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Evaluation of a novel topical formulation of liposome-encapsulated bleomycin as a therapy for non-melanoma skin cancers in veterinary species

Background

Bleomycin is a chemotherapeutic agent, used in human and veterinary medicine, against several types of malignancies, including non-melanoma skin cancers (NMSC). It works by producing both single- and double-strand DNA breaks. However, the large molecular structure of bleomycin hampers a free passage through the plasma membrane to reach the inside of the cancer cells. Bleomycin has been proposed to work in a tumour-type dependent manner but to date its mechanism of action and the mechanism of cellular resistance is poorly understood. Bleomycin is also characterized by high intrinsic systemic toxicity. To overcome these issues, we are testing a novel topical formulation of the drug, made by encapsulating the bleomycin inside ultra-deformable liposomes, called Bleosome. This is potentially a novel topical non invasive tool for the treatment of NMSC in animal and human patients.

Project aims

This study is three-tiered: 1. to investigate the molecular effects of Bleosome *in vitro* on a panel of cancer cell lines; 2. to assess the skin penetration of Bleosome *ex vivo*; and 3. to evaluate the efficacy and side-effects of Bleosome *in vivo.*

1. Cellular uptake and molecular response to Bleosome *in vitro*

We assessed the effect of Bleosome on cell viability (fig 1) and colony formation ability (data not shown) on a panel of canine, feline and human cancer cell lines, comparing the Bleosome with the free bleomycin. Both the compound achieved 100% of cell mortality, but a different drug concentration, and decreased the ability in forming colonies in each cell line. This experiment allowed us to establish IC50s of Bleosome and free bleomycin for the cell lines treated (data not shown).

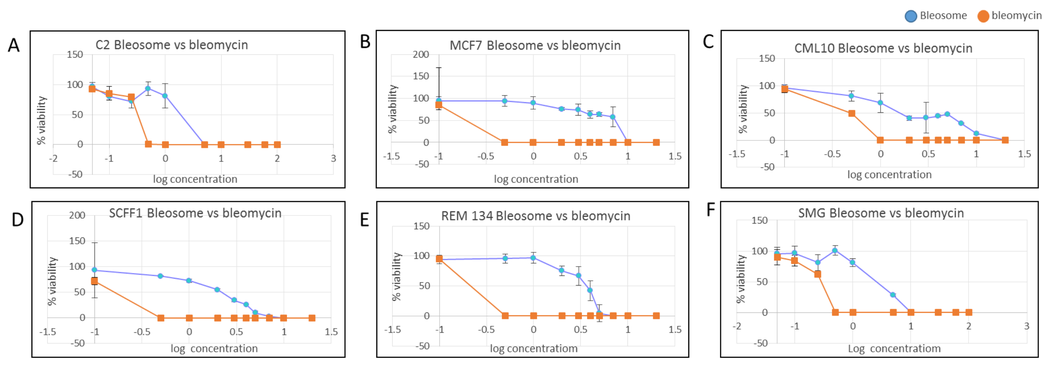

Figure 1 *Comparison of the effects of Bleosome and free bleomycin on (A) C2-canine mast cell tumour, (B) MCF7- human mammary tumour, (C) CML10-canine melanoma, (D) SCFF1- feline laryngeal squamous cell carcinoma, (E) REM134-canine mammary carcinoma and (F) SMG-feline oral squamous cell carcinoma cell lines, after 48 hours of treatment at growing drug concentrations.*

Subsequently, we investigated whether liposomes can improve cellular uptake of bleomycin. We labelled bleomycin with a green fluorophore, then encapsulated into liposomes (F-Bleosome). Canine melanoma cells (CML10) were treated with either F-Bleosome or F-free bleomycin. Two hours post-treatment F-Bleosome molecules were found within cells, while the free drug was not detected, indicating that Bleosome is taken up by cancer cells more readily than free bleomycin (fig 2).

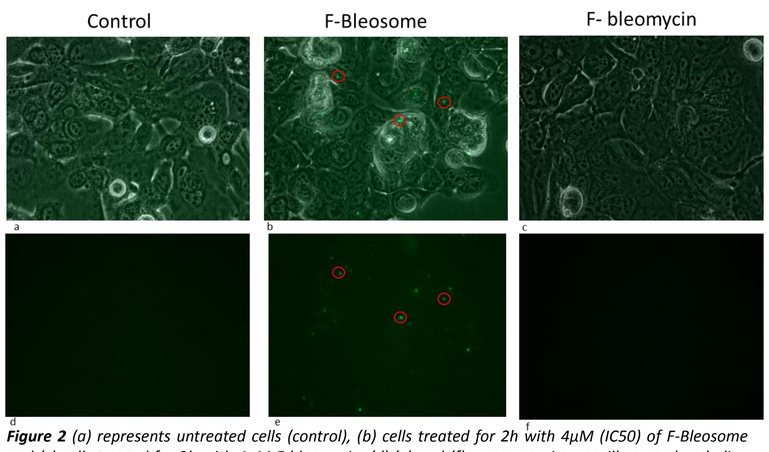

Figure 2 *(a) represents untreated cells (control), (b) cells treated for 2h with 4μM (IC50) of F-Bleosome and (c) cells treated for 2h with 4μM F-bleomycin. (d),(e) and (f) are same pictures, illustrated excluding the phase contrast, using the green channel only. Relevant representative green fluorescent bleomycin molecules are circled in red*

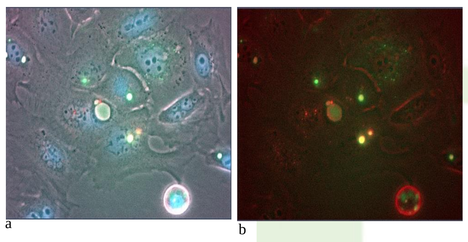

Figure 3 *CML10 treated with 4μM of F-bleomycin for 24h. Cells were pre-stained with Hoechst 33342 (blue nuclear staining) and LysoTracker Deep Red (red lysosomal staining). (a) is the merged images, (b) is the same picture taken excluding the blue channel (nuclear staining is not shown)*

Using a blue nuclear staining (Hoechst 33342) and a red lysosomal staining (LysoTracker Deep Red), we found that green drug particles in both treatments were co-localised and sometimes circled by the red lysosomal marker, suggesting a likely involvement of the endocytic pathway in the Bleosome and bleomycin cellular uptake (fig 3). The following step will be to investigate the fate of the drug within cancer cells after longer time of treatment.

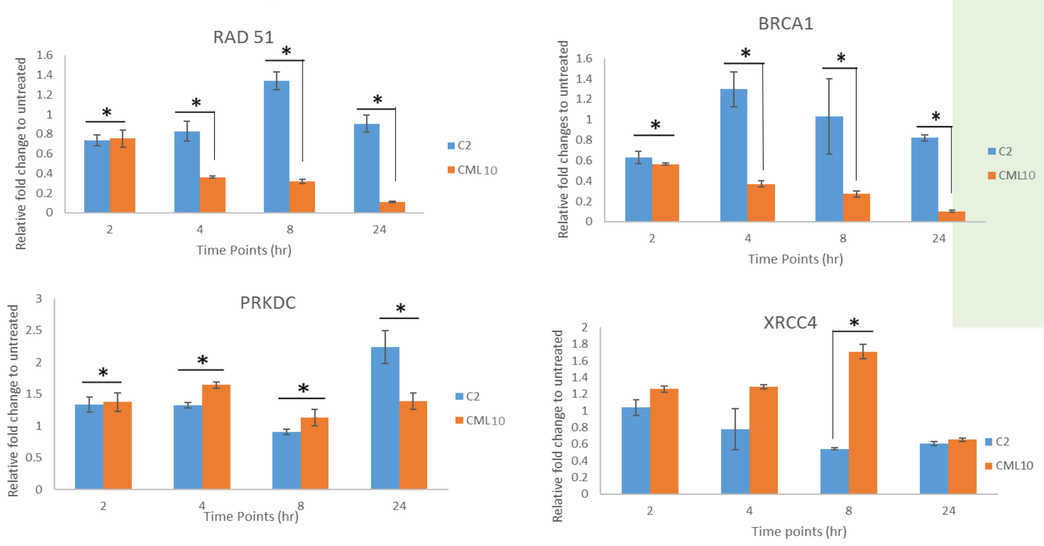

Figure 4 *Gene expression profiles of HR pathway (Rad51 and BRCA1), and NHEJ pathways genes (PRKDC and XRCC4) after treatment with Bleosome. Cells were treated with relative Bleosome IC50s (1.52μM for C2 and 3.81μM for CML10) for 2, 4, 8 and 24 h. Fold change in expression is relative to untreated cells. Bar charts indicate the differences in gene expression between CML10 (orange) and C2 (blue) cell lines at each time points.* * indicates statistically significant difference (p≤ 0.004)

We proved that Bleosome can efficiently produce double-strand DNA breaks (data not shown); to investigate the mechanism of resistance to the treatment we evaluated preliminarily, by qRT-PCR, the gene expression of key players in DNA repair machinery (HR and NHEJ pathways) (fig 4).
Response to the treatment was cell-type dependent: Bleosome activated HR pathway in C2, while CML10 showed preferential activation of the NHEJ signalling.

2. Grade and mechanism of skin penetration of Bleosome *ex vivo*

Canine and equine skin explants were treated with Bleosome and free bleomycin. Transmission Electron Microscope (TEM) allowed the visualisation of the liposomes but not the entrapped drug. Even after an extended period of six hours, liposomes were only found within the outermost *stratum corneum* (fig 4).

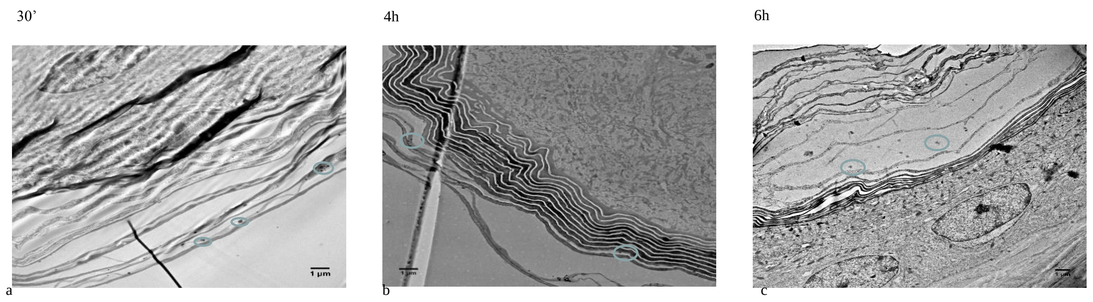

Figure 4 *(a) Canine skin explants treated with Bleosome for 30', (b) 4h and (c) 6h. visualised by the TEM. Representative liposomes within the keratinocytes are circled in blue*

However, the multiphoton microscope detected the fluorescent entrapped and free bleomycin. Imaris software analysis indicated that F-Bleosome penetrated significantly deeper through the skin that F-free bleomycin in both canine and equine skin (data not shown), that the Bleosome penetration is changing according to the length of time of treatment and that, at six hours post-treatment, F-Bleosome molecules have crossed the *stratum corneum* and reached the nucleated deeper layers of the skin (fig 5 and fig 6).

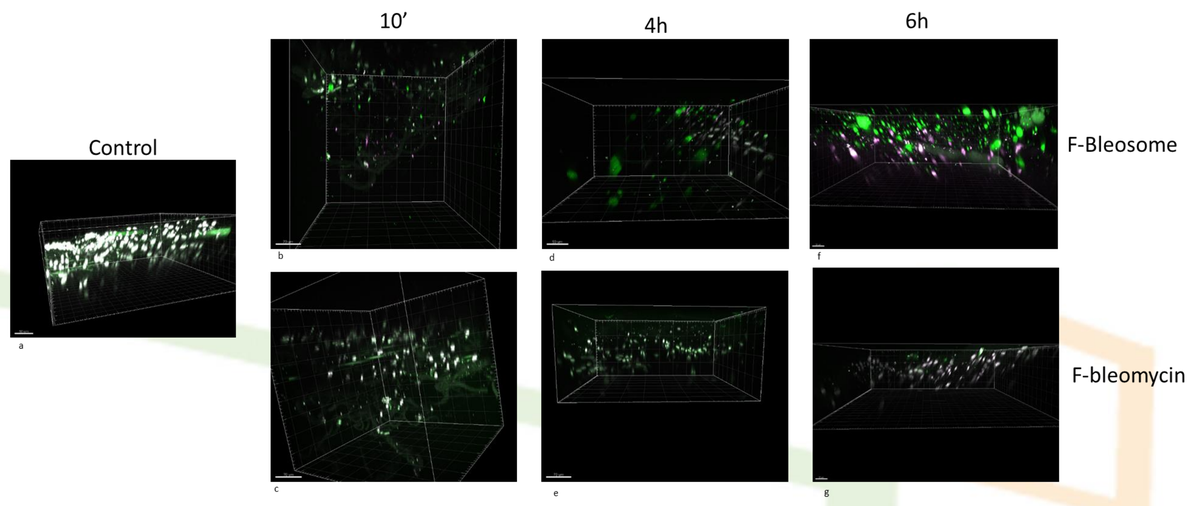

Figure 5 *Canine skin sections treated with F-Bleosome for (b) 10', (d) 4h and (f) 6h, compared with F-bleomycin treatment for (c) 10', (e) 4h, (g) 6h. (a) is the untreated skin sections. Side views of 3-D images gained by the multiphoton microscope and analysed by Imaris.*

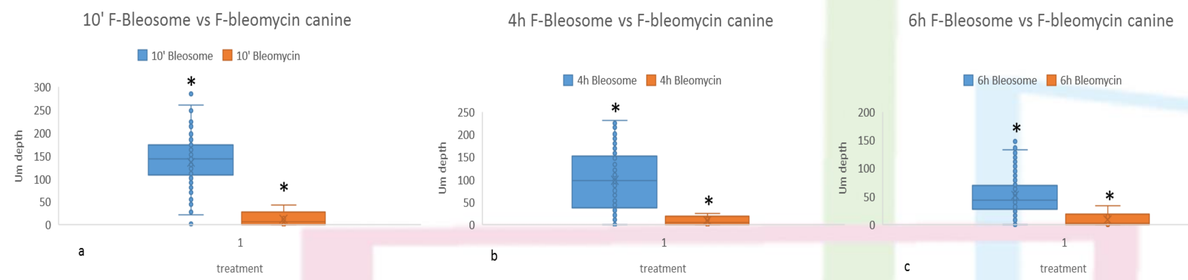

Figure 6 *(a) represents the different z-position (depth) of drug molecules in the canine skin sections treated with F-Bleosome (blue plot) and F-bleomycin (orange plot) after 10'. (b) after 4h and (c) after 6h. *represents statistically significant difference (p≤0.001).*

We propose that liposomes improve the penetration of bleomycin through animal skin acting as penetration enhancers: they might interact with the outermost keratinocytes, disrupting intercellular lipid lamellae and allowing the release and consequently the penetration of the entrapped bleomycin through the skin.

3. Efficacy and safety of Bleosome as adjuvant treatment for equine sarcoids *in vivo*

We have translated this research into the clinic, treating horses bearing several types of cutaneous sarcoids. All the patients recruited underwent surgical excision of the bulk disease using a CO2 laser. Subsequently, Bleosome was applied topically, twice a day, for a median length of 4 weeks

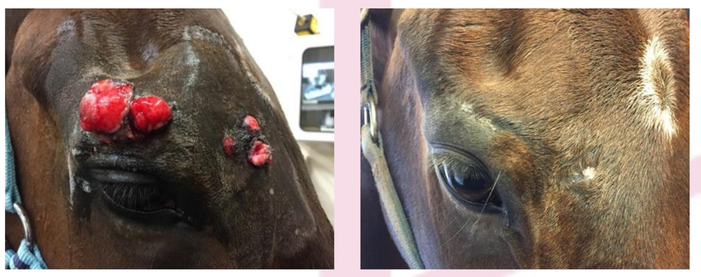

Figure 7 *(a) multiple periocular fibroblastic sarcoids prior C02 laser excision and Bleosome treatment (27.06.18). (b) surgical scar (11.10.18), 3.5 months post surgery and 2.5 months after Bleosome treatment.*

To date, we observed that 75% (6 out of 8) of equine patients, treated topically after surgical excision, showed no relapse or adverse reactions after an average of 3 months follow-up; while 2 horses experienced a recurrence, one of them is currently treated with a second course of Bleosome. This study is ongoing.

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