Human papillomavirus (HPV) infection is strongly associated with the development of anogenital neoplasia, particularly cervical cancer. It has been estimated that 99.7% of all cervical carcinomas are attributable to infection with HPV, and types 16 and 18 account for the vast majority of such cases. Both of these high-risk HPV types encode the oncoproteins E6 and E7, which exert multiple effects on many proteins involved in cell-cycle regulation, including p53. The nuclear export protein inhibitor leptomycin B (LMB) has been shown to cause the nuclear sequestration of p53 in cervical carcinoma cells. We demonstrate that LMB induces apoptosis in monolayer and organotypic raft cultures of transduced PHKs expressing HPV oncoproteins. Both monolayer and organotypic raft cultures of transduced PHKs were highly susceptible to treatment with LMB. By contrast, although LMB inhibited p53 accumulation in normal PHKs, no significant induction of apoptosis was detected on Western blots or immunostained monolayer/raft cells, or following pulsed exposure to the drug. Furthermore, topical application of pM concentrations of LMB to mouse skin was non-toxic. These data suggest that the application of LMB to HPV-infected intra-epithelial lesions may represent a specific and effective therapeutic strategy against HPV-associated anogenital neoplasia.

Key words: human papillomavirus; leptomycin B; keratinocytes; oncogenes; apoptosis

Leptomycin B (LMB), a secondary metabolite produced by Streptomyces spp., inhibits the export of proteins containing a nuclear export signal (NES) from the nucleus to the cytoplasm. This is achieved through inactivation of the nuclear export receptor protein CRM1 (exportin 1) by covalent modification. LMB therefore promotes nuclear accumulation of NES proteins, including the tumour suppressor protein p53. Furthermore, LMB has been shown to cause nuclear sequestration and reactivation of p53 in cervical carcinoma cell lines containing human papillomavirus (HPV) and this was associated with induction of apoptosis. By contrast, LMB treatment resulted in the reversible cell-cycle arrest of both rat fibroblasts and normal human primary fibroblasts. Although the effect of LMB on CRM1 is well documented, the specific molecular mechanism(s) by which LMB induces cell-cycle arrest and apoptosis still remain unclear.

Since the discovery of LMB in the early 1980s, there has been considerable interest in the use of the compound as a cancer therapeutic. LMB (alternatively termed CI-940, elactocin or PD 114, 720) was shown to possess potent activity against 7 different tumour models. Based on these encouraging findings, a phase 1 clinical trial was initiated to assess the systemic administration of LMB. However, the trial was later terminated because of unwanted side effects. Nevertheless, although there are difficulties in using LMB systemically, topical therapeutic use may be feasible.

Cervical cancer is the second most common malignant neoplasm recorded in the female population worldwide, accounting for almost 10% of all cancers (excluding non-melanoma skin cancers). The main aetiological factor in the development of squamous neoplasia of the cervix is infection with HPV, with an estimated worldwide HPV prevalence in cervical carcinomas of 99.7%. Specific HPV types have also been linked as the causative agent in over 50% of other anogenital cancers. HPV types are termed ‘high risk’ (e.g., HPV 16) or ‘low risk’ (e.g., HPV 6) according to their prevalence in cancers. The ‘high risk’ HPV types encode 2 major oncoproteins, E6 and E7, which interact with multiple proteins involved in cell-cycle regulation including p53 and pRb and cause the eventual disruption of cell-cycle machinery and DNA repair mechanisms. During a productive HPV infection, the viral genome is maintained in an episomal form and the E6 and E7 genes are expressed at low levels. Integration of the virus into the host genomic DNA is an important step in neoplastic transformation. The integration process generally results in disruption of the HPV E1/E2 DNA region, leading to failure of the vegetative viral DNA replication process and constitutive expression of the E6 and E7 genes following the loss of transcriptional repression by E2.

Mutations in the p53 protein, with concomitant loss of wild-type p53 function, are found in over half of all human tumours. The p53 protein is a powerful transcription factor (affecting ~300 promoter elements within the genome) that can orchestrate cell-cycle arrest and/or apoptosis under appropriate situations of cell stress. Unlike many other types of human cancer, a wild-type p53 gene is usually retained in HPV-associated cervical tumours. Although the ‘high risk’ HPV E6 and E7 proteins have pleiotropic effects on apoptotic pathways within the host cell, a central role of the E6 protein is the degradation of p53 via the ubiquitin-proteasome pathway thus obviating the need for p53 mutation. Moreover, although p53 is stabilised by the E7 protein, the resulting p53 molecules appear to be transcriptionally inert. It is therefore possible that the p53 sequestering activity of LMB may be utilised to enhance the apoptotic effect of wild-type p53 in cells expressing E6 and E7 proteins. If this were the case, LMB could prove a useful therapeutic agent against cervical cancer and other HPV-associated neoplasms.

In this study, we have investigated the effects of LMB on human keratinocytes expressing HPV oncogenes to test the hypothesis that this compound can selectively induce apoptosis in primary cells expressing viral oncogenes. In addition to monolayer culture,

Abbreviations: act-casp-3, activated-caspase-3; AEC, 3-amino-9-ethylcarbazole; CK18, cytokeratin 18; DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; HPV, human papillomavirus; HRP, horse radish peroxidase; KBM-2, keratinocyte basal medium-2; LDS, lithium dodecyl sulphate; LMB, leptomycin B; MoMuLV, moloney murine leukaemia virus; NES, nuclear export signal; PBS, phosphate buffered saline; PHKs, primary human keratinocytes; SDS, sodium dodecyl sulphate.

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organotypic raft culture was employed to mimic a stratified squamous epithelium. The effect of LMB on normal mouse skin was also examined.

Material and methods

Cell lines and culture

Primary human keratinocytes (PHKs) from neonatal foreskin (Cambrex) were cultured in keratinocyte basal medium-2 (KBM-2) containing the supplied supplements (Cambrex). Retroviral packaging lines (PA317) containing wild-type human papillomavirus (HPV) 16 E7, 16 E6, 16 E6/E7, 6 E6 and 6 E7 genes cloned into the pLXSN vector and under the control of the Moloney murine leukemia virus (MoMuLV) promoter–enhancer sequences24 were obtained from the ATCC, and were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

Organotypic raft culture of PHKs was carried out as described previously.25 After culture for 12 days, the rafts were fixed in 10% neutral buffered formalin for 24 hr, and then embedded in paraffin wax.

Leptomycin B

Leptomycin B (LMB) was a gift from Novartis, and was stored as a 10 mM stock solution in absolute ethanol. LMB was diluted as appropriate in medium before addition to cell cultures.

Generation of transduced PHKs

Packaging lines were left overnight in KBM-2 to allow viral particles to accumulate. The virus-containing supernatants were filtered (0.45 μm cellulose acetate filter), and then combined with an equal volume of KBM-2 containing hexadimethrine bromide (polybrene) at 1 μg/ml. Five millilitres of this solution was added to monolayer PHKs at ~10% confluency in 25 cm² flasks, and left in direct contact with the cells for 7 hr to allow infection to occur. Fresh medium was then added and the supernatant was discarded. Cells were allowed to recover for 24 hr, followed by selection with 150 μg/ml G418 for 4 days. After this time, all non-infected control cells exposed to the same concentration of G418 were dead.

Western blot analysis

Subconfluent cell cultures were washed twice in ice-cold phosphate buffered saline (PBS), then scraped into PBS and pelleted. Cell pellets were resuspended in 1% lithium dodecyl sulphate (LDS) buffer (Invitrogen) plus 1 mM dithiothreitol (DTT), heated for 5 min at 70°C and then sonicated 3 times for 10 sec, before centrifugation at 13,000g for 15 min. The protein concentrations within the supernatants were determined according to Bradford.26 Equivalent amounts of protein were separated on sodium dodecyl sulphate (SDS)/polyacrylamide gels (Bio-Rad) and transferred to Immobilon-P filters (Millipore). Proteins were detected using the following primary antibodies: p53 DO-1 mouse monoclonal (1:1000, from the laboratory of D.P. Lane, University of Dundee);27 M30 mouse monoclonal (1:500, Roche) and cytokeratin 18 (CK18) mouse monoclonal (1:500, from the laboratory of E.B. Lane, University of Dundee). Horse radish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody (1:1000, Dako) was added before detection with enhanced chemiluminescence (ECL from Amersham BioSciences). Application of an actin mouse monoclonal primary antibody (1:5000, Oncogene Research) was used to confirm equal loading of proteins across the blot.

Immunocytochemistry

For monolayer culture, cells were grown in Lab-Tek chamber slides (Nalgene) at 1 × 10⁵ cells/ml for 72 hr, washed in PBS and then fixed in ice-cold methanol for 30 min at −20°C.Slides were rinsed twice in PBS, and then blocked with PBS + 1% BSA for 10 min, before incubating with primary antibody for 60 min. The primary antibodies used were M30 mouse monoclonal (Roche); cleaved caspase-3 (Asp175) (act-casp-3) rabbit polyclonal (Cell Signaling) and CK18 mouse monoclonal (from the laboratory of E.B. Lane). Slides were rinsed twice in PBS, then incubated with either biotinylated rabbit anti-mouse F(ab′)² fragment (Dako) or biotinylated swine anti-rabbit F(ab′)² fragment (Dako) for 30 min. Antibodies were applied at the dilutions recommended by the manufacturer; CK18 was diluted 1:5 prior to use. A further 2 washes in PBS were carried out before incubation of cells with streptavidin-HRP (1:150, Dako) for 30 min. Detection was carried out using 3-amino-9-ethylcarbazole (AEC) (Sigma) after a final rinse in PBS. Cells were counterstained with haematoxylin (VectorLabs) and coverslips were mounted in glycerol buffer.

Positively stained cells were counted systematically from 1 edge of the slide using a 10 × 10 grid at 400× magnification. Cells were counted until the proportion of positive cells reached a plateau, as assessed using the running mean method.

For organotypic raft culture, antigen retrieval was performed on 5-μm thick sections of wax-embedded material. After dewaxing, slides were washed briefly in tap water before placing in 0.01 M citrate buffer (pH 6) in a pressure cooker heated in a microwave (850 W) at full power for 15 min. Slides were then washed in tap water and then subjected to immunocytochemistry as described earlier for monolayer cells. The primary antibodies used were p53 DO-1 mouse monoclonal (1:50, from the laboratory of D.P. Lane); p21WAF1/CIP1 118 mouse monoclonal (1:100, Dako);28 act-casp-3 rabbit monoclonal (1:50, Cell Signaling) and CK18 mouse monoclonal (1:50, from the laboratory of E.B. Lane). Biotinylated horse anti-mouse or goat anti-rabbit (Vector Elite) were used as secondary antibodies and the signal was developed using the Vector Elite staining kit with 3,3'-diaminobenzidine (DAB) as chromogen.

DAPI staining of cells

Monolayer cells were grown on slides at 5 × 10⁵ cells/ml for 72 hr, treated with LMB and then mounted in 4'-6-diamidino-2-phenylindole (DAPI) solution (Vysis) before analysis using fluorescence microscopy.

Treatment of cells

To assess the recovery of cells after LMB treatment, subconfluent PHK cultures were exposed to LMB for 40 hr. The medium plus LMB was then removed and cells were washed twice with PBS and twice with medium, and then fresh medium was applied. Cultures were examined daily for 7 days. For Western analysis, immunocytochemistry and DAPI staining of cells, the PHK cultures were treated with LMB for 38–44 hr.

Organotypic raft cultures were left in contact with medium containing the appropriate concentration of LMB for 72 hr before fixation, processing and analysis.

Treatment of mouse skin

LMB was diluted in ethanol and applied to the skin (20 mm² area) of nude mice (Harland, UK). After the treatments, mice were sacrificed and treated areas were fixed in formalin and embedded in paraffin wax. Sections were stained with CM-5 rabbit serum against murine p53 (from the laboratory of D.P. Lane). All experiments were performed in accordance with the guidelines of the UK Co-ordinating Committee on Cancer Research (UKCCCR).

Results

LMB induces apoptosis in monolayer PHKs expressing HPV oncoproteins

Phase contrast microscopy of PHKs transduced with HPV oncoproteins indicated that apoptosis was induced within these cells following treatment with 2 nM LMB (Fig. 1a, panels 3–8), however little evidence of apoptotic activity was detected in uninfected cells under the same conditions (Fig. 1a, panels 1 and 2). Cell necrosis and detachment were clearly visible in PHKs containing HPV 16 E7, with a less marked apoptotic effect in cells expressing...
HPV 16 E6/E7. Cells expressing HPV 16 E6 were the least affected by LMB. Immunofluorescence imaging of DAPI-stained cells confirmed these findings; enlarged cell nuclei/nuclear fragmentation were present in the LMB-treated transduced lines (Fig. 1b, panels 5 and 6). By contrast, this cytopathic effect was not evident in normal PHKs under the same treatment conditions (Fig. 1b, panels 7 and 8). Following the withdrawal of 2 nM LMB after a 40 hr period of exposure, the majority of normal PHKs survived; cell division was stalled for 96 hr, after which time cell growth appeared to resume and progress until the experiment was discontinued (7 days post-LMB treatment). However, in PHKs expressing HPV 16 E7 and 16 E6/E7, universal apoptosis, as determined by detachment and lysis of all the cells, was observed 24 and 96 hr, respectively, following removal of LMB (Fig. 2).

The M30 antibody identifies a specific cleavage product of the intermediate filament protein CK18 produced during the early stages of apoptosis.29 The act-casp-3 antibody detects a cleavage product of one of the key executioners of apoptosis, activated-caspase-3.30 M30 and act-casp-3 antibodies were used to detect the relevant markers of apoptosis within monolayer transduced PHKs using immunocytochemistry (Fig. 1b, panels 1–4 and Fig. 3). Both the M30 and act-casp-3 antibodies produced comparable results. M30 as well as p53 and CK18 levels were analysed in keratinocytes expressing HPV 16 oncogenes by Western blotting (Fig. 4a). LMB treatment resulted in a moderate increase in p53 levels in HPV 16 E7-containing PHKs, with a much more moderate p53 elevation in cells expressing both high risk HPV genes and only very slight accumulation of p53 in cells infected with HPV 16 E6. The LMB-induced p53 elevation present in PHKs expressing the HPV16 E7 gene occurred within 12 hr of treatment and remained at a relatively constant level until 48 hr post-LMB treatment (Fig. 4b). CK18 was constitutively expressed within all of the cell lines tested, regardless of treatment with LMB. The M30 target protein was detected in PHKs + HPV 16 E7, and to a lesser extent in PHKs + HPV16 E6/E7, with only a very faint band detected in the lane corresponding to PHK +16 E6 cells. Although the amount of p53 present on Western blots was elevated in normal PHKs following treatment with LMB, M30 was not detected (Fig. 4a).

Immunocytochemistry on monolayer PHKs revealed that expression of the apoptotic marker act-casp-3 was appreciably increased in PHKs transduced with HPV16 E7 or E6/E7 following treatment with LMB (Fig. 3). In PHKs + HPV 16 E7, the number of act-casp-3 staining cells increased from 0.6 to 19.7% (19.1% increase) after LMB treatment. In PHKs transduced with HPV16 E6/E7, LMB caused an increase in the number of cells staining positive for act-casp-3 from 0.6 to 8.9% (8.3% increase). No substantial increase in the percentage of cells expressing act-casp-3 was recorded in any of the other cells tested (normal PHKs, PHK + pLXSN vector only, PHK with HPV 16 E6 and PHK with HPV 6 E7) following LMB treatment with increases of between 0.9 and 2.2% recorded. The M30-specific antibody was also tested on the cell lines (data not shown). As was observed for act-casp-3, no appreciable increase in apoptosis was observed in the normal and vector only cells (normal PHKs, increase of 1.5%; PHK + pLXSN, increase of 1.8%) but the number of PHK + 16 E7 cells staining positive for M30 was noticeably increased (20.9% increase) after LMB exposure.

Apoptotic response of transduced PHK raft cultures to LMB

The use of organotypic raft cultures allows the effect of LMB to be tested on a cellular structure more closely resembling human skin. After exposure to 20 nM LMB, the stratified epithelium pres-
Raft culture of PHKs containing the pLXSN vector alone produced a thin epithelium containing differentiated cells, which was morphologically indistinguishable from raft culture of normal PHKs. By contrast, the organotypic rafts produced by growing keratinocytes containing the HPV 16 E6/E7 genes exhibited a thick epithelium with undifferentiated cells present throughout. The morphological differences exhibited by these 2 types of raft complicates comparison, but no noticeable disruption of the epithelial layer was apparent in the pLXSN raft following exposure to 50 nM LMB, as exemplified by the even expression of CK18 throughout both treated and untreated sections (Fig. 5b). Although clear images were unobtainable because of the flattened appearance of the pLXSN-expressing epithelia, no obvious increased expression of act-casp-3 was recorded after treatment with 50 nM LMB.

Topical application of micromolar concentrations of LMB to mouse skin is non-toxic

The toxicity of LMB was tested on normal mouse epidermis. To facilitate the application procedure, these experiments were carried out with hairless mice. 5 μl of LMB at the indicated concentrations (and dissolved in ethanol) was applied to a skin surface area of ~20 mm². After a single application of LMB at 250 μM, drug activity was monitored using p53 induction. At 24 and 48 hr after LMB application, an increase in the levels of p53 was consistently observed by immunostaining (Fig. 6). At 96 hr after LMB treatment, p53 levels decreased to initial levels. The appearance of the skin was normal during the full course of the experiment. The effect of repeated applications of LMB was also tested. With 250 μM LMB, experiments had to be terminated after the second application as this dose caused the appearance of areas of ulceration in the skin. Application of 25 μM LMB twice per week over a 5-week period did not, however, lead to any detectable lesions.

Discussion

In the present study, we provide evidence that LMB can selectively induce apoptosis in keratinocytes expressing HPV 16 oncoproteins, as shown by morphological assessment, Western analysis and immunostaining on monolayer and rafted cells. Keratinocytes expressing the HPV 16 E7 gene exhibited the greatest induction of both p53 and apoptosis. The increased p53 activation triggered by LMB was augmented by the presence of E7, possibly as a consequence of p53 stabilisation, and/or the many other biological effects of the HPV 16 E7 polypeptide.

Only very slight induction of p53 was detected in PHKs transduced with HPV 16 E6, with no appreciable apoptosis identified by immunostaining or Western analysis. It is probable that any LMB-stimulated p53 production has been thwarted by E6-mediated degradation via the ubiquitin-proteasome pathway. Indeed, a central function of the E6 protein is to inactivate pro-apoptotic proteins such as p53, Bak or Bax proteolytically, thereby protecting against cell death. It has been demonstrated that the HPV 16 E6 protein preferentially localises to the nucleus of cervical cancer cells and that HPV 18 E6 is co-expressed with p53 from the nucleus to the cytoplasm. LMB treatment of cells expressing 'high risk' HPV E6 results in a block of p53 nuclear export and stabilisation of p53, however this LMB-induced reduction in HPV 18 E6-mediated degradation was shown to be only partial, suggesting that both nuclear and cytoplasmic proteasomes can target p53 for degradation. Our data indicate that despite any predicted LMB-induced E6-mediated p53 stabilisation, the level of E6-mediated p53 degradation observed following LMB treatment is still strong enough to allow a significant protective effect to cells against LMB-induced apoptosis. This evidence provides a strong incentive for the use of LMB in a clinical environment.
The expression of HPV 16 E6 and E7 in combination most closely resembles the situation present in high grade intraepithelial lesions and invasive carcinomas. In PHKs expressing both of these genes, treatment with LMB resulted in considerable levels of p53 induction and apoptosis, in monolayer and raft culture. Thus, it would appear that, following LMB treatment, the opposing effects of E7-mediated p53 stabilisation and proteolytic p53 degradation mediated by E6 produce net p53 activation and induction of apoptosis, when compared with normal/vector-only keratinocytes. It is unknown whether the apoptotic response observed in PHKs + HPV 16 E6/E7 is triggered specifically by p53 or by a combination of p53 with other proteins but, given the general cellular action of LMB as an inhibitor of NES protein nuclear export, we might hypothesise that the latter option is more likely. However, the cytotoxicity of LMB on human neuroblastoma cells and normal human fibroblasts was attenuated by the inhibition of wild-type p53 (through overexpression of a dominant negative p53 protein), indicating that p53 induction has an important function in LMB-mediated cell death.5

No obvious induction of apoptosis was detected in monolayer cells expressing the HPV 6 E6 and E7 genes following treatment with 2 nM LMB. In contrast to the oncogenes from ‘high risk’ HPV types, the equivalent ‘low risk’ molecules have low transforming activity.37,38 Compared to HPV 16 E7, the equivalent HPV 6 protein has a lower affinity for pRb and is phosphorylated to a lesser extent.39,40 Furthermore, although HPV 6 E6 can bind to the C-terminus of p53, it cannot bind to the p53 core region, which is required for the induction of p53 degradation.41 The inability of LMB to facilitate widespread apoptosis in PHKs transduced with HPV 6 oncogenes may be related to such differences in activity on key cell-cycle regulators such as p53 and pRb. The failure of 2 nM LMB to induce appreciable apoptosis in monolayer cells expressing HPV 6 E6 and E7 genes suggests that LMB would not be appropriate as a treatment for warts, which usually represent benign hyperproliferation of epithelial cells infected with ‘low risk’ types, including HPV 6.42,43

Although p53 levels were induced in normal cells, no evidence of apoptosis was detected by Western analysis. In addition, immunocytochemistry on monolayer pLXSN-only and normal cells showed only a small increase in the expression of act-casp-3, suggesting that LMB would not be appropriate as a treatment for warts, which usually represent benign hyperproliferation of epithelial cells infected with ‘low risk’ types, including HPV 6.42,43

Although p53 levels were induced in normal cells, no evidence of apoptosis was detected by Western analysis. In addition, immunocytochemistry on monolayer pLXSN-only and normal cells showed only a small increase in the expression of act-casp-3 and this increase was markedly less than that observed after LMB treatment of HPV16 E7 or E6/E7-containing cells. Morphologically, normal PHKs remained viable following the withdrawal of LMB after a defined period of exposure. This was in sharp contrast to the situation observed with HPV 16 E7 and 16 E6/E7-containing cells, in which universal apoptosis was observed 24 and 96 hr, respectively, after removal of LMB. These findings are in agreement with previous studies on normal rat2 and human fibroblasts in which treatment with LMB resulted in the promotion of a reversible cell-cycle arrest instead of apoptosis. Smart et al.5

**Figure 3**– Proportion of LMB-treated transduced PHKs expressing act-casp-3. Immunocytochemistry was carried out on monolayer cells after treatment with 2 nM LMB (+). Cells which were not exposed to LMB (−) were subjected to identical experimental procedures to act as controls. Bars represent ±2× standard error of the proportion (SEP) and n represents the total number of cells counted.

**Figure 4**– (a) Effect of LMB on protein levels in PHKs expressing HPV16 oncogenes. Western blot analysis was performed on proteins extracted from monolayer cells plus (+) or minus (−) treatment with 2 nM LMB. (b) Time course of p53-expression in LMB-treated PHKs transduced with HPV 16 E7. Monolayer cells were harvested for Western analysis following treatment with LMB for the durations listed.
reported that normal fibroblast cultures exhibited a senescence-like phenotype following treatment with LMB, which was overcome after removal of the drug and the subsequent trypsinisation of cells.

The structural integrity of organotypic raft cultures of HPV 16 E6/E7-containing keratinocytes was severely disrupted following treatment with 50 nM LMB. At this concentration, there was almost complete breakdown of the stratified epithelium and widespread apoptosis was apparent, both morphologically and by act-casp-3 expression. This effect was not induced by LMB at 2 nM, which was highly effective at inducing apoptosis in the less robust monolayer PHK cells. It is probable that the therapeutic use of LMB to treat lesional tissue in vivo would require >50 nM concentrations of the drug as a topical application, however mouse...
skin remained unaffected by the repeated application of 25 μM LMB, suggesting that normal tissue would be able to tolerate exposure to concentrations well above the 50 nM range, which induced widespread apoptosis in organotypic culture of keratinocytes expressing HPV 16 E6/E7.

Although act-casp-3 was induced in HPV 16 E6/E7-containing rafts by 50 nM LMB, p53 expression was not detected in rafts at this LMB concentration, and only very few cells were weakly positive for p53 following treatment with 20 nM LMB. This may be indicative of a temporal lag in sampling time so that the p53 expression induced by exposure to 50 nM LMB has been exhausted by the time the raft is fixed and cells are no longer expressing this molecule. Alternatively, a p53-independent cellular mechanism may be involved in the induction of apoptosis by LMB. As previously mentioned, p53 appears to play an important role in the mechanism of LMB-induced apoptosis, but this does not exclude the possibility that other molecules are involved. p21 was expressed throughout the HPV 16 E6/E7-containing PHK raft, particularly in the supra-basal layers, at all concentrations of LMB and without LMB exposure. This is not unexpected given that HPV 16 E7 increases cellular p21 protein levels, and this may mask any additional LMB-induced p21 effect.

In an ideal therapeutic situation, HPV 16-positive cells expressing the E6 and E7 oncoproteins would be preferentially targeted by LMB, causing disruption of the abnormal epithelium, which would ultimately shed, leaving the surrounding tissue relatively unscathed. No structural damage or obvious activation of apoptosis was observed in the pLXSN-expressing PHK raft or 50 nM LMB. LMB was non-toxic when applied to mouse skin at concentrations well above those capable of inducing widespread apoptosis in PHKs expressing HPV 16 E6 and E7. Moreover, as determined by Western analysis, the p53 accumulation induced by LMB occurs at, or before, 12 hr exposure to the drug. This suggests that keratinocytes expressing the HPV 16 E7 gene respond rapidly and implies that short drug-exposure times would be therapeutically effective.

In conclusion, the selective induction of apoptosis by LMB in keratinocytes expressing HPV 16 oncogenes suggests that this drug may represent an effective and specific topical treatment for HPV-associated anogenital neoplasia.

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