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Ultra-deformable liposomes containing bleomycin: In vitro stability and toxicity on human cutaneous keratinocyte cell lines

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Abstract

Formulations of ultra-deformable liposomes containing bleomycin (Bleosome^{TM}) have previously been described and proposed for topical treatment of skin cancer [Lau, K.G., Chopra, S., Maitani, Y., 2003. Entrapment of bleomycin in ultra-deformable liposomes. S. T. P. Pharm. Sci. 13, 237–239]. In this study, the stability of various Bleosome^{TM} formulations was characterised and a purification process was established to isolate Bleosome^{TM} for testing on cultures of either human cutaneous keratinocytes (NEB-1) immortalised by human papilloma virus (HPV)-type 16, or a spontaneously immortalised human squamous cell carcinoma (SCC) from a primary tumour. Bleosome^{TM} facilitated entrapment of high concentrations of active bleomycin and samples purified by gel-filtration chromatography remained stable during 7 days of storage at 4 °C or at room temperature. Serially-diluted samples of this purified, high-strength product, 'high dose' were applied onto keratinocyte cell cultures to elucidate Bleosome^{TM} LD_{50} profiles.

In vitro data revealed that the LD_{50} of bleomycin encapsulated in Bleosome^{TM} was approximately three-fold higher than free bleomycin solution for SCC cells, and nearly 30 times higher for NEB-1 cells. However, Bleosome^{TM} containing 30 μg/ml of active bleomycin killed more than twice as many SCC cells than NEB-1 cells. At that concentration, the potency of liposomal bleomycin on causing cell death of SCC cells was found to be similar to that of free bleomycin solution. This effect was not seen on NEB-1 cells. It seems that SCC cells were particularly susceptible to Bleosome^{TM} containing high levels of bleomycin. Results from these experiments promote the development of a novel product for the topical treatment of skin cancer.
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Keywords: Ultra-deformable liposome; Bleomycin; Keratinocyte cell; Squamous cell carcinoma; Toxicity; In vitro stability

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1. Introduction

Non-melanoma skin cancer (NMSC) is the most commonly diagnosed cancer in the UK. The incidence of NMSC is rising rapidly and is an important cause of morbidity in the general population and is an increasing burden on healthcare resources.

Ultra-deformable liposomes have shown potential as a carrier for topical drug delivery systems because they can penetrate the skin intact (Cevc et al., 2002). They can be loaded with a wide variety of different therapeutic agents, such as low-molecular weight drugs like 5-fluorouracil (El Maghraby et al., 2001a) as well as larger molecules, such as oestradiol (El Maghraby et al., 2001b), triamcinolone acetonide (Cevc and Blume, 2003), interleukin-2 and interferon-α (Hofer et al., 1999), and even insulin (Cevc et al., 1998) and plasmid DNA (Kim et al., 2004). Bleomycin is an established anti-tumour drug used in the treatment of NMSC. Recent work has shown that bleomycin can be encapsulated in ultra-deformable liposomes and it has been suggested that this preparation may be useful for topical chemotherapy of NMSC (Lau et al., 2003). It is possible to investigate whether encapsulation of bleomycin in ultra-deformable liposomes might alter the lethal potency of this anti-tumour drug by in vitro testing on immortalised, human keratinocyte cell culture model systems. Furthermore, the stability assessment of candidate preparations would provide valuable evidence of which preparations might be viable and suitable for progression to clinical testing.

Several changes to the methodology of the previous study (Lau et al., 2003) have been pursued in these experiments in order to evaluate the possible benefits they might confer. The determination of bleomycin concentration has been conducted with a different solvent system and ion-pairing agent (Fiedler and Wachter, 1991, cf. Aszalos et al., 1981) that dramatically reduces the duration required to analyse each sample, thus allowing higher throughput and rapid monitoring of Bleosome^{TM} preparations. Gel-filtration chromatography was used in this study to isolate and purify Bleosome^{TM} preparations for keratinocyte toxicity studies. This technique is less invasive than ultra-centrifugation and would avoid the possibility of irreversible compaction and aggregation of Bleosome^{TM}. Nonetheless, ultra-centrifugation was still a suitable isolation technique in the course of determining bleomycin concentration in Bleosome^{TM} because liposomal integrity would ultimately be breached by addition of methanol in order to release the drug for assaying.

It was essential to test Bleosome^{TM} preparations on human cell types similar to the targeted diseased tissue because the response of human keratinocytes to drug treatment may be dissimilar from that of keratinocytes from murine or other animal sources. Cell cultures of human papilloma virus (HPV)-type 16 immortalised human keratinocytes (NEB-1) were used as a model for an immortalised human keratinocyte cell line that is not tumourigenic in nude mice (Morley et al., 2003). Cell cultures of spontaneously immortalised human squamous cell carcinoma (SCC) were grown from primary lesions (Purdie et al., 1993) and utilised as a model of human SCC. These two types of keratinocytes formed the basis of initial testing of Bleosome^{TM} toxicity in vitro and calculation of LD_{50} responses. Potency of Bleosome^{TM} could then be compared against the toxic profiles of free bleomycin solution at an equivalent concentration.

The aim of this study was to evaluate the in vitro bioefficacy of Bleosome^{TM} on immortalised and malignant human keratinocytes. Another objective was to assess the physical stability of such preparations in vitro, with regards to particle size and bleomycin retention. The results from these experiments will facilitate development of a clinical preparation suitable for testing on human subjects.

2. Materials and methods

2.1. Materials

Bleomycin hydrochloride for injection was purchased from Nippon Kayaku Co. (Tokyo, Japan). Egg phosphatidycholine (ePC) was purchased from Avanti Polar Lipids (Alabaster, AL). Sodium cholate was obtained from Sigma Chemicals (St. Louis, MO). Sodium perchlorate and other chemicals used were of reagent grade and purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Preparation of ultra-deformable, multi-lamellar liposomes containing bleomycin

Multi-lamellar liposomes (MLVs) were prepared using a modified, dry-film method. Briefly, formu-

lations of ePC, with (5, 10 or 16%, w/w) or without sodium cholate, were dissolved in ethanol, which was removed by rotary evaporation, in a nitrogen atmosphere. The dry-lipid film was hydrated with 10% (w/v) bleomycin hydrochloride solution and diluted with the appropriate strength of phosphate-buffered saline (PBS, pH 7.4) to yield a liposomal suspension of 1% bleomycin hydrochloride for a preliminary stability test, and with 1/10-diluted PBS (1/10 PBS) to yield 'low' dose Bleosome^{TM} for cytotoxicity test. Final lipid concentration was 0.1 mM. The 'high' dose Bleosome^{TM} was prepared by purification of by gel chromatography described in Section 2.5.

2.3. Determination of bleomycin entrapment efficiency

Bleomycin MLV suspensions were centrifuged at 100,000 × *g* for 360 min to separate the free bleomycin in the supernatant from the liposomal bleomycin. Upon removal, the liposome pellet was dissolved using methanol and the amount of active bleomycin congeners A2 and B2, and inactive A2 in 10 μl of the supernatant or pellet was determined by ion-pair high-performance liquid chromatography (HPLC), 717 plus autosampler, 600S controller, 616 pump (Waters; MA, USA) and UV detector at 240 nm (L-4000, Hitachi, Tokyo, Japan) with sodium perchlorate. Based on a method by Fiedler and Wachter (1991), a 5% (v/v) acetonitrile in 0.1 M phosphoric acid and 10 mM sodium perchlorate, pH 4.3 was increased to 25% (v/v) with line gradient over 20 min and then increased to 100% for 2 min and hold for a 2 min at room temperature and at a rate of 1 ml/min through a C_{18} (ODS) column (TSK-GEL, Tosoh, Tokyo, Japan) of 25 cm × 4.6 mm. The first two primary peaks represent active bleomycin A2 and B2, respectively, and the third major peak relates to inactive bleomycin A2 (Fig. 1). A calibration curve was produced using commercial bleomycin in the range of 1–1000 μg/ml.

2.4. Particle size and zeta-potential measurements

The average diameters of MLV vesicles were determined by dynamic light scattering using a laser light-scattering instrument (ELS 800, Otsuka Electronics Co. Ltd., Osaka, Japan). The samples were diluted in distilled water in order to avoid multiple scattering. The measurements were performed at 25 ± 0.1 °C, at an angle of 90° between laser and detector.

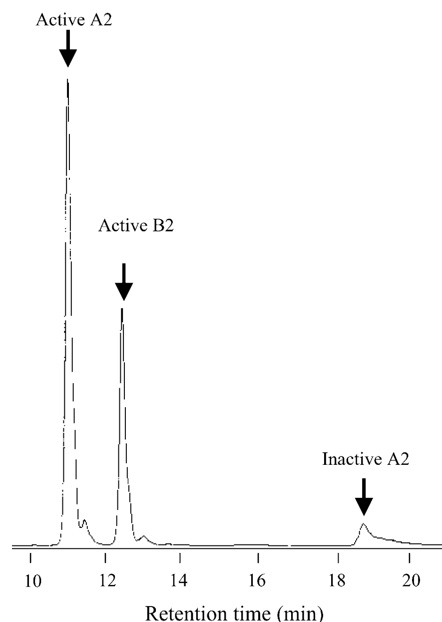

Fig. 1. High-performance liquid chromatography (HPLC) separation of a commercial sample of bleomycin hydrochloride revealing three major peaks: active bleomycin A2 (time = 10.9 min); active bleomycin B2 (time = 12.3 min) and inactive demethyl-bleomycin A2 (time = 18.6 min).

2.5. Liposome purification

Bleosome^{TM} containing bleomycin was separated from free bleomycin by gel-filtration chromatography. A column of Sephadex G-50 was prepared in a burette (25 cm × 1 cm) and equilibrated with 1/10 PBS. Eluent fractions of 1 ml/min were collected for 50 min. A UV-spectrophotometric trace revealed which fractions contained the highest concentrations of Bleosome^{TM}. The most concentrated three fractions (each fraction 1 ml) were pooled to form a stock suspension for testing on keratinocytes.

2.6. Stability study

A preliminary study of individual Bleosome^{TM} preparations in PBS was evaluated at two different

temperatures (room temperature at 25 °C and refrigerated at 4 °C) for 8 days. Samples were taken daily for analysis, as noted in Sections 2.3 and 2.4 above. Subsequent studies were only carried out on the preferred candidate preparation (10% (w/w) sodium cholate), which was kept in 1/10 PBS, at room temperature (25 °C), and sampled daily for 7 days. Due to batch-to-batch variation, it was necessary to monitor the stability of the actual preparation that was used for the keratinocyte toxicity tests.

2.7. Cell culture

Two types of keratinocytes were tested with 'high' (1091.5 ± 25.8 μg/ml active bleomycin; $n = 3$) and 'low' (22.3 ± 8.2 μg/ml active bleomycin; $n = 3$) dose Bleosome™. Firstly, NEB-1 cells were cultured and used as a model for human papilloma virus (HPV)-associated disease. Secondly, SCC cells were grown and used as a model for NMSC in general. These cell lines were maintained in standard keratinocyte tissue culture medium: Dulbecco's modified Eagle's medium with 25% Ham's F12 medium, 10% fetal calf serum, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, 10^{-10} mol/l cholera toxin, 5 μg/ml transferrin, 2×10^{-11} mol/l lyothyronine, 1.9×10^{-4} mol/l adenine and 5 μg/ml insulin, supplemented with 3T3 fibroblast feeder cells (Morley et al., 2003). Cells that remained alive after 2 days exposure to Bleosome™ or bleomycin solution were counted using WST-8 reagent solution (Dojindo, Kumamoto, Japan) and a UV-spectrophotometer.

3. Results

3.1. Stability studies

The quantity of sodium cholate in each 'low dose' Bleosome™ formulation had an effect on bleomycin entrapment levels and average particle size (Figs. 2 and 3), but refrigeration at 4 °C over a time period of 8 days did not improve liposome stability when compared to similar samples stored at room temperature. Control liposomes without sodium cholate exhibited the largest size and the lowest capacity for bleomycin entrapment, whereas liposomes featuring 16% sodium cholate yielded the smallest size, but their capability for entrapping bleomycin was still relatively poor. Although liposomes containing 5 or 10% sodium cholate exhibited similar tendencies with respect to size and bleomycin entrapment, in the absence of further refinement, it was considered that Bleosome™ preparations featuring 10% sodium cholate merited selection for further study since they encapsulated the most bleomycin.

It had been noted that the average particle size of the various Bleosome™ preparations incorporating sodium cholate was significantly larger than the data seen in a previous study (Lau et al., 2003). This was thought to be a result of storage in PBS and the possible occurrence of ion–charge interactions that increased the inter-lamellar distance, and consequently caused an expansion within liposome particles. In order to avoid this effect, thereafter, Bleosome™ preparations were stored in 1/10 PBS and their average particle

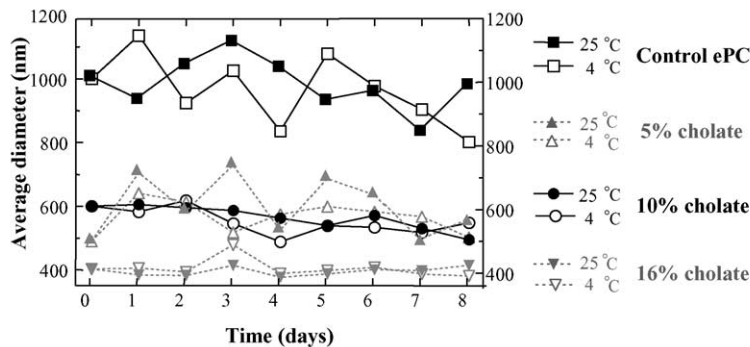

Fig. 2. Comparing average particle size (nm) of 'low dose' Bleosome™ formulations incorporating various concentrations of sodium cholate against control liposomes without any sodium cholate, whilst being stored in PBS, at refrigerated (4 °C) and room temperatures (25 °C) over 8 days ($n = 1$).

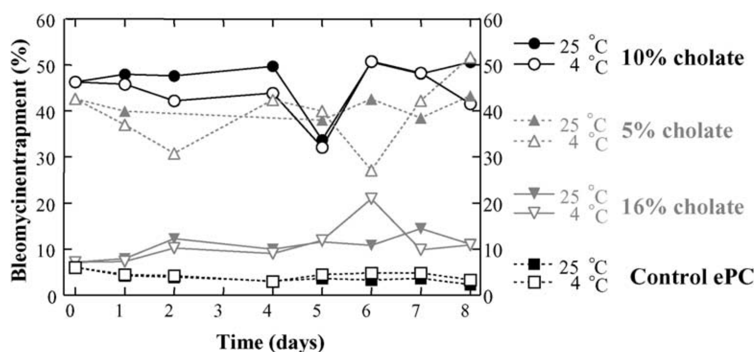

Fig. 3. Comparing bleomycin entrapment (%) in 'low dose' Bleosome^{TM} formulations incorporating various concentrations of sodium cholate against control liposomes without any sodium cholate, whilst being stored in PBS, at refrigerated (4 °C) and room temperatures (25 °C) over 8 days ($n = 1$).

sizes (low dose 163.5 ± 20.1 nm, $n = 3$; empty vesicles 189.9 ± 7.3 nm, $n = 3$; high dose 139.4 ± 5.7 nm, $n = 3$) (data not shown) were seen to have returned to the expected range of 150–200 nm. Subsequent stability tests were carried out at room temperature (25 °C) on three separate preparations of Bleosome^{TM} incorporating 10% (w/w) sodium cholate and data from each preparation have been individually plotted to clearly demonstrate their relatively unchanged characteristics after 7 days. It was observed that initial entrapment levels afforded by the same formulation fluctuated between 12 and 32% (Fig. 4). However, only the 32% preparation (first preparation) was used for purification and cell toxicity testing, procedures, which were carried out on the same day as Bleosome^{TM} production. Hence, it was possible to accurately compare the precise data on initial entrapment concentration and efficiency and average particle size (Fig. 4) of the original Bleosome^{TM} preparation against their respective values after purification (as above and in Section 3.2), prior to cell toxicity testing.

3.2. Purification by gel chromatography

Fig. 5 shows a bimodal distribution (□) with the peak on the left-hand representing the elution of bleomycin entrapped in Bleosome^{TM} and the right-hand peak signifying elution of free bleomycin in the solution. Also shown is the corresponding average particle diameter (nm) for each 1 ml fraction (right axis) from fractions 10–25 (●) of the eluent. A significant reduction in average particle size was observed

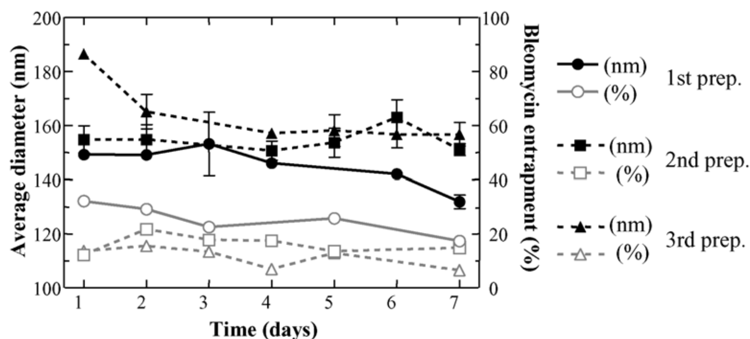

Fig. 4. Average diameter (nm) (mean ± S.D., $n = 3$) and bleomycin entrapment levels (%) ($n = 1$) of three separate preparations of 'high dose' Bleosome^{TM} featuring 10% (w/w) sodium cholate, in 1/10 PBS during storage over 7 days at room temperature (25 °C). Each trace represents the bleomycin retention or particle size profile of 'high dose' Bleosome^{TM} in one preparation.

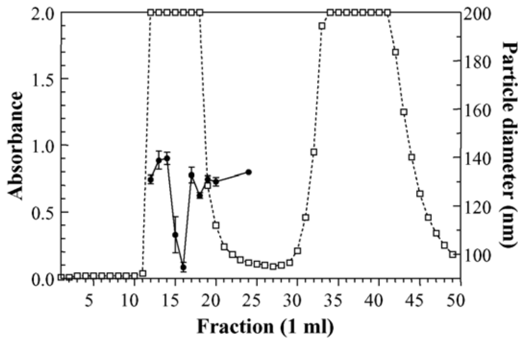

Fig. 5. Gel-filtration chromatography separation (□) of bleomycin entrapped in 'low dose' Bleosome^{TM} incorporating 10% (w/w) sodium cholate and unentrapped, free bleomycin. Fractions 14–16 were pooled to form a stock supply of 'high dose' Bleosome^{TM} for testing on keratinocytes ($n = 1$). Average particle diameter (nm) for fractions 10–25 (●) have been plotted to show size variation within the liposomal component of the eluent (mean ± S.D., $n = 3$).

for fraction 15 (108.1 ± 7.4 nm, $n = 3$) and fraction 16 (94.6 ± 2.0 nm, $n = 3$). Theoretically, the largest particles ought to elute first and smaller particles would be retarded the most, eluting thereafter. It can be seen that fraction 16 possessed the smallest average size compared to the general range of 130–140 nm. When fractions 14, 15 and 16 were pooled together to form a stock suspension of 'high dose' Bleosome^{TM}, the resulting average size returned to the median of approximately 140 nm (Section 3.1). However, if it was necessary to supply only the smallest liposomes, then fraction 16 could be isolated and specifically used for this purpose.

3.3. Toxicity studies on human keratinocyte lines

Two different concentrations of liposomal bleomycin were prepared for keratinocyte toxicity studies: Bleosome^{TM} preparations containing a low concentration (low dose) of bleomycin (22.3 ± 8.2 μg/ml; $n = 3$) and a high concentration (high dose) of bleomycin (1091.5 ± 25.8 μg/ml; $n = 3$). The average particle size of these high-dose preparations did not differ significantly from the low-dose Bleosome^{TM} and both versions lay within the desired range of 150–200 nm (Section 3.1).

'Low dose' Bleosome^{TM} were tested on NEB-1 and SCC cells, but it was found that the levels of bleomycin inside these liposomes were of a concentration that was too low to exert a clearly lethal action on both cell types (data not shown). Subsequently, it was necessary to manufacture a new batch of Bleosome^{TM}, and by substantially increasing the initial amount of bleomycin used for entrapment, liposomal bleomycin levels were elevated 50-fold in order to obtain a 'high dose' for eliciting lethal effects in keratinocytes.

Fig. 6 compares the toxicity of bleomycin in Bleosome^{TM} against free bleomycin solution on NEB-1 and SCC cells. It was clear that the liposomal form of bleomycin does have a progressively lethal effect on both types of keratinocytes as the concentration of liposomal bleomycin was increased. In order to quantatively compare the degree of toxicity, it was convenient to compare the dose required to kill half the population of cells present (LD_{50}). From Fig. 6, the estimated LD_{50}

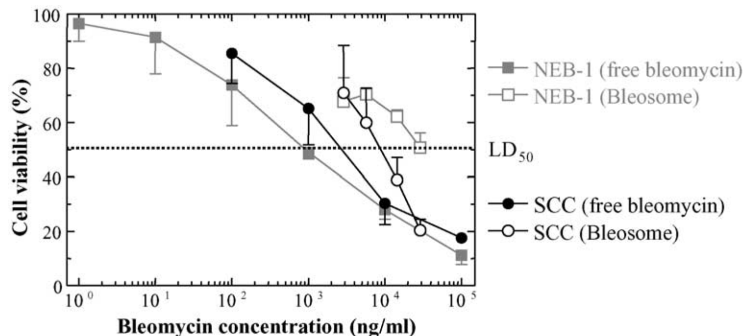

Fig. 6. LD_{50} toxicity study of 'high dose' Bleosome^{TM} incorporating 10% (w/w) sodium cholate (empty symbols) and free bleomycin in culture medium (solid symbols) on NEB-1 human keratinocytes, immortalised with HPV-16 (Storey et al., 1988) (NEB-1, square symbols) and keratinocytes derived from human squamous cell carcinoma (SCC, circle symbols) (mean ± S.D., $n = 3$).

values for immortalised keratinocytes (NEB-1) were found to be 30 μg/ml for Bleosome™ and 1 μg/ml for free bleomycin solution. From Fig. 6, the estimated LD_{50} values for SCC cells were found to be 9 μg/ml for Bleosome™ and 3 μg/ml for free bleomycin solution.

4. Discussion

Bleosome™ preparations generally seemed to be less potent than free bleomycin solution against both NEB-1 and SCC cell lines, except for the 30 μg/ml dose of liposomal bleomycin on SCC cells. Encapsulating bleomycin molecules inside vesicles naturally partitioned them away from as well as hindered their presentation to keratinocytes. This compartmentalisation of the cytotoxin most likely limits the amount of drug available to the keratinocytes in vitro. Although minor, some leakage was seen to occur from Bleosome™ samples during storage at room temperature (Fig. 4). It has yet to be determined whether cell death was caused by bleomycin that had leaked out, or from extensive drug release when the liposomes themselves had been destabilised, whilst adsorbing onto cell surfaces or during ingestion by keratinocytes. The 30-fold difference in LD_{50} between Bleosome™ and free bleomycin solution on NEB-1 cells (Fig. 6) suggests that even a small amount of drug leakage from a 30 μg/ml preparation of Bleosome™ might release sufficient bleomycin to potentially cause a substantial reduction in the population of NEB-1 cells. However, this factor seems to be diminished when SCC cultures were treated with Bleosome™ since the LD_{50} value was only three times higher than that of unencapsulated drug (Fig. 6). This indicates that SCC cells were more susceptible to bleomycin and Bleosome™ treatment than NEB-1 cells. Since both cell lines were treated from the same batch of Bleosome™, it may be assumed that their difference in sensitivity to drug treatment might be due to other factors, as discussed below.

The origins of SCC and NEB-1 cells are dissimilar. The SCC tumour was excised and dissociated samples of malignant keratinocytes were then cultivated and the cells that subsequently emerged exhibited immortality, possibly as a result of genetic changes such as a chromosomal loss. In contrast, NEB-1 cells were immortalised in vitro by the co-operative transforming properties of E6 and E7 genes of the HPV-16 genome (Barbosa and Schlegel, 1989; Hawley-Nelson et al., 1989): E6 disrupts p53 tumour suppressor protein expression (Scheffner et al., 1990; Werness et al., 1990) and E7 disrupts the PKB pathway (Dyson et al., 1989). These pathways had become inactivated in both instances, but the mechanisms behind their ability for uncontrolled proliferation may be inhibited by bleomycin to varying degrees. It has yet to be determined whether the activity of Bleosome™ on keratinocytes led to cell death by way of apoptosis or necrosis. Nonetheless, the E6 protein of a number of different cutaneous HPV-types has been found to block the epidermal apoptotic response to UV radiation (Jackson and Storey, 2000) and HPV-positive NMSC displayed greatly reduced apoptotic rates when compared to HPV-negative NMSC (Jackson et al., 2002). However, despite the high expression of p53 in many tumours, patients with cutaneous SCC had low prevalence of anti-p53 antibodies (Moch et al., 2001). This may be related to the prevalence of wild-type p53 over mutant p53, where the tumour is HPV-positive. These findings suggest that part of the signalling pathway responsible for initiating apoptosis may be absent or substantially impaired in one or both of the cell lines.

Bleomycin hydrolase is a member of the papain superfamily of cysteine proteases (Takeda et al., 1996b). It is responsible for the intracellular inactivation of bleomycin (Umezawa et al., 1974). Bleomycin hydrolase activity can be measured by quantifying metabolites of active bleomycin B2, and significantly it has been demonstrated that bleomycin hydrolase is expressed in normal skin, but not in squamous cell carcinoma (Lazo et al., 1982; Takeda et al., 1999). Moreover, it has been directly demonstrated that overexpression of human bleomycin hydrolase protected Chinese hamster ovary cells against bleomycin-induced chromosomal damage in G2 phase of the cell cycle (Lefterov et al., 1998). Whether these differences in Bleosome™ toxicity between the NEB-1 and SCC cells was due to a difference in expression and/or activity of bleomycin hydrolase remains to be determined. Bleomycin hydrolase activity can be inhibited by iodoacetic acid, *N*-ethylmaleinimide (NEM) and *p*-chloromercuribenzoic acid (pCMB) as well as divalent cations such as Cu^{2+}, Cd^{2+}, Hg^{2+} and Zn^{2+} (Takeda et al., 1996a). As yet, relatively little is known about metabolic factors that might influence

chromosomal DNA damage caused by bleomycin and further investigations will be required to ascertain the determining intracellular operations that elevate NEB-1 cell resistance to liposomal bleomycin to three times that of SCC cells (Fig. 6).

Purification of Bleosome™ by gel-filtration chromatography resulted in a product suitable for research purposes, but the yield was low and the liposomal concentration of bleomycin was diluted at least five-fold. It became necessary to employ much higher quantities of lipids and drug at the outset of production in order to obtain a viable product after elution from the gel column. The expense of the raw materials may rule out this method of purification when scaling up batch production for clinical trials. Ultra-centrifugation is an obvious choice, but further testing would be required in order to fine-tune this method to achieve a better yield of stable highly loaded liposomes.

The fact that storage of Bleosome™ at 4 °C did not change their stability over storage at room temperature (Figs. 2 and 3) has an important practical aspect in any subsequent commercial manufacturing and storage of Bleosome™ preparations: Bleosome™ can be kept at room temperature for at least a week without significant detriment. Mild storage conditions may suffice and no further optimisation of the formulation with respect to stability is required. These circumstances may need to be reconsidered if the Bleosome™ suspension will be diluted into a solid or semi-solid preparation, such as a gel/cream. Compatibility assessment of gel/cream excipients on liposome stability would then be required.

5. Conclusions

Ultra-deformable liposomes containing bleomycin (Bleosome™) were stable at room temperature and did not exhibit significant changes to their drug load or average particle size for at least 7 days. Bleosome™ exerted a lethal effect on human keratinocytes cell lines and a cell line derived from a primary carcinoma in vitro when loaded with sufficient bleomycin. The cell line derived from squamous cell carcinoma seemed to be more susceptible to Bleosome™ than HPV-immortalised keratinocytes (NEB-1). Potency of Bleosome™ loaded with 30 μg/ml bleomycin on SCC cells was similar to that of free bleomycin solution, but significantly fewer NEB-1 cells than SCC cells were killed at this concentration. This distinction suggests that Bleosome™ containing high levels of bleomycin may possess a passive specificity for the in vitro demise of SCC over NEB-1 cells.

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References

Aszalos, A., Crawford, P., Vollmer, P., Kantor, N., Alexander, T., 1981. High-performance liquid chromatographic determination of components of bleomycin preparations. J. Pharm. Sci. 70, 878–880.

Barbosa, M.S., Schlegel, R., 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. Oncogene 4, 1529–1532.

Cevc, G., Blume, G., 2003. Biological activity and characteristics of triamcinolone-acetonide formulated with the self-regulating drug carriers, transfersomes. Biochim. Biophys. Acta 1614, 156–164.

Cevc, G., Gebauer, D., Stieber, J., Schatzlein, A., Blume, G., 1998. Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. Biochim. Biophys. Acta 1368, 201–215.

Cevc, G., Schatzlein, A., Richardsen, H., 2002. Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. Biochim. Biophys. Acta 1564, 21–30.

Dyson, N., Howley, P.M., Munger, K., Harlow, E., 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243, 934–937.

El Maghraby, G.M., Williams, A.C., Barry, B.W., 2001a. Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro. J. Pharm. Pharmacol. 53, 1069–1077.

El Maghraby, G.M., Williams, A.C., Barry, B.W., 2001b. Skin hydration and possible shunt route penetration in controlled estradiol delivery from ultradeformable and standard liposomes. J. Pharm. Pharmacol. 53, 1311–1322.

Fiedler, H.-P., Wachter, J., 1991. High-performance liquid chromatographic determination of bleomycins. J. Chromatogr. 536, 343–347.

Hawley-Nelson, P., Vousden, K.H., Hubbert, N.L., Lowy, D.R., Schiller, J.T., 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. 8, 3905–3910.

Hofer, C., Gobel, R., Deering, P., Lehmer, A., Breul, J., 1999. Formulation of interleukin-2 and interferon-alpha containing ultradeformable carriers for potential transdermal application. Anticancer Res. 19, 1505–1507.

Jackson, S., Storey, A., 2000. E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. Oncogene 19, 592–598.

Jackson, S., Ghali, L., Harwood, C., Storey, A., 2002. Reduced apoptotic levels in squamous but not basal cell carcinomas correlates with cutaneous human papillomavirus expression. Br. J. Cancer 87, 319–323.

Kim, A., Lee, E.H., Choi, S.H., Kim, C.K., 2004. in vitro and in vivo transfection efficiency of a novel ultradeformable cationic liposome. Biomaterials 25, 305–313.

Lau, K.G., Chopra, S., Maitani, Y., 2003. Entrapment of bleomycin in ultra-deformable liposomes. S. T. P. Pharm. Sci. 13, 237–239.

Lazo, J.S., Boland, C.J., Schwartz, P.E., 1982. Bleomycin hydrolase activity and cytotoxicity in human tumors. Cancer Res. 42, 4026–4031.

Lefterov, I.M., Koldamova, R.P., King, J., Lazo, J.S., 1998. The C-terminus of human bleomycin hydrolase is required for protection against bleomycin-induced chromosomal damage. Mutat. Res. 421, 1–7.

Moch, C., Moysan, A., Lubin, R., de la Salmoniere, P., Soufir, N., Galisson, F., Vilmer, C., Venutolo, E., Le Pelletier, F., Janin, A., Basset-Seguin, N., 2001. Divergence between the high rate of p53 mutations in skin carcinomas and the low prevalence of anti-p53 antibodies. Br. J. Cancer 85, 1883–1886.

Morley, S.M., D'Alessandro, M., Sexton, C., Rugg, E.L., Navsaria, H., Shemanko, C.S., Huber, M., Hohl, D., Heagerty, A.I., Leigh, I.M., Lane, E.B., 2003. Generation and characterization of epidermolysis bullosa simplex cell lines: scratch assays show faster migration with disruptive keratin mutations. Br. J. Dermatol. 149, 46–58.

Purdie, K.J., Sexton, C.J., Proby, C.M., Glover, M.T., Williams, A.T., Stables, J.N., Leigh, I.M., 1993. Malignant transformation of cutaneous lesions in renal allograft patients: a role for human papillomavirus? Cancer Res. 53, 5328–5333.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., Howley, P.M., 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63, 1129–1136.

Storey, A., Pim, D., Murray, A., Osborn, K., Banks, L., Crawford, L., 1988. Comparison of the in vitro transforming activities of human papillomavirus types. EMBO J. 7, 1815–1820.

Takeda, A., Higuchi, D., Yamamoto, T., Nakamura, Y., Masuda, Y., Hirabayashi, T., Nakaya, K., 1996a. Purification and characterization of bleomycin hydrolase, which represents a new family of cysteine proteases, from rat skin. J. Biochem. (Tokyo) 119, 29–36.

Takeda, A., Masuda, Y., Yamamoto, T., Hirabayashi, T., Nakamura, Y., Nakaya, K., 1996b. Cloning and analysis of cDNA encoding rat bleomycin hydrolase, a DNA-binding cysteine protease. J. Biochem. (Tokyo) 120, 353–359.

Takeda, A., Nonaka, M., Ishikawa, A., Higuchi, D., 1999. Immunohistochemical localization of the neutral cysteine protease bleomycin hydrolase in human skin. Arch. Dermatol. Res. 291, 238–240.

Umezawa, H., Hori, S., Sawa, T., Yoshioka, T., Takeuchi, T., 1974. A bleomycin-inactivating enzyme in mouse liver. J. Antibiot. 27, 419–424.

Werness, B.A., Levine, A.J., Howley, P.M., 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248, 76–79.