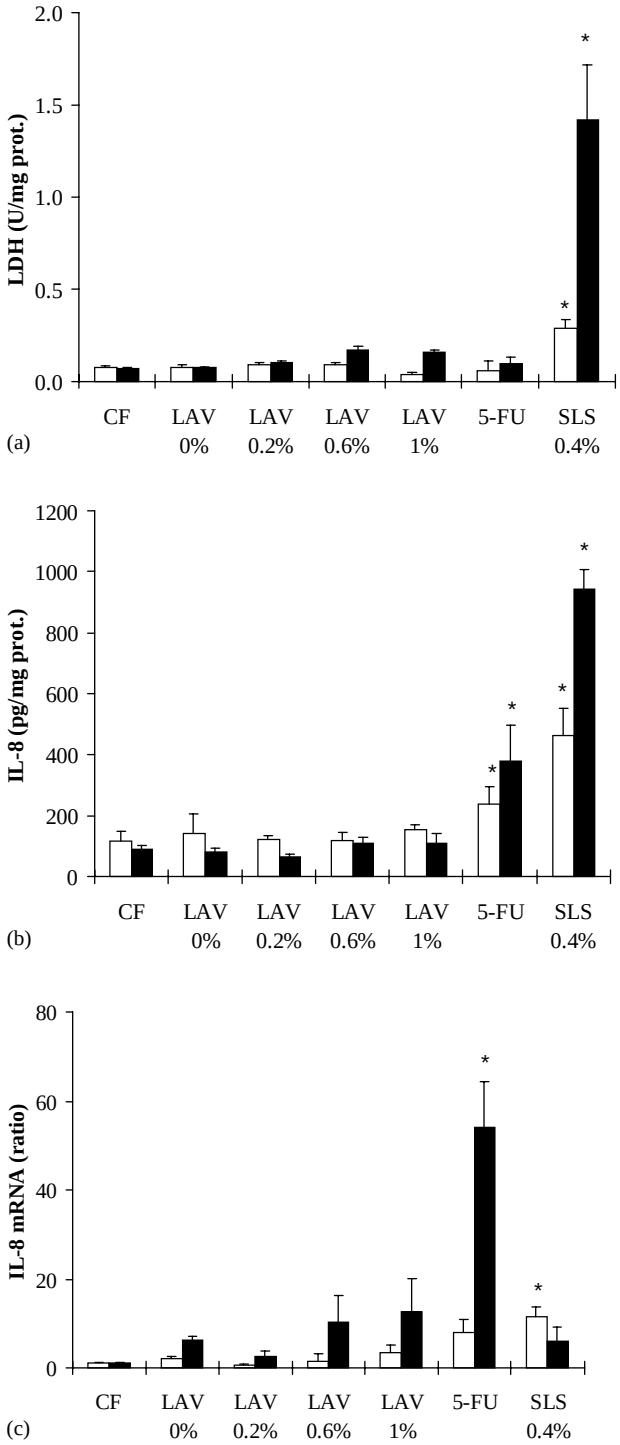

Fig. 1. 5-FU and SLS, but not LAV694, increased biochemical markers of irritation in the HRE. Following topical treatment of the epidermal tissues for 24 hr (open bars) or 72 hr (closed bars), LDH release (a) was determined as a marker of cytotoxicity, and IL-8 release (b) and mRNA expression (c) were quantified as markers of pro-inflammatory activity. Results represent the mean ± SD of three determinations. Statistical analysis by Student's *t*-test. $^{*}P < 0.05$ vs. CF at the corresponding time point.

As shown in Fig. 2, morphological changes indicative of the induction of skin irritation were observed on histopathological examination of the HRE following application of 5-FU and SLS, whereas only the highest tested

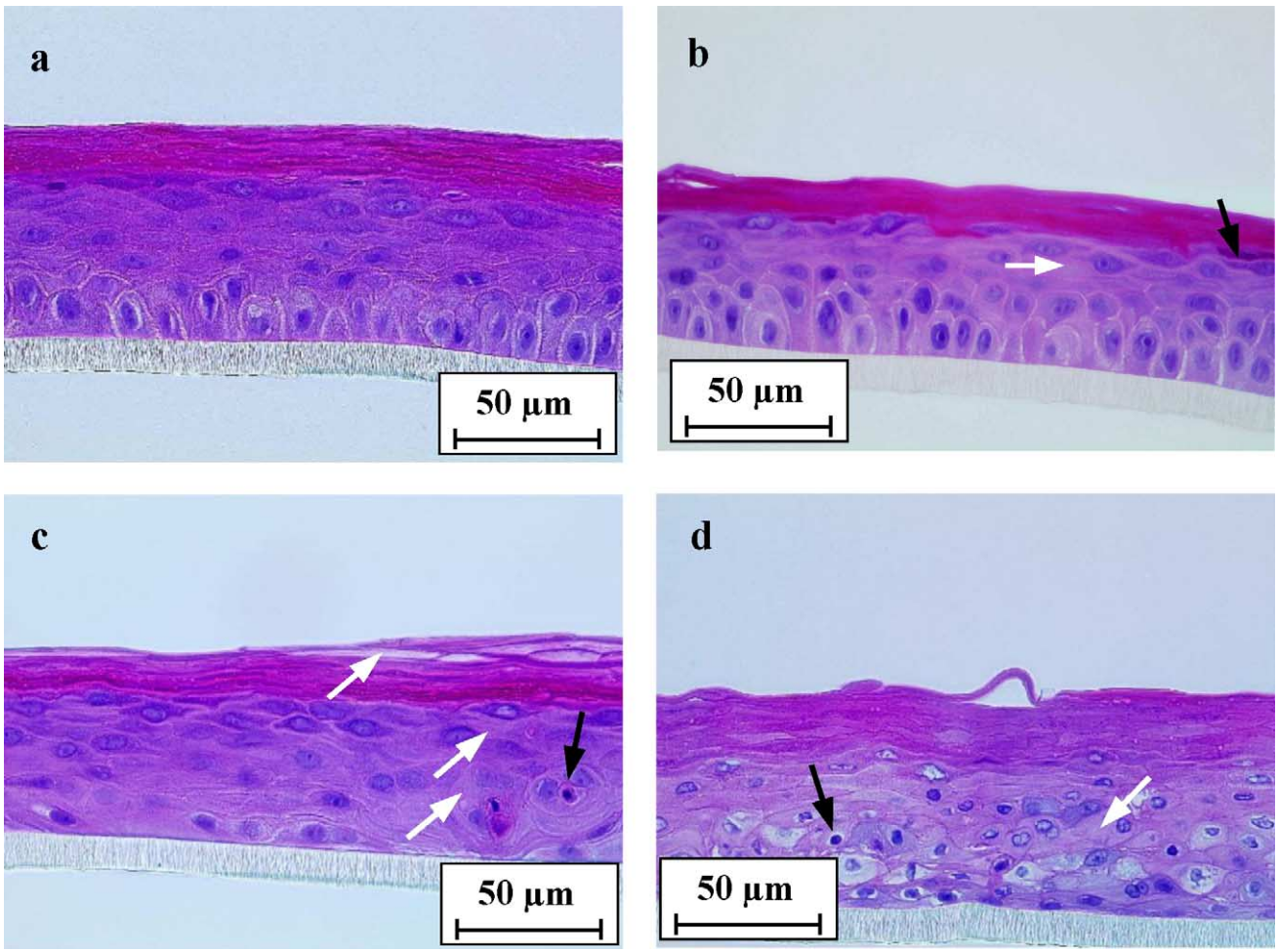

Fig. 2. Topical application of 5-FU (c) or SLS (d), but not of LAV694 (b) or the placebo (a) for 72 hr resulted in histopathological signs of skin irritation in the HRE. (a) Placebo: the HRE presented the aspect of a normal epidermis with well-differentiated basal, spinous, granular and horny layers. (b) 1% LAV694: a few pyknotic nuclei (black arrow), in the most superficial layer and a diffuse but minimal edema (white arrow) in all layers were observed. (c) 5% 5-FU: pyknotic nuclei (black arrows) were observed not only in the granular layer, but also in the spinous layer, as well as intracellular edema in the spinous, granular and horny layers (white arrows). (d) 0.4% SLS: abundant pyknotic nuclei in all layers (black arrow) associated with moderate edema (white arrow) were noted.

concentration of LAV694 (1%) resulted in mild morphological changes, namely, the appearance of a few pyknotic nuclei in the granular layer and minimal intracellular edema. The control formulation induced no effects.

These findings indicate that under the conditions of the experiments, neither LAV694 nor its vehicle induced any significant signs of skin irritation, whereas 5-FU did, as suggested by the production of the pro-inflammatory chemokine IL-8 (onset of an inflammatory reaction) and morphological changes consistent with an irritative process.

3.2. Evaluation of the skin tolerability of LAV694 in minipigs

Topical exposure of minipigs to 0.5% podophyllotoxin and 5% 5-FU had to be stopped early on days 4 or 8, after 6 or 14 applications, respectively, due to severe skin lesions (Table 1). LAV694 induced the first signs of weak skin reddening on day 5.

Skin treated with 0.5% podophyllotoxin showed an increase in reddening in the range of very slight on day 2 to severe on day 3. In addition, necrotic tissue and formation of crusts were observed on day 4. The low measured values for skin reddening in contrast to the visual skin observations with 0.5% podophyllotoxin were considered to be related to interferences *due to* the formation of crusts at the application sites. Similar changes, although slightly less pronounced, developed with 5% 5-FU within 7 days of treatment. With LAV694, skin reddening occurred in a concentration-dependent manner from very slight on day 5 to moderate on day 10. In addition, a focal ulceration was evident in the male minipig on one LAV694 1%-treated site and in the female minipig on both sites with the 1% cream and on one site with the 0.6% cream on day 10.

Histologically, after application of 5-FU and podophyllotoxin, epidermal necrosis associated with a moderate to severe inflammatory infiltrate extending from the epidermis to the hypodermis was observed, signs which were not

Table 1
Minipig skin reflectance and maximal visual score after topical treatment with LAV694, 5-FU or podophyllotoxin

	M/F							
	Placebo	LAV694 (0.2%)	LAV694 (0.6%)	LAV694 (1%)	5% 5-FU	0.5% podophyllotoxin	Sham	Control
Predose	$7.4^0/8.0^0$	$6.5^0/6.0^0$	$5.7^0/6.6^0$	$6.1^0/6.3^0$	$7.6^0/8.1^0$	$6.9^0/8.9^0$	$6.4^0/7.3^0$	$5.7^0/6.9^0$
Day 2	$8.6^0/9.8^0$	$7.5^0/7.8^0$	$8.3^0/9.1^0$	$8.8^0/12.4^0$	$9.9^1/12.6^1$	$7.8^2/10.2^2$	$7.0^0/8.3^0$	$5.2^0/7.6^0$
Day 3	$10.8^0/9.3^0$	$7.3^0/7.6^0$	$8.4^0/8.3^0$	$8.7^0/8.5^0$	$13.6^2/11.6^1$	$7.2^3/15.8^3$	$5.9^0/6.2^0$	$5.1^0/6.3^0$
Day 4	$9.2^0/10.6^0$	$7.9^0/9.5^0$	$8.0^0/8.3^0$	$8.9^0/10.9^0$	$15.2^2/18.1^2$	$-^4/-^4$	$5.7^0/8.3^0$	$5.8^0/7.3^0$
Day 5	$13.0^0/11.4^1$	$10.7^0/9.2^1$	$9.9^0/8.5^1$	$10.3^1/9.1^1$	$14.7^2/16.7^2$	–/–	$5.2^0/6.8^0$	$3.6^0/5.2^0$
Day 6	$17.2^2/12.5^2$	$13.8^1/9.3^2$	$12.4^1/9.4^2$	$13.8^2/11.8^2$	$8.4^2/16.1^2$	–/–	$5.0^0/5.5^0$	$5.2^0/4.7^0$
Day 7	$13.8^1/9.8^2$	$12.3^2/12.5^2$	$13.6^2/11.2^2$	$14.3^2/14.7^2$	$11.8^4/11.5^4$	–/–	$6.1^0/5.0^0$	$4.2^0/5.3^0$
Day 8	$14.3^1/10.3^2$	$14.6^2/12.9^2$	$16.1^2/13.3^2$	$18.2^2/18.2^2$	–/–	–/–	$4.6^0/5.3^0$	$3.7^0/5.2^0$
Day 9	$13.8^1/12.3^1$	$16.2^2/16.1^2$	$17.8^3/16.9^3$	$19.7^3/21.7^3$	–/–	–/–	$4.4^0/5.9^0$	$4.8^0/5.8^0$
Day 10	$12.2^1/10.9^1$	$16.3^2/13.4^1$	$19.1^2/19.1^2$	$20.2^3/19.6^2$	–/–	–/–	$4.2^0/5.1^0$	$3.2^0/5.1^0$

The test *compounds* were topically applied under occlusion to the back of two minipigs, *twice a day*, for a maximum of 9 days. The skin reactions were monitored by chromometry and visual scoring before treatment start and approximately 14 hr after the second administration on each day. Skin reflectance is expressed as "a" Units, as defined by the "Commission Internationale d'Èclairage". Values are the mean of both treated sides. Visual scores (superindices): (1) no erythema, (2) very slight erythema (barely perceptible), (3) well-defined erythema, (4) moderate to severe erythema, (5) severe erythema to eschar formation. M: male; F: female.

observed following treatment with LAV694 or the placebo (Fig. 3). After treatment with LAV694, apoptotic nuclei in the basal cell layer were seen in a frequency that increased with the dose. A focal or extensive dermal–epidermal detachment was seen after application of 0.6 or 1% LAV694, respectively, the latter associated with the appearance of a mild inflammatory reaction in the epidermis and superficial dermis.

In order to investigate the molecular events leading to the observed skin alterations in minipigs, the mRNA levels of the pro-inflammatory cytokines IL-1α and TNFα in the biopsies were assessed by RT-PCR. LAV694 induced only minimal, non-significant changes in the levels of the mentioned cytokines after 9 days of treatment (Fig. 4). However, 5% 5-FU, as well as 0.5% podophyllotoxin markedly increased IL-1α mRNA expression in minipig skin when the treatment had to be stopped due to severe skin lesions (after 7 days of treatment with 5-FU and after 3 days of treatment with podophyllotoxin).

Table 2
Cell cycle parameters in the skin of minipigs topically treated with LAV694, 5-FU or podophyllotoxin

	M/F		
	% G_1	% G_2	% M
LAV694 (0%) (placebo)	77.3/79.0	5.9/6.4	1.3/1.1
LAV694 (0.2%)	67.2/69.2	8.9/11.6	1.3/4.6
LAV694 (0.6%)	71.6/76.4	9.8/8.9	2.5/5.3
LAV694 (1.0%)	71.3/74.2	10.6/9.0	2.4/3.2
5% 5-FU	n.d.	n.d.	n.d.
0.5% podophyllotoxin	n.d.	n.d.	n.d.
Control (sham)	83.2/87.3	5.4/5.4	0.6/0.9
Untreated site	78.8/81.1	4.6/5.0	0.6/0.4

The test *compounds* were topically applied under occlusion to the back of two minipigs, *twice a day*, for a maximum of 9 days. Cell cycle parameters were determined by LSC in the biopsies of the treated sites. Data relative to untreated site; n.d.: not determined, due to extensive tissue necrosis of the treated sites. M: male; F: female.

5-FU, but not podophyllotoxin, enhanced TNFα mRNA expression. Finally, IL-8 mRNA expression was markedly induced by 5-FU (more than 90-fold with respect to non-treated control), whereas 1% LAV694 induced no increase at all (data not shown). These findings reflect the severe inflammation induced by 5-FU and podophyllotoxin, and are consistent with the visual observations described above.

3.3. Investigation of cell cycle parameters in minipig skin after application of LAV694

Although tested in only a limited number of minipigs in this study, a trend towards accumulation of cells in the M and G_2 phases of the cell cycle, as well as a reduction in the number of cells in the G_1 phase, were evident following topical application of LAV694, in comparison with the placebo, the sham control and the untreated site (Table 2).

3.4. Investigation of apoptosis in the HRE in vitro and in minipigs

The mRNA expression of several genes involved in apoptosis was measured in the HRE, after 24 hr treatment with LAV694 or 5-FU. Table 3 shows that 1% LAV694 induced a significant increase in mRNA expression of the pro-apoptotic genes Bax, p53, caspases 3 and 9 and granzyme B, when compared to the control formulation. A similar effect was not observed with the placebo. In addition, an increased number of cells undergoing apoptosis in the HRE tissue upon application of LAV694 when compared to control was observed at histological analysis with laser scanning cytometry. These findings indicate that LAV694 induced apoptosis of keratinocytes in the HRE. 5-FU also increased the expression of the mentioned genes, but to a much lower extent than 1% LAV694. As shown in

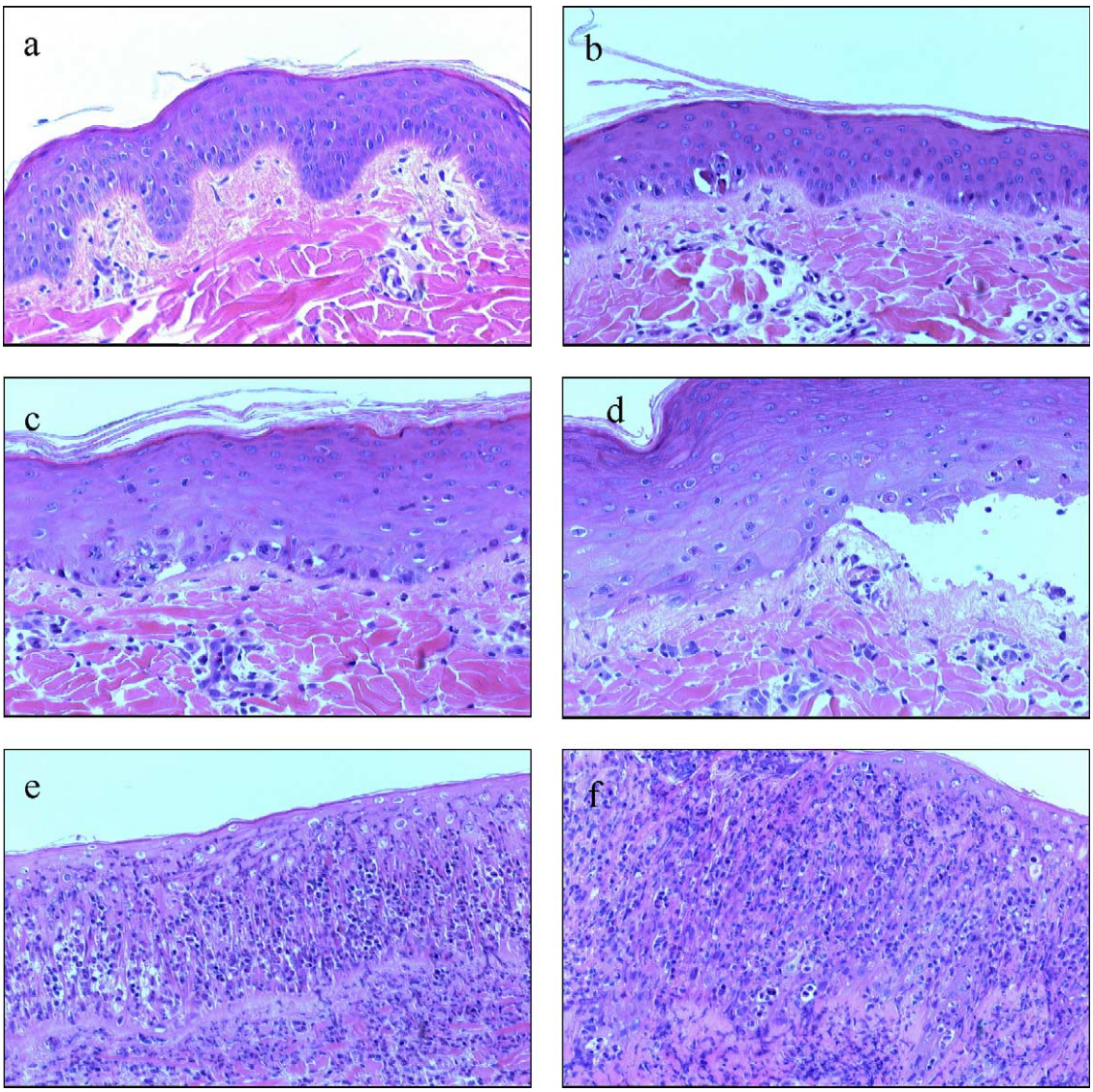

Fig. 3. Topical exposure of minipig skin to 5-FU (e) or podophyllotoxin (f), but not to LAV694 (b, 0.2%; c, 0.6%; d, 1%) or placebo (a) resulted in histopathological signs of marked skin irritation. (a) Placebo: the skin showed a normal aspect. In contrast, LAV694 induced the appearance of apoptotic nuclei (black arrows) in the basal cell layer, in a dose-dependent manner: (b, 0.2%; c, 0.6%; d, 1%). LAV694 0.6% (c) also induced focal detachment of the epidermis (arrow-head) without inflammatory reaction, and LAV694 1% (d) resulted in a large area of dermo-epidermal detachment (arrow-head) associated with a slight inflammatory reaction in the epidermis and superficial dermis. (e) 5% 5-FU: epidermal necrosis associated with a moderate to marked inflammatory reaction (white arrows) in the epidermis, which was extended in the dermis until the hypodermis (not shown in the picture). No dermo-epidermis detachment was observed. (f) 0.5% podophyllotoxin: similar observations to those induced by 5-FU (white arrows, inflammatory infiltrate).

Fig. 5, the local density of the pro-apoptotic protein Bax in minipig skin was increased after treatment with LAV694, in comparison with the untreated site, the sham control and the placebo. The presence of apoptotic nuclei in minipig skin treated with LAV694, as seen at histological examination, was also confirmed by confocal laser scanning microscopy (Fig. 6). This indicates that minipig skin cells specifically undergo apoptosis after LAV694 treatment.

Table 3
Messenger RNA expression of genes involved in apoptosis in the HRE after 24-h treatment with LAV694 or 5-FU

	Bax	p53	Caspase 3	Caspase 9	Granzyme B
CF	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
LAV 0%	0.8 ± 0.1	0.6 ± 0.1	1.1 ± 0.0	1.0 ± 0.1	n.d.
LAV 1%	$9.1 \pm 0.7^{*}$	$8.7 \pm 1.0^{*}$	$85.2 \pm 6.4^{*}$	$102.5 \pm 11.4^{*}$	$702.7 \pm 105.4^{*}$
5-FU	5.1 ± 3.1	2.3 ± 1.8	$21.9 \pm 11.1^{*}$	$22.8 \pm 11.9^{*}$	$270.0 \pm 54.0^{*}$

Following topical treatment of the epidermal tissues for 24 hours, the mRNA expression of pro-apoptotic genes was quantified by Real Time PCR. Results represent the mean ± SD of three determinations. CF: Control Formulation. LAV: LAV694; 5-FU: 5-fluoro-Uracil. n.d.: not determined. Data are expressed as the mean ± S.D. of 3 experiments; fold increases vs. values obtained with CF. $^{*}P < 0.05$ vs. CF.

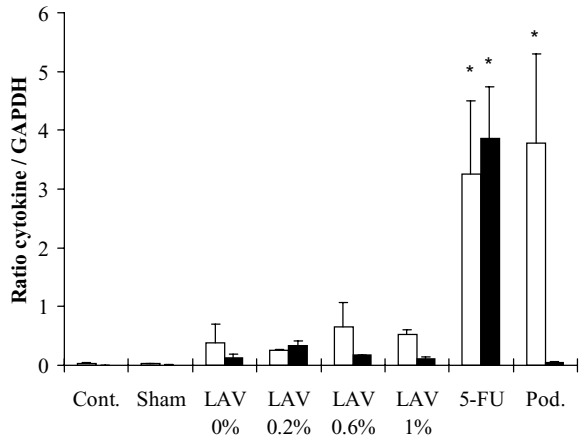

Fig. 4. Effects of LAV694, 5-FU and podophyllotoxin on the mRNA expression of pro-inflammatory cytokines in minipig skin. Following topical treatment of minipigs with the test compounds as described in the methods section, the mRNA expression of IL-1α (open bars) and TNFα (closed bars) was determined by RT-PCR. The skin biopsies used for the determinations were collected on day 4 for podophyllotoxin, on day 7 for 5-FU, and on day 10 for all other treatments. Results represent the mean ± SD of determinations performed in triplicate with the biopsies of the left and right treatment sites. Statistical analysis by Student's *t*-test. $^{*}P < 0.05$ vs. control.

4. Discussion

The response of the human epidermal tissue to a topically applied cream containing LAV694 was evaluated *in vitro* in terms of direct cytotoxicity, pro-inflammatory activity and morphology. LAV694 did not decrease cell viability of keratinocytes nor did it increase the release and mRNA expression of IL-8 in a HRE that has been shown to correctly predict the skin irritation potential of topical products for humans when utilized under defined experimental conditions [9]. Our data indicate therefore that LAV694 was well tolerated. Conversely, the 5% 5-FU cream induced the production of IL-8 at the mRNA and protein levels. Keratinocytes produce, release and respond to cytokines and chemokines such as IL-8 [18–23], which plays an important role in the initiation and development phases of skin inflammatory reactions. Therefore, our data suggest the initiation of an inflammatory reaction by 5-FU. The well-known severe skin irritant used for comparison (0.4% SLS) was markedly cytotoxic, as indicated by leakage of LDH. These results were confirmed by the

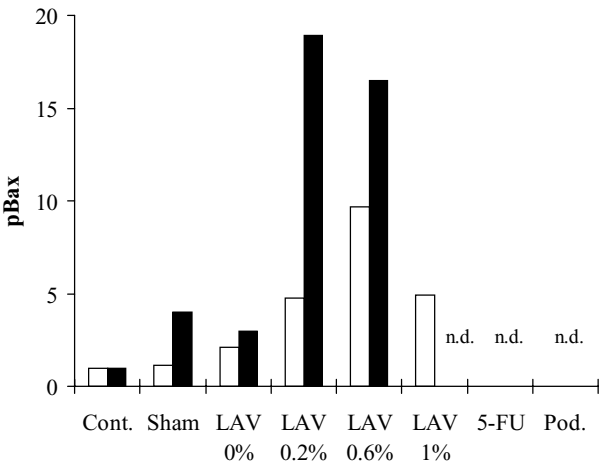

Fig. 5. Levels of the pro-apoptotic protein Bax in minipig skin topically treated with LAV694, 5-FU or podophyllotoxin. Following topical treatment of minipigs with the test compounds as described in Section 2, the levels of Bax were determined by LSC. Results are expressed relative to the level of Bax in untreated sites; n.d.: not determined, due to extensive tissue necrosis or superficial skin wounds of the treated sites. Open bars, male; closed bars, female.

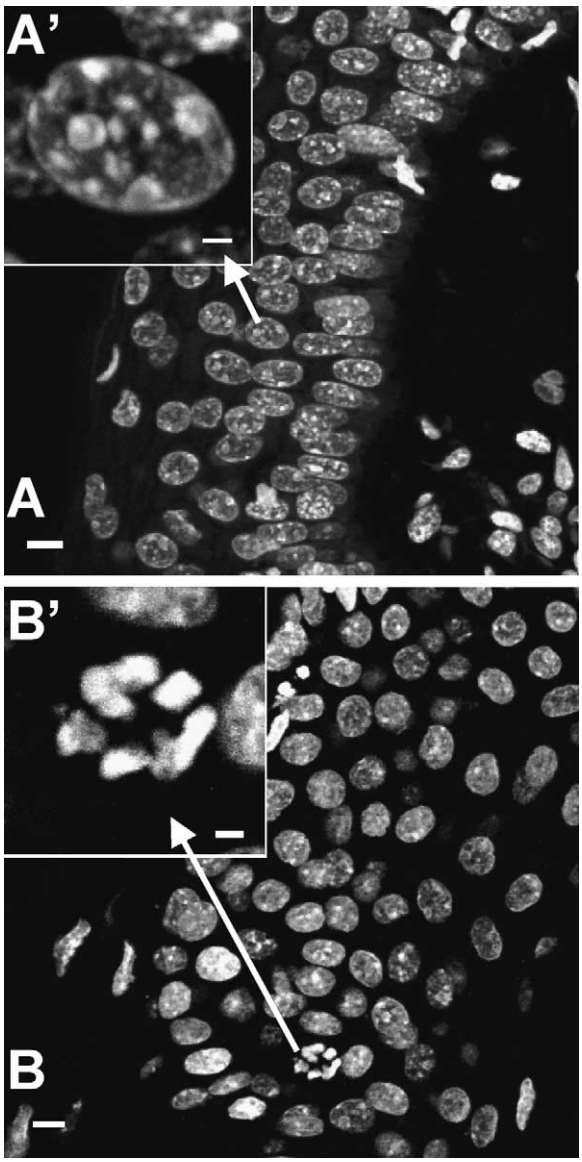

Fig. 6. Confocal laser scanning microscopy images of paraffin sections stained for DNA using Draq5 (Alexis) dye from minipig skin topically treated with LAV694 1%. Following topical treatment of minipigs with the test compounds as described in Section 2, the presence of fragmented nuclei (chromatin condensation) was evaluated by confocal microscopy after stacking successively 20 XY images of 0.405 μm thick (A) and 0.365 μm thick (B). (A) Untreated animal; (B) LAV694 1%-treated animal showing the fragmented nucleus of an apoptotic cell. Bar: (A) and (B) = 5 μm; (A') and (B') (inserts) = 1 μm.

observation of morphological signs of irritation under conventional histopathological examination of 5-FU or 0.4% SLS-treated epidermis.

Exposure of minipig skin to LAV694 for 4 days resulted in weak skin reddening, whereas application of 0.5% podophyllotoxin had to be stopped after 3 days due to severe skin lesions, and 5% 5-FU already showed signs of moderate irritation. The application of 5% 5-FU had to be stopped after 7 days of treatment, whereas administration of LAV694 could be continued until day 10 (end of the experimental period). To clarify whether the compound was effective under the conditions of the study, investigation of cell cycle parameters was performed. Indeed, an accumulation of cells in the M phase of the cell cycle was observed, as expected from a compound that inhibits tubulin polymerisation, thereby blocking mitosis. Taken together, these results clearly represent an improvement in tolerability vs. standard clinical treatments at doses with equivalent pharmacological efficacy.

Growth-arrested cells are deemed to die. However, the death pathway undergone by the cells determines whether an inflammatory reaction is elicited: cell death by necrosis is typically associated with inflammation. On the contrary, apoptosis is not followed by inflammation, similarly to what occurs during natural replicative senescence of irreversibly growth-arrested keratinocytes. The cellular and molecular mechanisms underlying this difference are known in part: although apoptotic cells can be eliminated by neighbouring cells, they can also undergo phagocytosis by macrophages. Several receptors are involved, thereby allowing for their efficient clearance [24]. Concomitantly, macrophages are primed to produce immunoregulatory factors such as IL-10, TGF-β, and PGE_2 while the production of proinflammatory cytokines (e.g. IL-12, TNFα) is suppressed [25–27]. Moreover, macrophages degrade rather than process antigens contained within apoptotic cells, as they fail to induce antigen-specific cytotoxic T lymphocytes when injected *in vivo* [28] and are not recognised as targets of cytotoxic T lymphocytes *in vitro* [29]. Altogether, these data suggest that macrophages modulate the immune response after phagocytosis of apoptotic cells through the release of immunosuppressive factors and failure to present the antigen. On the other hand, exposure of macrophages to necrotic cells leads to macrophage activation. Based on this evidence, the reason for the reduced inflammation and consequent improved tolerability observed upon treatment with LAV694 in comparison to other agents might be due to a potential difference in the death pathway undergone by the growth-arrested keratinocytes. Our initial investigations showed that application of LAV694 to the HRE resulted in the appearance of cells presenting apoptotic-like nuclei. Therefore, it was decided to investigate the effects of LAV694 on several genes that play a key role in different pathways leading to apoptotic cell death: Bax, caspases 3 and 9, p53 and granzyme B.

LAV694 markedly increased Bax expression in the HRE, with respect to controls. The Bcl-related protein Bax forms channels in lipid membranes, thus leading to leakage of cytochrome *c* from mitochondria into the cytosol [30,31], which in turn activates caspases [32,33]. Caspases are proteases that are required for intracellular protein degradation and execution of apoptotic cell death [34–36]. They inactivate inhibitors of proapoptotic proteins, destroy cell structures (such as lamina) and dysregulate proteins by separating regulatory and catalytic domains, resulting in loss or gain of function [14]. Caspase 3 is an effector caspase and caspase 9 an initiator caspase functioning upstream of effector caspases. Our data may therefore reflect an upregulation of the expression of caspases in keratinocytes as a regulatory mechanism to compensate for their enhanced usage resulting from the activation of the Bax apoptotic pathway by LAV694. 5-FU also increased the expression of caspases 3 and 9, but to a much lower extent than LAV694. Since Bax exerts its actions upstream of this apoptotic pathway, we selected it as a marker of apoptosis in minipigs skin following application of LAV694 and the comparators. Indeed, LAV694 induced a marked increase in the local density of Bax in minipig skin *in vivo*, as compared with control, thus providing conditions that facilitate activation of the apoptotic execution machinery in epidermal cells. This was confirmed by the observation of apoptotic cells in the biopsies. It must be noted that some differences were also observed in pBax levels between male and female values following LAV694 treatment. This is consistent with the higher susceptibility observed for the female minipig at clinical examination. Since there is no evidence of the involvement of any gender-specific mechanism that could explain this difference, it should be interpreted as inter-individual variability in the response.

Additional investigations with a larger number of animals of each sex were performed in further minipig studies with single or multiple (2 weeks) dermal administrations of 0.2, 0.6 and 1% LAV694 cream under semi-occlusive conditions. All these studies have shown similar results with respect to the above described pharmacological effects of LAV694 (data not shown; manuscript in preparation).

LAV694 also enhanced the mRNA levels of the tumour suppressor gene p53 in the HRE. The DNA-binding protein p53 plays a key role in apoptosis [37] by inducing genes such as Bax [38], Fas/APO-1 [39,40], and KILLER/DR5 [41], genes with less well defined signalling roles (positive and negative) [42,43], unknown genes that have been recently discovered by microarray techniques [44] and other p53-induced genes (PIGs) which were isolated on the basis of their induction following DNA damage by reactive oxygen species which may also function in p53-dependent apoptosis [45].

LAV694 strongly increased granzyme B mRNA levels in the HRE, whereas 5-FU induced a much weaker increase.

Granzyme B has recently been described to be involved in keratinocyte apoptosis [46]. Granzymes are serine proteases expressed almost exclusively by cytotoxic T cells and NK cells, and stored with perforin, a protein that forms pores in lysosome-like secretory granules [47]. Human keratinocytes have also been shown to express granzyme B [46] in confluent cultures. Granzyme B activates caspases and mimics the cleavage of their downstream substrates, thereby contributing to the induction of perforin-dependent apoptosis [48,49].

We have therefore identified the activation by LAV694 of several apoptotic pathways in the epidermis, involving Bax, p53, caspases 3 and 9 and granzyme B. Thus, the main novelty value of LAV694 is the selective induction of apoptosis, rather than necrosis, in those keratinocytes whose growth is inhibited after treatment, as shown *in vitro* using a HRE and *in vivo* in minipigs. This mode of action results in an improved skin tolerability in comparison with standard AK therapies.

References

[1] Salasche SJ. Epidemiology of actinic keratoses and squamous cell carcinoma. J Am Acad Dermatol 2000;42:4–7.

[2] Cockerell CJ. Histopathology of incipient intraepidermal squamous cell carcinoma. J Am Acad Dermatol 2000;42:11–7.

[3] Dinehart SM. The treatment of actinic keratoses. J Am Acad Dermatol 2000;42:25–8.

[4] Nussbaumer P, Winiski A, Cammisuli S, Hiestand P, Weckbecker G, Stuetz A. Novel antiproliferative agents derived from lavendustin A. J Med Chem 1994;37:4079–84.

[5] Cammisuli S, Winiski A, Nussbaumer P, Hiestand P, Stutz A, Weckbecker G. SDZ 281-977: a modified partial structure of lavendustin A that exerts potent and selective antiproliferative activities *in vitro* and *in vivo*. Int J Cancer 1996;65:351–9.

[6] Ure I, Niederecker C, Nussbaumer P. SDZ LAP 977, a potent and selective anti-proliferative agent indicates therapeutic efficacy in AK. Skin Pharmacol 1996;9:166–7.

[7] Nussbaumer P, Billich A, Meingassner JG, Winiski AP. SDZ LAV 694: a novel antiproliferative agent for topical use (Abstract). Drug Future 2002;27(Suppl A):61.

[8] Rosdy M, Clauss LC. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air–liquid interface. J Invest Dermatol 1990;95:409–14.

[9] De Brugerolle de Fraissinette A, Picarles V, Chibout S, Kolopp M, Medina J, Burtin P, Ebelin M, Osborne S, Mayer FK, Spake A, Rosdy M, De Wever B, Ettlin RA, Cordier A. Predictivity of an *in vitro* model for acute and chronic skin irritation (SkinEthic®) applied to the testing of topical vehicles. Cell Biol Toxicol 1999;15:121–35.

[10] Danpure CJ. Lactate dehydrogenase and cell injury. Cell Biochem Funct 1984;2:144–8.

[11] Barker JN, Jones ML, Mitra RS, Crockett-Torabe E, Fantone JC, Kunkel SL, Warren JS, Dixit VM, Nickoloff BJ. Modulation of keratinocyte-derived interleukin-8, which is chemotactic for neutrophils and T-lymphocytes. Am J Pathol 1991;139:869–76.

[12] Boelsma E, Tanojo H, Boddé HE, Ponec M. Assessment of the potential irritancy of oleic acid on human skin: evaluation *in vitro* and *in vivo*. Toxicol In Vitro 1996;10:729–42.

[13] Boelsma E, Tanojo H, Boddé HE, Ponec M. An *in vivo–in vitro* study of the use of a human skin equivalent for irritancy screening of fatty acids. Toxicol In Vitro 1997;11:365–76.

[14] Griffiths CEM, Barker JNWN, Kunkel S, Nickoloff BJ. Modulation of leucocyte adhesion molecule, a T cell chemotaxin (IL-8) and a regulatory cytokine (TNF-α) in allergic contact dermatitis (rhus dermatitis). Br J Dermatol 1991;124:519–26.

[15] Mohamadzadeh M, Muller M, Hultsch T, Enk A, Saloga J, Knop J. Enhanced expression of IL-8 in normal human keratinocytes and human keratinocyte cell line HaCaT *in vitro* after stimulation with contact sensitizers, tolerogens and irritants. Exp Dermatol 1994;3:298–303.

[16] Schröder JM. Cytokine networks in the skin. J Invest Dermatol 1995;105:20S–4S.

[17] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.

[18] Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322–6.

[19] Evan G, Littlewood T. A matter of life and cell death. Science 1998;281:1317–22.

[20] Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312–6.

[21] Svendsen O. The minipig in toxicology. Scand J Lab Anim Sci 1998;25:1–15.

[22] Xing QF, Lin S, Chien YW. Transdermal testosterone delivery in castrated Yucatan minipigs: pharmacokinetics and metabolism. J Control Release 1998;52:89–98.

[23] Kamentsky LA, Burger DE, Gershman RJ, Kamentsky LD, Luther E. Slide-based laser scanning cytometry. Acta Cytologica 1997;41:123–43.

[24] Savill J. Recognition and phagocytosis of cells undergoing apoptosis. Br Med Bull 1997;53:491–508.

[25] Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR. Immunosuppressive effects of apoptotic cells. Nature 1997;390:350–1.

[26] Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptosis cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF. J Clin Invest 1998;101:890–8.

[27] Gao Y, Herndon JM, Zhang H, Griffith TS, Ferguson TA. Antiinflammatory effects of CD95 ligand (FasL)-induced apoptosis. J Exp Med 1998;188:887–96.

[28] Ronchetti A, Rovere P, Iezzi G, Galati G, Heltai S, Protti MP, Garancini MP, Manfredi AA, Rugarli C, Bellone M. Immunogenicity of apoptotic cells *in vivo*: role of antigen load, antigen-presenting cells, and cytokines. J Immunol 1999;163:130–6.

[29] Albert ML, Pearce SFA, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N. Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta_5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med 1998;188:1359–68.

[30] Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, Culhane AC, Williams GT. Apoptosis: molecular regulation of cell. Eur J Biochem 1996;236:1–26.

[31] Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC. Inhibition of Bax channel-forming activity by Bcl-2. Science 1997;277:370–2.

[32] Lazebnik YA, Cole S, Cooke CA, Nelson WG, Earnshaw WC. Nuclear events of apoptosis *in vitro* in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. J Cell Biol 1993;123:7–22.

[33] Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 1996;86:147–57.

[34] Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. FASEB J 1996;10:587–97.

[35] Stennicke HR, Salvesen GS. Caspases—controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 2000;1477:299–306.

[36] Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326:1–16.
[37] Amundson SA, Myers TG, Fornace Jr AJ. Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. Oncogene 1998;17:3287–99.
[38] Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293–9.
[39] Owen-Schaub LB, Zhang W, Cusack JC, Angelo L, Santee SM, Fujiwara T, Roth A, Deisseroth A, Zhang W, Kruzel E, Radinsky R. Wild-type human p53 and a temperature - sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol 1995;15:3032–40.
[40] Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med 1998;188:2033–45.
[41] Wu GS, Burns TF, McDonald Jr ER, Jiang W, Meng R, Krantz ID, Kao G, Gan DD, Zhou JY, Muschel R, Hamilton SR, Spinner NB, Markowitz S, Wu G, el-Deiry WS. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997;17:141–3.
[42] Yin Y, Terauchi Y, Solomon GG, et al. Involvement of p85 in p53-dependent apoptotic response to oxidative stress. Nature 1998;391: 707–10.
[43] Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR, Kley N. Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 1995;377:646–9.
[44] Saller E, Tom E, Brunori M, Otter M, Estreicher A, Mack DH, Iggo R. Increased apoptosis induction by 121F mutant p53. EMBO J 1999; 18:4424–37.
[45] Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. Nature 1997;389:300–5.
[46] Berthou C, Michel L, Soulie A, Jean-Louis F, Flageul B, Dubertret L, Sigaux F, Zhang Y, Sasportes M. Acquisition of granzyme B and Fas ligand proteins by human keratinocytes contributes to epidermal cell defense. J Immunol 1997;159:5293–300.
[47] Smyth MJ, Trapani JA. Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol Today 1995;16:202–6.
[48] Shi L, Kraut RP, Aebersold R, Greenberg AH. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J Exp Med 1992;175:553–66.
[49] Trapani JA, Browne KA, Smyth MJ, Jans DA. Localization of granzyme B in the nucleus. A putative role in the mechanism of cytotoxic lymphocyte-mediated apoptosis. J Biol Chem 1996;271: 4127–33.