Original Research Article [Veterinary and Comaprative Oncology]

Glycolysis inhibition improves photodynamic therapy cure rates for equine sarcoids

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Running title: Photodynamic therapy with glycolysis inhibition

ABSTRACT

Photodynamic therapy (PDT) holds great promise in treating veterinary and human dermatological neoplasms, including equine sarcoids, but is currently hindered by the amount of photosensitiser and light that can be delivered to lesions thicker than around 2 mm, and by the intrinsic antioxidant defences of tumour cells. We have developed a new PDT technique that combines an efficient transdermal penetration enhancer solution, for topical delivery of 5-aminolevulinic acid (ALA) photosensitiser, with acute topical post-PDT application of the glycolysis inhibitor Ionidamine. We demonstrate that the new PDT combination treatment selectively kills sarcoid cells in vitro, with repeated rounds of treatment increasing sarcoid sensitisation to PDT. In vivo, ALA PDT followed by 600μM Ionidamine substantially improves treatment outcomes for occult, verrucous, nodular and fibroblastic sarcoids after 1 month (93% treatment success in 27 sarcoids), compared to PDT using only ALA (14% treatment success in 7 sarcoids).

Keywords: PDT, sarcoid, skin, tumour, glycolysis inhibition, combination therapy.

INTRODUCTION

Photodynamic therapy (PDT) is an evolving, cost-effective, and easy to administer non-surgical treatment for a variety of dermatological cancers and pre-cancers in humans (Morton et al 2008, 2013). PDT combines tumour-selective photosensitiser dyes and targeted illumination to generate cytotoxic reactive oxygen species (ROS) within the tumour. The pro-drug 5-aminolevulinic acid (ALA) is administered either topically or orally and becomes selectively concentrated and metabolised in tumours cells to the red-fluorescent photosensitiser, protoporphyrin IX (ppIX). In addition to directly acting on tumour cells, PDT damages and restricts tumour microvasculature, and causes local inflammation that stimulates an immune response against the tumour (Castano 2006). Unlike surgery, because the surrounding extracellular matrix is unaffected, tissue healing and cosmesis is excellent following PDT, making it popular for cancers of the head and neck (Morton et al 2008, 2013). In veterinary practice, topically-applied ALA PDT has been used successfully to treat cancers of the feline nasal planum (Bexfield et al 2008) and as a monotherapy or post-surgical adjunctive therapy to treat equine sarcoids (reviewed by Giuliano 2010; Kemp-Symonds 2012).

Sarcoids are non-metastatic but locally aggressive cutaneous fibroblastic tumours; the most common neoplasia diagnosed in horses (35-90% of skin tumours) and noted for a high tendency for local recurrence (Knottenbelt, 2003, 2005; Scott & Miller, 2011). They are most likely caused by bovine papillomaviruses 1 and 2 (Nasir & Brandt, 2013). Equine sarcoids can be grouped into six broad categories: mild (1) occult and (2) verrucous forms, more severe (3) nodular and (4) fibroblastic, highly proliferative (5) malevolent forms and (6) mixed combinations of the other types; with their size and location dictating their clinical significance, treatment feasibility and

prognosis. Several treatment options have been described, including: PDT, surgical excision, cryotherapy, radiofrequency hyperthermia, immunomodulation, radiation brachytherapy, intralesional cisplatin, and topical chemotherapy (Knottenbelt 2000, Martens 2000, 2003, Carr 2009, Giuliano 2010, Scott & Miller 2011, Kemp-Symonds 2012). Each treatment has its benefits and drawbacks, such as treatment cost, availability, cosmesis, and the type and size of the lesion that can be treated. Brachytherapy gives the best cure rate (near 100%), but is also the most expensive and has limited availability (Wyn-Jones 1983, Knottenbelt 2000).

Currently, surgical excision with an adjunctive therapy gives the best long-term prognosis. For instance, CO_2 laser excision with adjunctive PDT was reported to achieve a 93% one-year disease-free rate (Kemp-Symonds 2012). However, excision is only suitable for relatively small sarcoids, whilst PDT can be used to treat larger areas (Morton 2008, 2013). Moreover, sarcoids in some anatomical locations, such as periocular or sheath, are challenging to treat surgically (Knottenbelt 2000) but are amenable to PDT.

An ideal treatment should be: inexpensive, portable, easy to administer, not require general anaesthesia, and require few repeat applications. PDT satisfies all of these requirements, but currently has a variable cure rate depending on the depth of the tumour. Deeper tumours receive less photosensitiser and less light from topical administrations. This leads to less ROS production and lower cytotoxicity during PDT. Moreover, antioxidant defences that destroy excess ROS are upregulated in many cancers (Tracootham et al. 2009), undermining the full potential of PDT.

In order to overcome the antioxidant defences of cancer cells, we have been investigating combination therapies of antioxidant inhibitors plus PDT (Kimani et al 2012) or glycolysis inhibitors plus PDT (Golding et al 2013). Both of these

modifications to standard PDT deplete cellular antioxidants and significantly improve PDT cytotoxicity against human cancer cells in vitro, with glycolysis inhibition providing the most impressive, 10-fold, improvement (Golding et al 2013).

Aerobic glycolysis is the preferred energy metabolism pathway in most cancers (Vander Heiden et al 2009) including sarcoids (Potocki et al 2014), with the extent of reliance on glycolysis generally correlating with tumour malignancy and poorer clinical prognosis (Chen 2012). Glycolysis inhibitors rapidly deplete ATP levels and elevate ROS in cancer cells, leading to tumour cell death (Tracootham et al. 2009). Glycolysis inhibition, using drugs such as 2-deoxyglucose and lonidamine, has proved a successful clinical treatment for many human tumours, either as monotherapy or in combination with radiotherapy (Brawer 2005, Coleman et al. 2008, Zhang & Yang 2012).

In this study, we find that single-treatment PDT, followed by acute topical application of glycolysis inhibitors, dramatically improves the 1-month treatment response rate for equine occult, verrucous, nodular and fibroblastic sarcoids from 14% with ALA-only to 93% with ALA followed by 600μM lonidamine (defined according to RECIST guidelines as complete response (CR) plus partial response (PR); Eisenhauer, 2009).

MATERIALS AND METHODS

Reagents

5-aminolevulinic acid (ALA), as hydrochloride salt, was BioReagent grade >98% (Sigma-Aldrich catalogue number A7793). Two commercial cream formulations of photosensitiser were: 200 mg/g ALA hydrochloride cream (Mandeville Medicines,

Aylesbury UK) and Metvix cream (160 mg/g methyl aminolevulinic acid hydrochloride in a proprietary cream, Galderma. European marketing authorisation number PL 10590/0048). Photosensitisers were kept frozen until use. Glycolysis inhibitors were: 2-deoxy-D-glucose (Sigma-Aldrich D8375) and lonidamine (Sigma-Aldrich L4900, kept frozen as a 6 mM stock in ethanol). Penetration enhancer solution was made as 60 mg N-lauroylsarcosine (Sigma-Aldrich L5125) plus 40 mg Span20 (Sigma-Aldrich S6635) dissolved in 10ml in PBS/ethanol (1:1) (Karande 2004). Other reagents were obtained from Sigma-Aldrich unless otherwise stated.

Cell culture

Equine sarcoid cell line EqS04b and primary equine foetal palate fibroblasts EqPalF were gifts of Professor Lubna Nasir (University of Glasgow) (Yuan et al. 2008). Cells were maintained in DMEM, supplemented with 10% horse serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, in a humidified incubator with 5% CO_2 at 37°C.

Application of PDT in vitro

Cells were seeded at a density of 150,000 cell/well in 24-well plates and allowed to adhere overnight, yielding around 90% confluent monolayers by the following day. Cells were incubated with a low-dose of ALA (21 µg/ml) in serum-free, phenol red-free low-glucose DMEM for 3 hr. For no-photosensitiser control experiments, ALA was not added. Cells were then subjected to PDT (10 J/cm^2, 32 mW/cm^2), using a heat-filtered 500W white halogen lamp, corresponding to the lower limit of current clinical doses, so as to simulate the conditions at the deeper margins of a sarcoid. Dark toxicity control experiments were performed under identical conditions, but were not exposed to light. Following PDT or dark exposure, the medium was changed to

phenol red-free low glucose DMEM with 10% horse serum and supplemented with either: nothing (drug control), or 180 mM 2-deoxy-D-glucose (2DG), or 150μM lonidamine (Lon) for 24 hr. Then, floating cells and adherent cells were pooled and percentage cell viability was assessed by propidium iodide exclusion flow cytometry (FACScalibur, Becton Dickinson).

To determine whether repeated rounds of PDT lead to treatment resistance, some cells were subjected to four rounds of PDT, in each case with or without subsequent 24hr glycolysis inhibition. Following PDT, surviving cells were allowed to repopulate the 24-well plate for around 1 week to 90% confluency before the next round of PDT. After the fourth round of PDT, percentage cell viability was assessed 24 hrs later by propidium iodide exclusion flow cytometry. All culture experiments were performed in triplicate. Statistical differences were determined by 2-way ANOVA with Dunnett or Bonferroni multiple comparisons post-tests using GraphPad Prism 6.

Skin biopsy studies ex-vivo

Full thickness fibroblastic sarcoid tissues were obtained from routine surgical CO_2 laser excisions. Full thickness mouse skin was obtained from the back of euthanized adult C57/BL6 mice and was shaved with a razor blade prior to use. For both types of skin, the epithelial surface was wiped with 70% ethanol and the skin biopsies were cultured on transwell supports in 6-well culture plates over serum-free DMEM, such that the skin sat at an air/medium interface. Biopsies were maintained in a humidified incubator with 5% CO_2 at 37°C.

For ALA uptake studies on live skin, ALA was prepared as either 20% (w/v) in penetration enhancer solution or 20% (w/w) in Cetraben emollient cream (Thornton & Ross, Huddersfield UK). A clinical cream formulation of 20% (w/w) ALA was obtained

from Mandeville Medicines. ALA in penetration enhancer was soaked into 2x2 mm square filter paper, which was then placed onto the skin biopsy. Creams were thinly applied to the skin in a similar 2x2 mm area. Vehicle-only controls were applied similarly. After incubating for 4hr, biopsies were fixed in 4% paraformaldehyde, embedded in 20% gelatine and 50 micron transverse sections were cut through the application site using a vibratome. Sections were mounted in Hoechst nuclear stain and viewed with an epifluorescence microscope using x20 objective.

For studies of glycolysis inhibitor penetration into live sarcoid skin, 300μM lonidamine or 180mM 2-deoxy-D-glucose in penetration enhancer were applied onto 2x2 mm filter paper squares, placed onto the skin, and incubated for 2 hr. Then the skin surface was rinsed and the biopsy was cut transversely through the application site with a scalpel blade. The two halves were placed into serum-free DMEM containing 335 μg/ml ALA and incubated for 1.5hr. The skin was then rinsed, fixed in 4% paraformaldehyde, gelatin embedded, and vibratome-sectioned parallel with the transverse surface that had been exposed to ALA. Sections were mounted in Hoechst nuclear stain and viewed with an epifluorescence microscope using x20 objective.

Clinical study design

The study protocol was approved by The Open University Animal Welfare Ethical Review Body (ref G4432), and by the University of Cambridge, Department of Veterinary Medicine Ethics & Welfare Committee (ref CR81).

Inclusion criteria were occult, verrucous, nodular or fibroblastic sarcoids less than 5 mm thick and not exceeding 110 mm length in any direction, measured with vernier calipers. Horses were excluded if they had a history of photosensitivity, or any renal

or hepatic impairment in the previous 12 months. Owners gave written informed consent for every horse.

Horses were enrolled into 4 study arms with the following post-PDT topical treatments: no-drug (control); lonidamine 192 µg/ml (600 µM) in penetration enhancer, 2-deoxy-D-glucose (2DG) 30 mg/ml (183 mM) in penetration enhancer. An additional but separate study used two treatment sessions of PDT with topical Metvix cream to provide a comparison with a licensed human clinical photosensitiser (European marketing authorisation PL 10590/0048) that has previously also been used to treat equine sarcoids (Kemp-Symonds, 2013).

Application of PDT in vivo

All procedures were performed on standing horses. Sarcoid skin was cleaned and de-greased with an alcohol wipe. ALA (256 mg/ml in penetration enhancer) was applied to the sarcoid, plus a ~5 mm margin, using a cotton bud every 30 mins for 3 hrs (6 applications in total). Horses were maintained away from strong lighting during this time and for 24 hr following PDT. Prior to PDT the horse was sedated, usually intravenously with a combination of detomidine 0.01mg/kg (Medesedan, Virbac) and butorphanol 0.02mg/kg (Torbugesic, Zoetis), and the area anaesthetised by a local nerve block, topical Lidocaine gel or topical spray, depending on the site to be treated.

A dose of 37 J/cm^2 630 nm red light was administered to the sarcoid from either a Lumacare LC122A light source with LUM E probe at an intensity of 70 mW/cm^2 (Jane Dobson), or Aktilite CL128 light source at an intensity of 77 mW/cm^2 (Jeremy Kemp-Symonds), both requiring around 8 mins illumination delivery time.

Immediately after PDT, glycolysis inhibitor drugs were reconstituted with penetration enhancer solution and mixed well. Glycolysis inhibitors were applied to the sarcoid with a cotton bud every 30 mins for 2 hrs, and then every 2 hrs for a further 10 hrs (10 applications in total).

For treatment with Metvix, sarcoids were cleaned with an alcohol wipe and a thin layer of Metvix cream was applied for 3 hr. PDT was delivered as described above for ALA treatments. However, unlike the ALA treatments, Metvix PDT was delivered in two sessions, 6-10 days apart.

Evaluation of treatment outcomes

One month after PDT, the sarcoid size was re-measured with vernier calipers and treatment response was evaluated according to RECIST guidelines (Eisenhauer et al 2009). Comparison of the pre-treatment and 1-month sarcoid size resulted in one of four possible outcomes: a complete response (CR, disappearance of lesion); partial response (PR, >30% decrease in lesion size); stable disease (SD, neither increase not decrease in lesion size); or progressive disease (PD, >20% increase in lesion size). Treatment success was defined as either CR or PR at 1-month, whilst treatment failure was defined as either PD or SD at 1-month. Statistical differences between the treatment success and treatment failure categories for each treatment arm were determined in 2x2 matrices using Fisher's exact test, 2-tailed, summing small *P* values, with 95% confidence interval (GraphPad Prism 6). Using the same pre-treatment and 1-month size measurements, sarcoid volume was estimated using the formula $V = \pi/6 \times 1.6 (\text{length} \times \text{width})^{1.5}$ (Feldman et al 2009). Wilcoxon matched-pairs signed rank test was used to compare pre-treatment and 1-month sarcoid volume in the same horses and Mann-Whitney test for comparison of percentage

volume changes between categories (GraphPad Prism 6). Some sarcoids were photographed at weekly intervals for 1 month.

RESULTS

In vitro cytotoxicity studies

Initially, we investigated whether PDT followed by glycolysis inhibition was more effective than PDT-only at killing equine sarcoid cells in vitro, and whether there was any selectivity for sarcoid cells over normal equine fibroblasts.

Cells were subjected to either one round of PDT or four successive rounds of PDT. Sarcoid cells were consistently more sensitive to PDT than fibroblasts, and glycolysis inhibition strongly potentiated the PDT-induced cell death (Fig. 1). Importantly, repeated rounds of PDT then glycolysis inhibition did not lead to the formation of PDT-resistant colonies, but instead further sensitised sarcoid cells to PDT (Fig. 1).

PpIX accumulation in sarcoid skin

To translate the in vitro work with glycolysis inhibitors to a clinical setting, we initially examined the accumulation of ppIX fluorescence in ex-vivo sarcoid skin, following 4hr topical application of ALA in a number of formulations. These included: a commercial cream preparation (Mandeville Medicines), a preparation in Cetraben emollient cream, and a liquid preparation in a penetration enhancer solution (Karande 2004). Then, transverse sections of sarcoid skin were examined by fluorescence microscopy. In every case, including the control treated with penetration enhancer only, we found a high red fluorescence background within the dermis (Fig.

2), possibly due to endogenous porphyrins (Borisova 2014). However, only in sarcoid skin treated with ALA in penetration enhancer was red fluorescence detected in the epidermis (asterisk in Fig. 2D), implying that this vehicle was the best at delivering ALA into the skin.

To verify these findings, we repeated the ex-vivo topical application experiment using normal mouse skin, which we find to have a much lower red fluorescence background. It now became apparent that the penetration enhancer preparation (Fig. 3A) delivered much more ALA into the superficial epidermis and hair bulbs than the Cetraben cream formulation (Fig. 3B).

Transdermal penetration of glycolysis inhibitors in sarcoid skin

It is technically difficult to measure the skin depth distribution of the glycolysis inhibitors using direct detection methods. However, lonidamine and 2DG both inhibit the metabolism of ALA into ppIX (Golding et al 2013). We took advantage of this effect to indirectly assess the ex-vivo skin penetration of glycolysis inhibitors by topically applying glycolysis inhibitors, then bisecting the skin through the application site and incubating the two halves in ALA. Fluorescence microscopy revealed strong ppIX red fluorescence in the epidermis and hair bulbs of control sections that had been topically treated with penetration enhancer only (Fig. 4A). By contrast, in sections topically treated with glycolysis inhibitors in penetration enhancer, the formation of red fluorescent ppIX within the epidermis and hair bulbs at the application site was greatly diminished (Fig. 4B). Within the same sections, but further away from the glycolysis inhibitor application site, strong ppIX fluorescence

was observed, similar to control sections (Fig. 4C). These results indicate that the glycolysis inhibitors fully penetrate the epidermis (about 200 microns thick).

Clinical data

Based on these encouraging ex-vivo tests, we used topical application of ALA in penetration enhancer, with or without post-PDT glycolysis inhibitors, to treat naturally occurring flat occult, verrucous, nodular and fibroblastic sarcoids in vivo.

The application of the ALA, illumination and subsequent application of glycolysis inhibitors was well tolerated in all cases. Within 12-24 hrs of illumination some localised erythema and oedema of the treated skin could be observed, this generally resolved within 5-7 days. Over the next 2-4 weeks (and longer in some cases) those sarcoids that responded to treatment became scabby with desquamation of the affected skin (Fig. 5), before finally healing. In some partial responders, the lesions became much flatter but the scabbing and crusting persisted.

Treatment outcomes after 1 month were determined on a categorical basis (RECIST, Eisenhauer 2009) and on a numerical basis (percentage change in sarcoid volume, Feldman 2009). In each study arm, treatment success was defined as a sarcoid that demonstrated either complete recovery (CR) or partial response (PR), whilst treatment failure was defined as a sarcoid that demonstrated either stable disease (SD) or progressive disease (PD) (Eisenhauer 2009).

The data are summarised in Tables 1 and 2 and the full data set is presented in Supplementary Table 1. Representative photographs of one horse, treated with ALA PDT then 600μM lonidamine, are presented in Fig. 6.

ALA-only PDT mainly resulted in a stabilisation of sarcoid growth progression (SD) after 1 month (4/7 cases) or progressive disease (PD, 2/7 cases) with one case showing a partial response. ALA-only PDT demonstrated a non-significant increase in mean sarcoid volume of 13.1±17.9% (±SEM; $P>0.9999$, n=7). By contrast, ALA PDT followed by either 600μM lonidamine or Metvix PDT gave significantly greater treatment successes than ALA-only PDT: $P=0.0001$ (ALA-Lon, 25/27) and $P=0.0047$ (Metvix, 7/7). ALA PDT followed by 180mM 2DG gave a treatment success that was only just non-significant ($P=0.0573$; 9/4) compared with ALA-only PDT. The mean sarcoid volume also significantly decreased 1 month after these treatments, by 80.6±6.8% $P<0.0001$ (ALA-Lon), 66.0±12.9% $P=0.0039$ (ALA-2DG), and 99.0±0.5% $P=0.0156$ (Metvix).

ALA PDT followed by 600μM lonidamine gave treatment successes that were indistinguishable from those of Metvix PDT ($P=1.0000$), and sarcoid volume decreases that were also indistinguishable from those of Metvix PDT ($P=0.6742$). This result is particularly important, since Metvix is applied as two separate PDT treatments about 1 week apart, whilst ALA PDT followed by 600μM lonidamine was applied as a single treatment. Like-for-like comparison of ALA and Metvix treatments was not possible, because single-treatment Metvix PDT was not ethically approved for this study. Similarly, glycolysis inhibitor-only controls were not ethically approved.

DISCUSSION

We have demonstrated that a combination treatment of ALA PDT followed by glycolysis inhibition selectively kills sarcoid cells in vitro. Our work further demonstrates that a single application of ALA PDT then glycolysis inhibition can

successfully treat most equine occult, verrucous, nodular and fibroblastic sarcoids up to 5mm thick. The most striking increase in treatment success was obtained with ALA PDT followed by 600μM lonidamine.

The advantage of PDT over more conventional therapies is that it is minimally invasive and provides very good cosmesis. Furthermore, as we have shown, PDT is well tolerated by horses, even those with multiple sarcoids. Whilst all horses in this study were hospitalised for application of lonidamine, the light sources used are portable and it would be quite feasible to routinely treat horses in their home yards.

The success of this new technique can be attributed mainly to glycolysis inhibition (as shown by our in vitro work), which selectively compromises cellular antioxidant defences and repair in cancer cells that preferentially rely on aerobic glycolysis (Vander Heiden et al 2009). Our choice of a particularly effective transdermal penetration enhancer (Karande 2004) is also likely to contribute to the overall success of this new combination PDT technique.

Existing guidelines for human therapy, advise against using PDT for relatively deep skin tumours, such as nodular BCCs or SCCs (Morton 2013).

For this reason, current work on improving PDT is focussed on achieving deeper skin penetration of ALA by changing the delivery formulation. For instance, new ALA formulations, such as BF-200 nanoemulsion, demonstrate improved skin penetration and are substantially better at treating pre-cancerous skin conditions with ALA PDT than Metvix PDT (Dirschka, 2012). However, improvements in the skin penetration of ALA and other photosensitisers will eventually reach a limit, after which the only way to further improve PDT will be by combination therapies, such as glycolysis inhibition, and this area requires further research.

To facilitate adoption of this new PDT technique in clinical practice, subsequent work will need to determine whether our treatment protocol can be simplified. For instance, can similarly good treatment responses be obtained by using a single topical application of ALA and a single post-PDT topical application of lonidamine? Furthermore, in order to make meaningful comparisons with the current two-treatment Metvix PDT protocol, treatment success rates need to be examined using a similar two-treatment regimen for ALA PDT followed by lonidamine. Indeed, our in vitro data indicate that repeated rounds of ALA PDT followed by lonidamine should sensitise any remaining sarcoid cells to subsequent PDT. This two-treatment protocol is currently being evaluated at the Cambridge equine hospital.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

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TABLES AND FIGURES

Table 1. Summary of sarcoid treatment responses (RECIST guidelines) after 1 month.

CR = complete recovery. PR = partial response. SD = stable disease. PD = progressive disease.

Treatment	**CR**	**PR**	**SD**	**PD**	**Total**	**Success (CR+PR)**	**Failure (SD+PR)**
ALA only	0	1	4	2	7	14%	86%
ALA then 2DG	7	2	4	0	13	69%	31%
ALA then Lon	17	8	1	1	27	93%	7%
Metvix	4	3	0	0	7	100%	0%

Table 2. Fisher's exact test, comparing treatment success (CR+PR) with treatment failure (SD+PD) after 1 month.

Treatment comparison	***P* value**	**Summary**
ALA vs ALA 2DG	0.0573	ALA 2DG is better than ALA
ALA vs ALA Lon	0.0001	ALA Lon is better than ALA
ALA vs Metvix	0.0047	Metvix is better than ALA
Metvix vs ALA Lon	1.0000	Metvix = ALA Lon

Figure 1. PDT followed by glycolysis inhibition selectively potentiates death of equine sarcoid cells.

Equine sarcoid cell line (SO4B) or normal equine fibroblasts (Eq) were subjected to either one round of PDT (x1) or four successive rounds of PDT (x4). An identical series of dark toxicity experiments was performed where cells were not exposed to light. Low doses of ALA (21 μg/ml, 3hr) and light (10 J/cm^2) were used, so as to simulate the conditions at the deeper margins of a sarcoid. A control in the absence of ALA (zero) was included. Cell death was quantified 24 hrs after the final PDT session by propodium iodide exclusion cytometry. Sarcoid cells were more sensitive to PDT than fibroblasts, and glycolysis inhibition potentiated this cell death. Importantly, repeated rounds of PDT then glycolysis inhibition did not lead to the formation of PDT-resistant sarcoid cell colonies, but conversely resulted in increased sensitivity of sarcoid cells to PDT then glycolysis inhibition.

For each condition n=3 and data are presented ±SEM. * denotes a significant difference versus respective zero control (Dunnett post-test). # denotes a significant difference Dark Toxicity versus PDT (Bonferroni post-test). § denotes a significant difference x1 treatment versus x4 treatments (Bonferroni post-test). In each case, a single symbol indicates $P<0.05$, two symbols indicate $P<0.01$, three symbols indicates $P<0.001$

Figure 2. Comparison of ex-vivo sarcoid skin penetration of three topically applied ALA formulations.

Horse sarcoid biopsies were incubated for 3 hrs with topical application of three ALA formulations (each 20% w/w or w/v), then sectioned through the application site and photographed. ALA is metabolised to red fluorescent protoporphyrin IX. Panels on the left show protoporphyrin IX (red) and Hoechst nuclear stain (blue). Panels on the right show the corresponding brightfield image. In each fluorescence image, the epidermis is marked with an asterisk.

A) No ALA control. B) Clinical ALA cream (Mandeville Medicines). C) ALA in Cetraben emollient cream. D) ALA in penetration enhancer liquid.

Only ALA in penetration enhancer gave strong protoporphyrin IX staining throughout the epidermis. However, note that deeper layers of skin have natural red autofluorescence.

Figure. 3. Comparison of normal mouse skin penetration of two topically applied ALA formulations.

Panels on the left show protoporphyrin IX (red) and Hoechst nuclear stain (blue). Panels on the right show the corresponding brightfield image. In each fluorescence image, the superficial epidermis is marked with an asterisk.

A) ALA in penetration enhancer gave strong red protoporphyrin IX staining in the superficial epidermis and hair root bulbs. B) ALA in Cetraben cream gave much less protoporphyrin IX red fluorescence.

Figure 4. Penetration of Lonidamine into horse sarcoid skin ex-vivo.

Lonidamine inhibits the metabolism of ALA into fluorescent protoporphyrin IX and this is used as a proxy for detecting lonidamine skin penetration. Sarcoids were treated with topical lonidamine, then bisected and incubated with ALA, and then photographed under fluorescence illumination (left panels) and brightfield (right panels). In each fluorescence image, the epidermis is marked with an asterisk.

A) No-lonidamine control demonstrates strong red fluorescence in the epidermis and hair root bulbs. B and C) Lonidamine-treated skin shows much less red fluorescence in the epidermis and hair bulbs at the lonidamine application site (B), but a return of red fluorescence further away from the lonidamine application site (C).

Figure 5. Crusting and desquamation on an occult sarcoid responding to ALA PDT then 600μM lonidamine after 7 weeks.

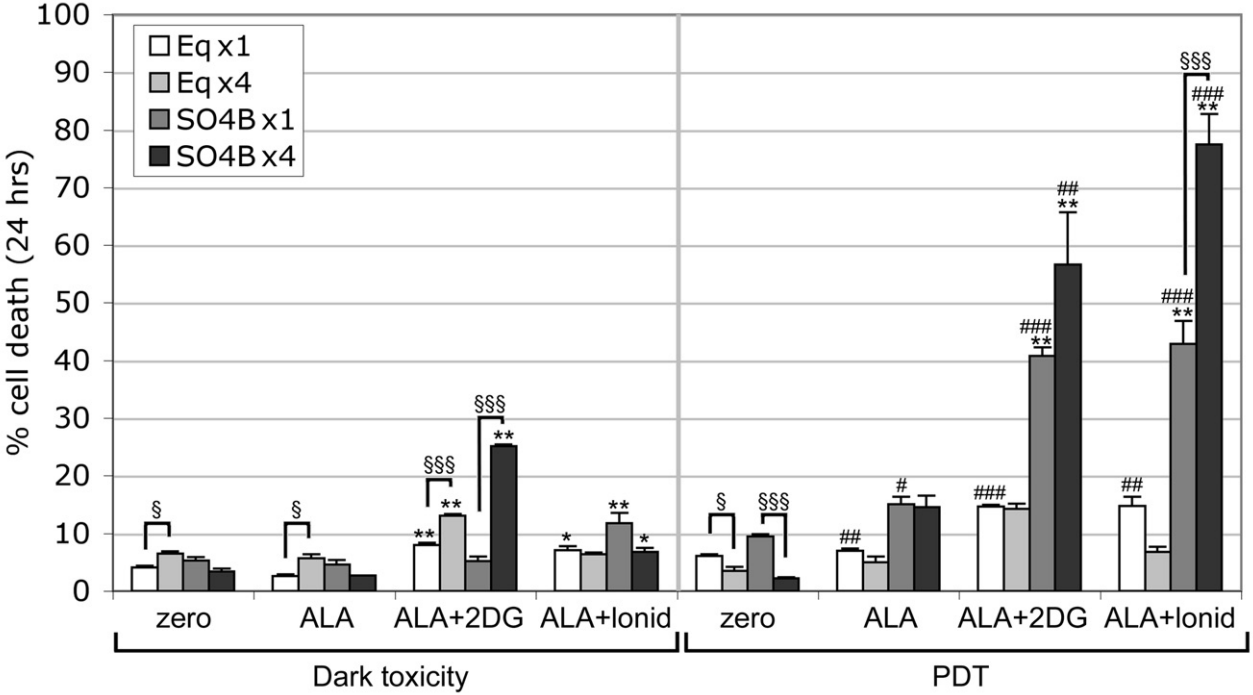
Figure 6. Temporal sequence of ALA PDT followed by 600μM lonidamine application on an occult sarcoid.

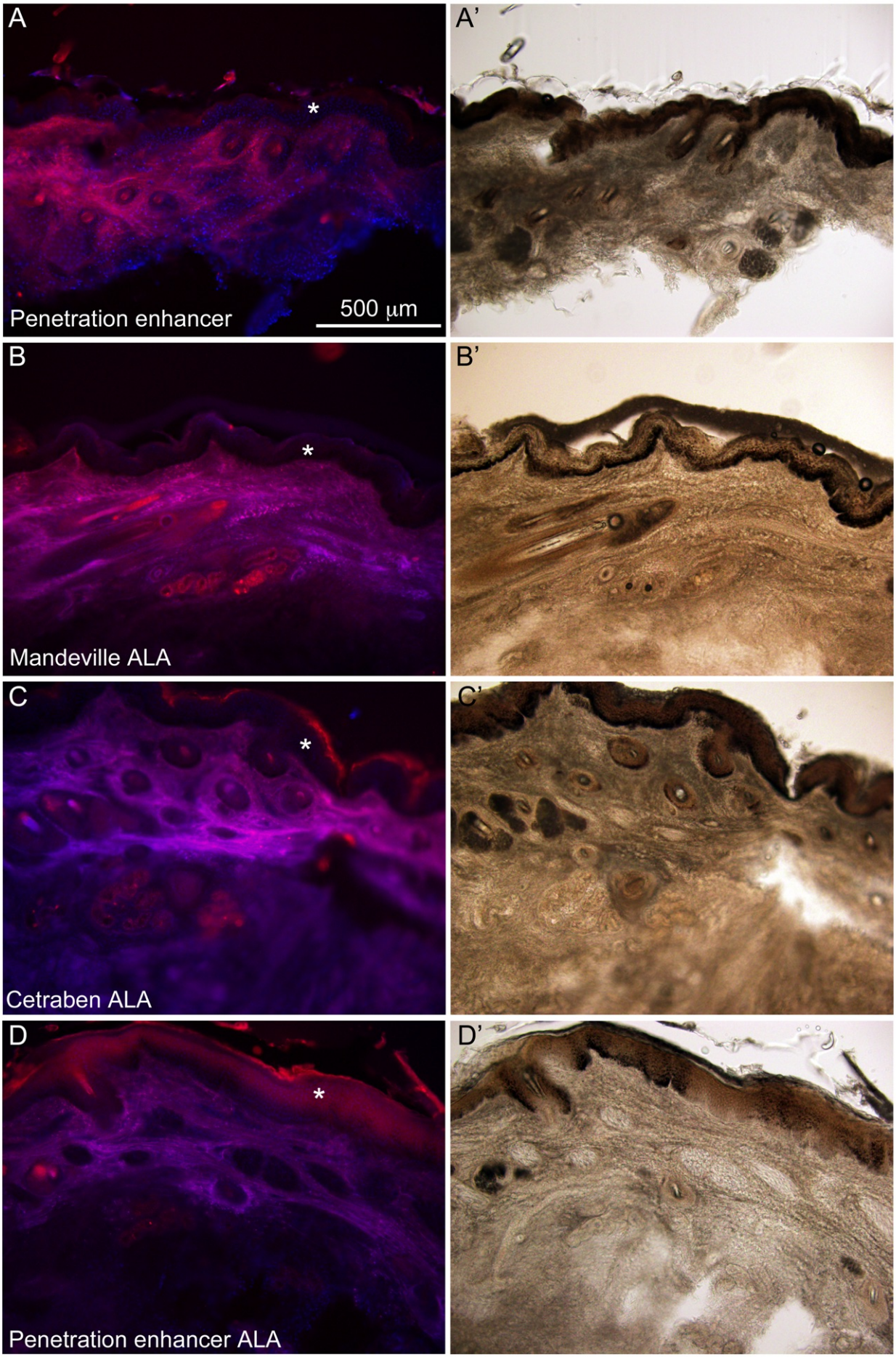
A) Pre-treatment. B) 1 week post-PDT showing crusting. C) 1 month post-PDT showing reduction in sarcoid size. D) xxxx months post-PDT showing complete cure.

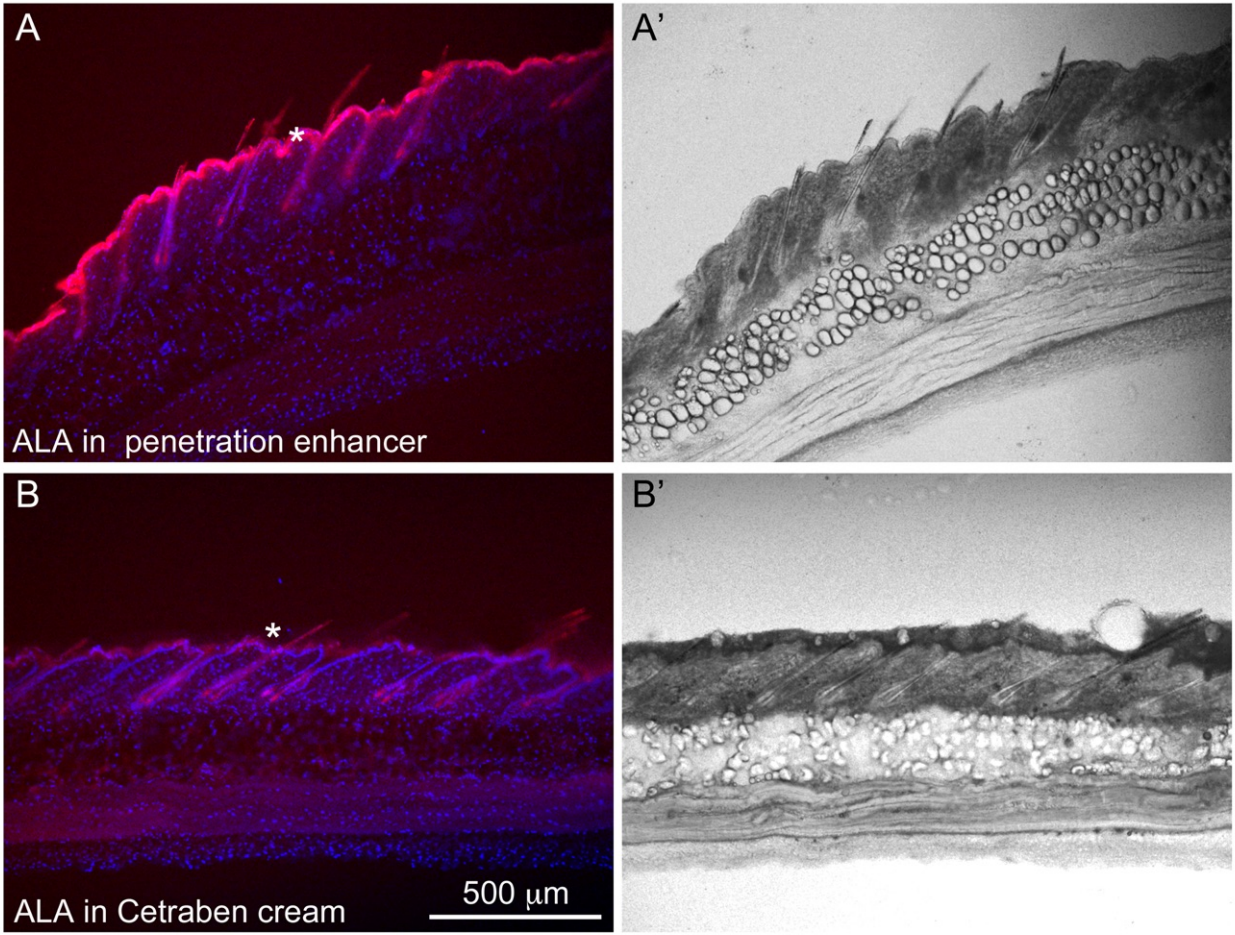
SUPPORTING INFORMATION

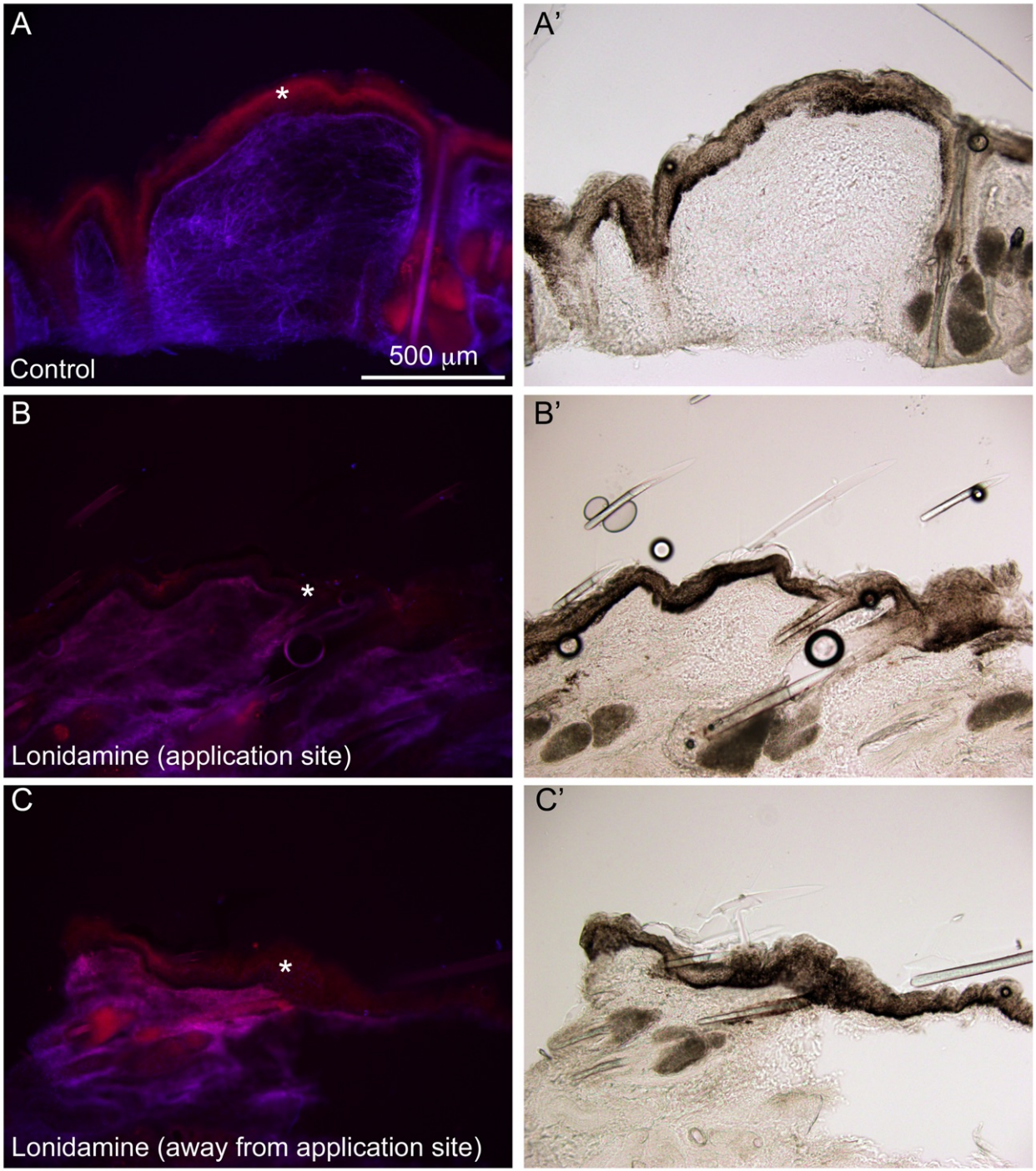
Supplementary Table 1

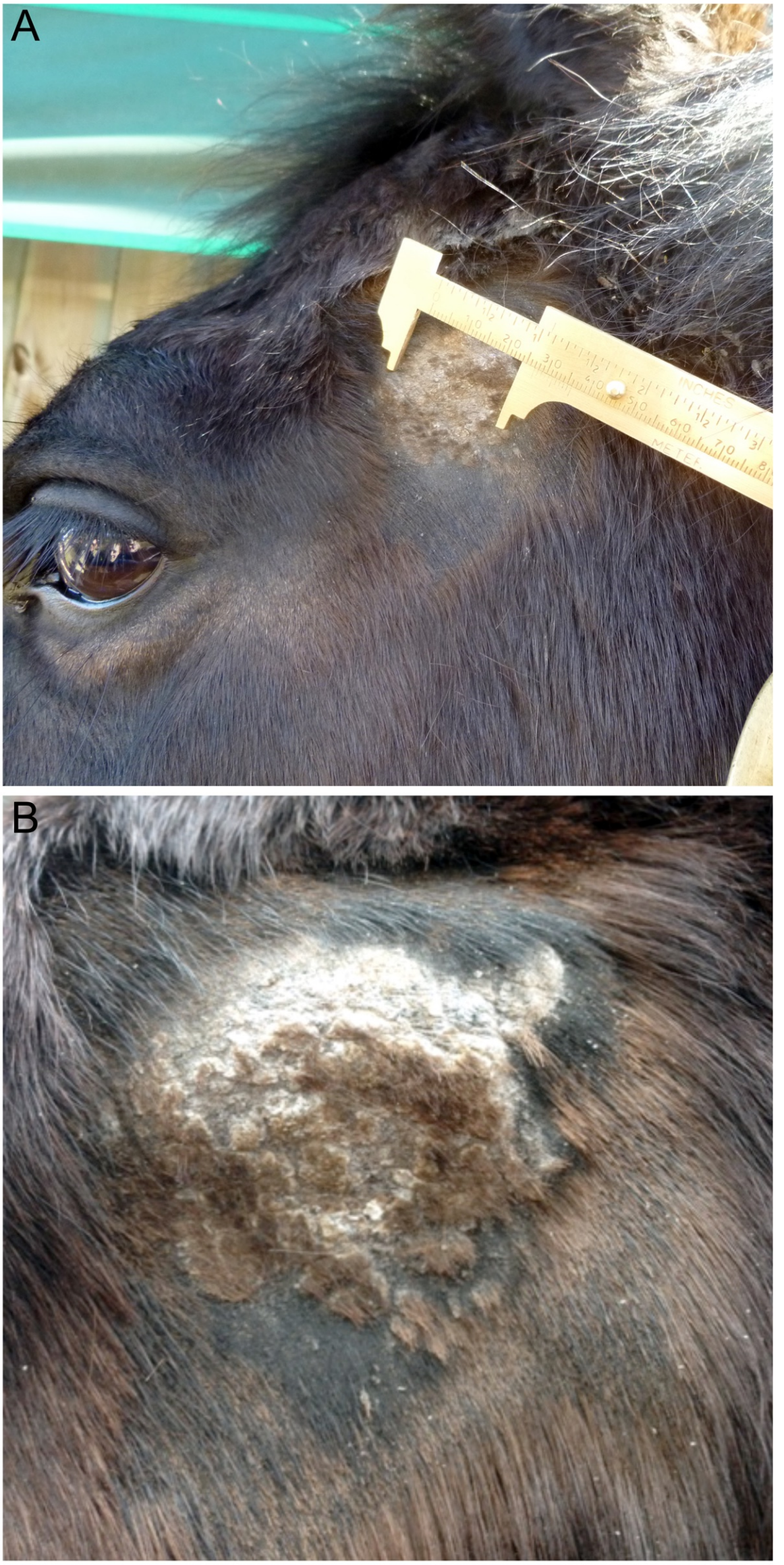
Excel spreadsheet showing full clinical details and results.

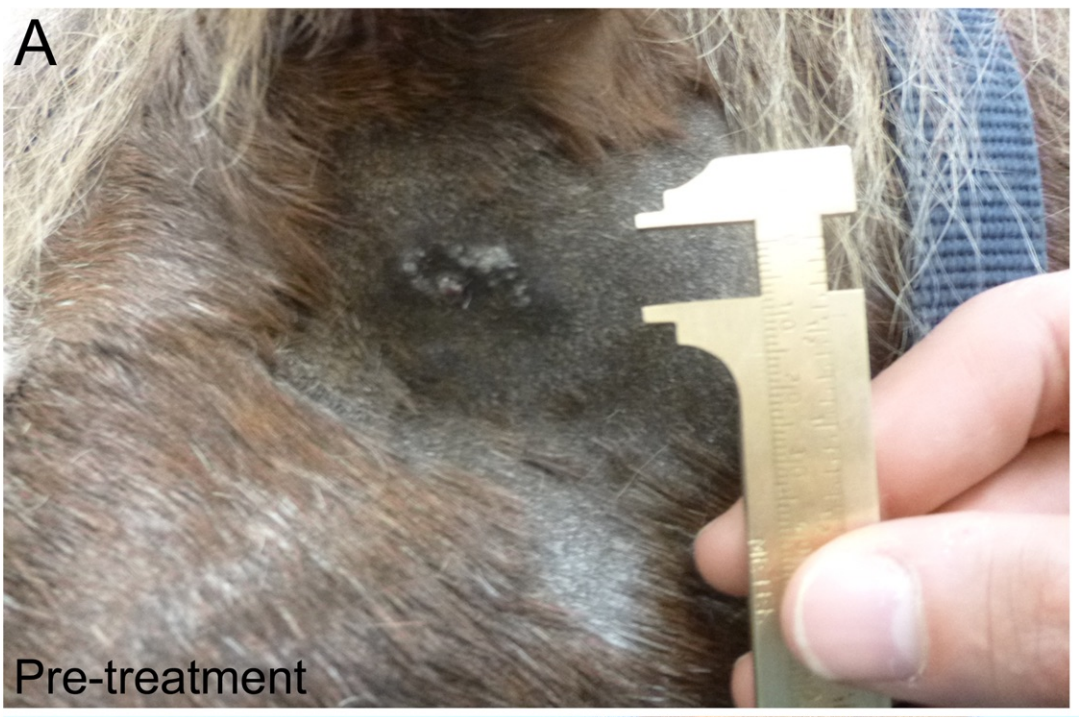

Eq x1
Eq x4
SO4B x1
SO4B x4
% cell death (24 hrs)
100
90
80
70
60
50
40
30
20
10
0
zero
ALA
ALA+2DG
ALA+Ionid
zero
ALA
ALA+2DG
ALA+Ionid
Dark toxicity
PDT


A
A'
*
Penetration enhancer
500 µm
B
B'
*
Mandeville ALA
C
C'
*
Cetraben ALA
D
D'
*
Penetration enhancer ALA

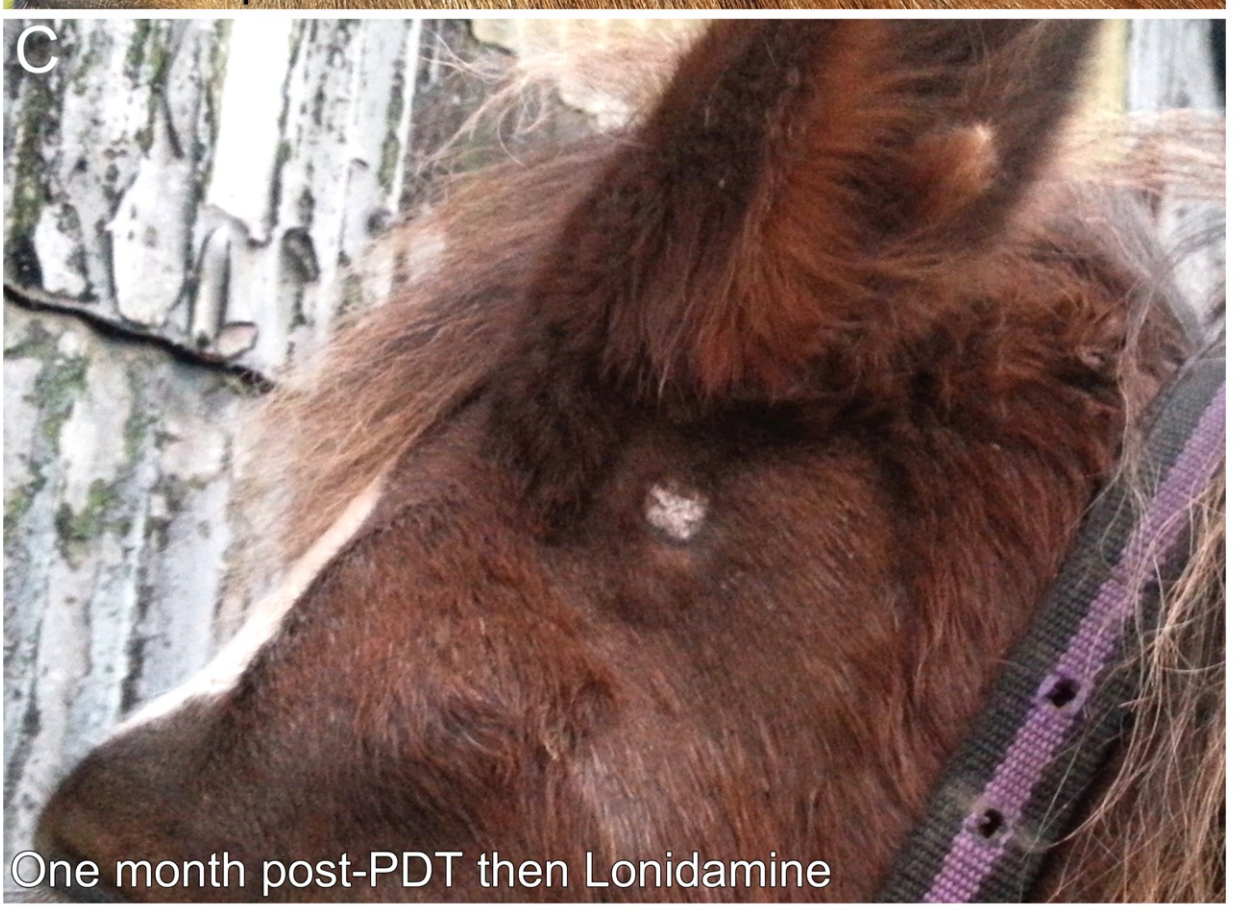

A
A'
ALA in penetration enhancer
B
B'
ALA in Cetraben cream
500 μm

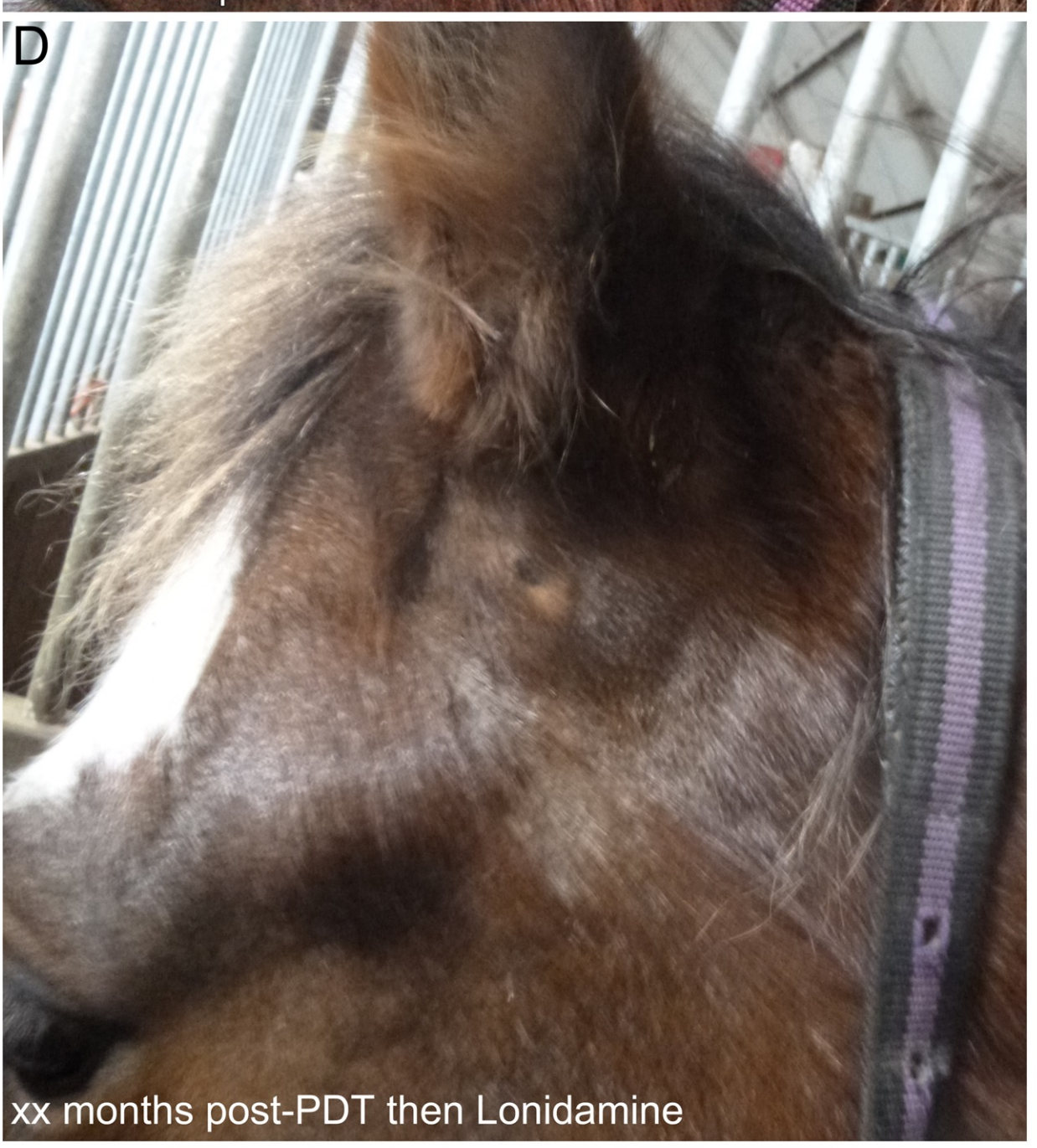

A
*
Control
500 μm
A'
B
*
Lonidamine (application site)
B'
C
*
Lonidamine (away from application site)
C'


A
B


A
Pre-treatment


B
One week post-PDT then Lonidamine


C
One month post-PDT then Lonidamine


D
xx months post-PDT then Lonidamine