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Macro-microscopic Fluorescence Imaging of Human NPC Xenografts in a Murine Model Using Topical vs. Intravenous Administration of 5-Aminolevulinic Acid

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Macro-microscopic fluorescence imaging of human NPC xenografts in a murine model using topical vs intravenous administration of 5-aminolevulinic acid

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Abstract. The use of 5-aminolevulinic acid to induce endogenous porphyrins for the purpose of detection of epithelial cancers is being studied extensively in many centres around the world. The challenge is to prepare an efficacious formulation of 5-ALA for the purpose of cancer detection. In this study, we compared two formulations of topical 5-ALA applications with intravenous administration in NPC/CNE-2 xenografts on balb/c nude mice. One of the formulations was a gantrez muco-adhesive patch and the other was a polyvinyl-pyrolidone muco-adhesive patch. The Karl Storz fluorescence endoscopy system was used to obtain macroscopic fluorescence images. Microscopic fluorescence imaging was done by laser confocal microscopy. The macroscopic images were further analysed for fluorescence intensity distribution. It was found that between the two formulations of topical application of 5-ALA; there was very little difference in the fluorescence biodistribution. When the topical applications were compared with the intravenous administration, the tumour to normal differential in biodistribution was significantly higher with the topical application compared to the intravenous application.

Introduction

The use of endogenous protoporphyrin IX (PpIX) as a photosensitizer to diagnose certain superficial cancers is under extensive study in many centres around the world. 5-Aminolevulinic acid (5-ALA) is a precursor in the heme biosynthetic pathway of nucleated cells. It is metabolized by certain endogenous enzymes to produce PpIX, which is an endogenous photosensitizer. PpIX is an intermediate by-product in the heme biosynthetic pathway (1). Survival of mammalian cells is crucially dependent upon the biosynthesis and metabolism of porphyrins (2). All mammalian nucleated cells have the capacity to synthesize heme. PpIX being the immediate precursor of heme will also be synthesized in equal amounts. However, a tight feedback loop control ensures that only a very small amount of PpIX is left at any one time (3). Accumulation of the endogenous porphyrins only happens under abnormal conditions such as in malignant or pre-malignant cell transformation. PpIX preferentially accumulates in tumour cells due to changes in the activity of two main enzymes, porphobilinogen deaminase (PBG) and ferrochelatase (FC) (4-6). In tumour cells, while the activity of PBG is increased, the activity of FC is decreased resulting in the build up of PpIX. In normal cells, FC catalyses the conversion of photosensitive PpIX to heme, which is not photosensitive. It chelates the Fe^{2+} into the porphyrin ring structure. The over production of PpIX can also be induced by the administration of exogenous 5-ALA or ALA esters (topically or intravenously). While 5-ALA is hydrophilic, which is a limitation as it restricts its penetration through cellular membranes; its ester derivatives have been developed to be of increased lipophilicity.

PpIX is a well-known photosensitizer that yields sufficient singlet oxygen when activated by light of appropriate wavelength and intensity. 5-ALA is already in use in many centres for photodynamic diagnosis (PDD) and photodynamic therapy (PDT) in various superficial diseases such as actinic keratosis, basal cell carcinoma and squamous cell carcinoma. 5-ALA induced PpIX is also highly fluorescent. Upon activation with blue/violet light, PpIX fluoresces in the red. 5-ALA is in use for the detection of neoplastic urothelial lesions, early stage lung cancer, cervical dysplasia and laryngeal neoplasm.

There have only been a few studies conducted with respect to pharmacokinetics of 5-ALA-induced porphyrins following topical applications. Most studies have been done on applications to the skin. It was observed that the normal looking skin overlaying the tumours constitutes a much

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lower barrier for 5-ALA penetration than the skin overlaying healthy tissues. It was also found that the 5-ALA induces more PpIX fluorescence than certain of its esters do. Systemic uptake is also reported to be high resulting in high accumulation of PpIX in other vital organs such as the liver, gut and skin. These studies were compared with intravenous or intraperitoneal administrations of 5-ALA (7-10). The aim of this study was to compare the uptake and retention pharmacokinetics of the topical applications of 5-ALA, in the form of muco-adhesive patches and intravenous administration. With the use of macroscopic and microscopic fluorescence imaging of endogenous PpIX, the optimal selectivity between tumour and normal regions was determined. Gantrez and polyvinylpyrolidone were chosen to prepare the patches for topical applications, respectively.

Materials and methods

Tumour model. CNE-2 is a cell line derived from a poorly differentiated nasopharyngeal carcinoma in human. It was a gift from Professor K.M. Hui, Cellular and Molecular Research, National Cancer Centre, Singapore. The cells were grown in culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1X L-glutamine, 100 units/ml penicillin and streptomycin (Gibco) and 1X sodium pyruvate in an atmosphere of 5% CO_2 at 37°C. Once confluent they were washed with 1X phosphate buffered saline and trypsinized with 0.05% Trypsin-EDTA (Gibco). The cells were then re-suspended in 1X Hanks balanced salt solution. Male Balb/c nude mice, 6-8 weeks old, about 20-25 g were obtained from Animal Resource Centre of Australia. The trypsinized cells were implanted subcutaneously into the flanks of the nude mice. The mice were housed in micro-isolator cages (5 per cage) fed with filtered air through air vents. Their food, water and bedding were sterilized. The tumour was allowed to grow over the next 7-10 days to a size of 5-8 mm in diameter.

5-ALA muco-adhesive patches. In the Gantrez patch series (GP), 5-ALA was dissolved in poly (methyl vinyl ether/maleic anhydride (Gantrez, ISP, USA)/poly (meth) acrylates (Eudragit, Rohm Pharma, Germany)/alcohol solution. In the Polyvinyl pyrolidone patch series (PP), 5-ALA was dissolved in polyvinyl-pyrolidone (Plasdone, ISP, USA)/alcohol solution. A low concentration of 1 mg/cm^2 of 5-ALA and high concentration of 2.5 mg/cm^2 of 5-ALA were made for both formulations of patches. The solution was then poured onto a calculated area of polythene sheets and left to dry. The patches were cut into 1.5 cm^2 pieces and sealed in bags and stored for further use. Both formulations have muco-adhesive properties.

Animal experiments. Three groups of animals were used. One group was used for the intravenous administration of 5-ALA; the other two were used for PP and GP applications. For topical application, 10 mice per group were used. The nude mice were anaesthetized with a cocktail of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml flaunisone, Janssen), Dormicum (5 mg/ml midazolam HCl, David Bull Laboratories) and deionised water. The skin overlaying the tumour was carefully removed to expose the tumour. Extreme care was taken to minimize bleeding as this can interfere with the imaging procedure. A white light image of the tumour was obtained using the Karl Storz fluorescence endoscopy system (FES). This is to help later in identifying the tumour and its margins when comparing with the fluorescence image. The patches were then applied on the tumour making sure that the patch covers the tumour well and part of the normal tissue surrounding the tumour. For the PP muco-adhesive patches, the patch was applied for 30 min and then removed. The area was rinsed well with 70% alcohol to remove any residual 5-ALA. The tumour was imaged by the FES, at different time points, post patch application, using filtered blue light at 460 nm. For the GP muco-adhesive patches, the patch was left on for 1 h, before it was imaged using the endoscopy system.

Intravenous administration. 5-ALA was dissolved in 0.9% sodium chloride solution and the pH was adjusted to 6.5. The mice were administered with a dose of 250 mg/kg-body weight by tail vein injections. Ten animals were used for each time point. They were then kept in darkness. The mice were imaged at 1 and 3 h, the skin overlaying the tumour was removed and the tumours were imaged using the FES. The mice were sacrificed, the tumours were removed and snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

Image analysis. The images thus obtained were analyzed using the image analysis software, MicroImage 4.0. Images obtained in blue light needed to be further processed to obtain normalized fluorescence intensity images. The processing involves contrast enhancement of the original image. This is followed by hue extraction where the fluorescence colour (red) that is needed is extracted. Following hue extraction, the thresholding and segmentation of the Area of Interest (AOI), which is the tumour in this case, was carried out. This gives us the red-black image. From this we obtained the relative intensity distribution over the tumour and the normal regions. Finally, the normalised intensity image, which is a ratio of the tumour fluorescence to the normal (blue) background, was obtained.

Microscopic imaging. Cross sectional cryo-sections of the frozen tumours were obtained by using the Microm Cryostat. The cryo-sections were sandwich sections, which means the thickness of the sections were in the order of 5-10 μm/15-20 μm/5-10 μm. The thinner sections were stained with haematoxylin and eosin (H&E). The thicker middle sections were imaged using the fluorescence laser confocal microscope (Carl Zeiss LSM510, Germany). An argon ion laser with a wavelength of 488 nm was used to excite the PpIX in the tissue and a 590 nm long pass filter was used to detect the PpIX fluorescence. An untreated tumour was used as a control and to subtract background fluorescence.

Statistical analysis. Statistical analysis was done using the SPSS 10.0 statistical package for windows. Analysis was done by comparing means (ANOVA). The mean difference was found to be statistically significant ($p<0.05$).

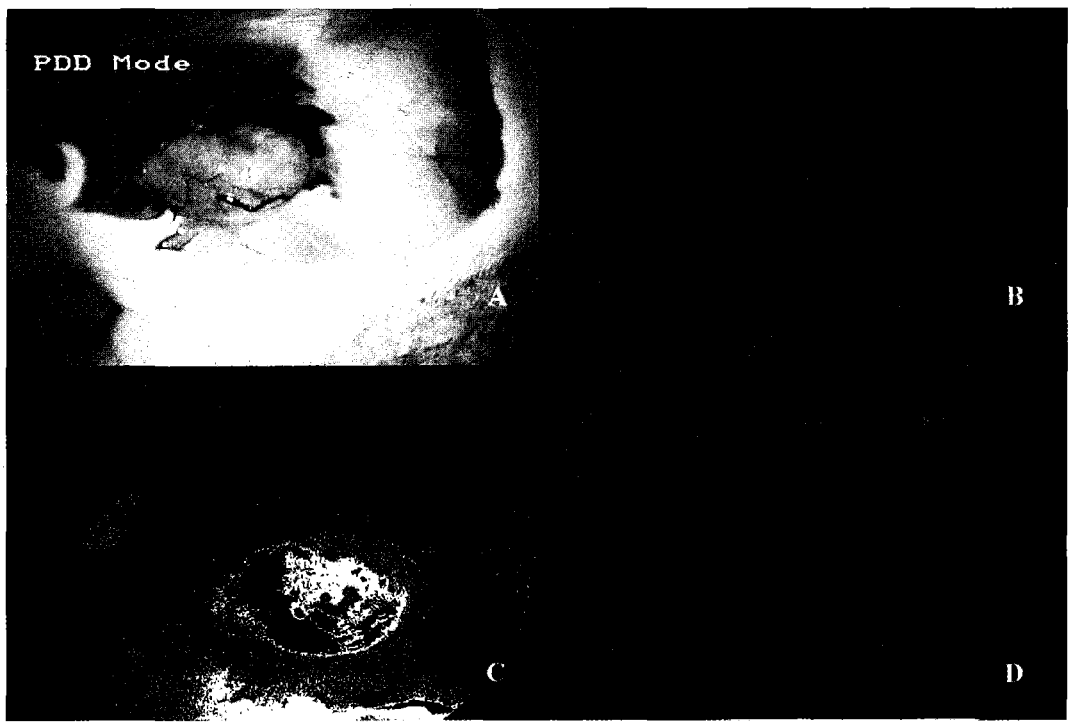

Figure 1. (A), White light image obtained by endoscopy system; (B), Red-black image obtained during image processing indicating fluorescent area of interest; (C), Fluorescence intensity distribution indicating low (green), medium (yellow) and high (red) regions within the tumour; (D), Enhanced fluorescence image of tumour.

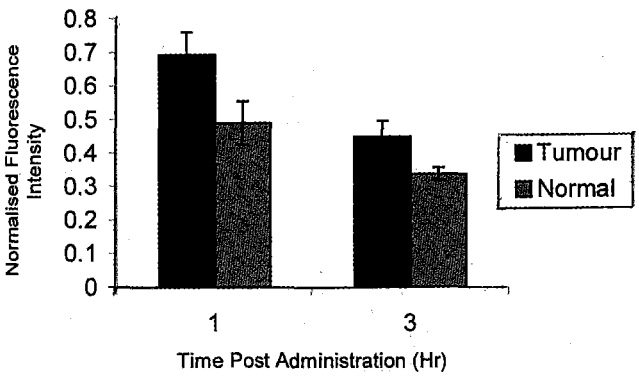

Figure 2. Normalised fluorescence intensity of tumour versus normal as a function of time for intravenous administration.

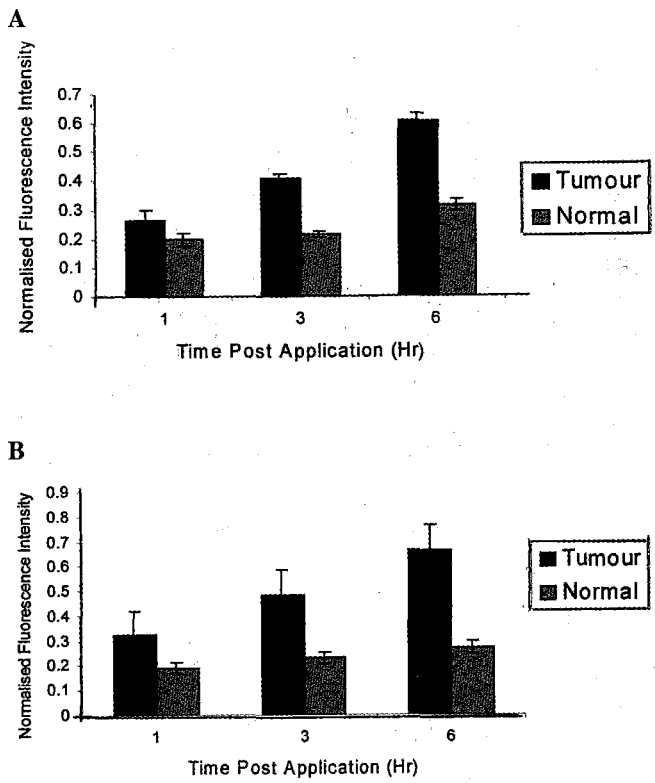

Figure 3. (A), Normalised fluorescence intensity of tumour versus normal as a function of time for 1 mg/cm^2 PP patch; (B), Normalised fluorescence intensity of tumour versus normal as a function of time for 2.5 mg/cm^2 PP application.

Results

The normalized intensity for the macroscopic fluorescence images was obtained by using the MicroImage 4.0. software. From the series of processed images obtained (GP patch) at 1-3 h post application (Fig. 1) it can be seen clearly that PpIX is highly selective to tumourous regions. When the normalized fluorescence intensities that were obtained from the tumours were plotted in histograms, both the PP and the GP patches were found to have tumour fluorescence increasing with time. The tumour to normal fluorescence ratio (T/N) also increased correspondingly. For the PP patches (1 mg/cm^2 and 2.5 mg/cm^2), by 3 h the T/N ratio was almost 2.6 ($p<0.05$). At 6-h post 5-ALA patch application, the T/N ratio was 2 ($p<0.05$). The GP patches (1 mg/cm^2 and 2.5 mg/cm^2), followed similar patterns as the PP patches (Figs. 3 and 4). By 3 h T/N ratio was also high, almost 2. When 5-ALA was intravenously administered, the background normal tissue fluorescence was high between

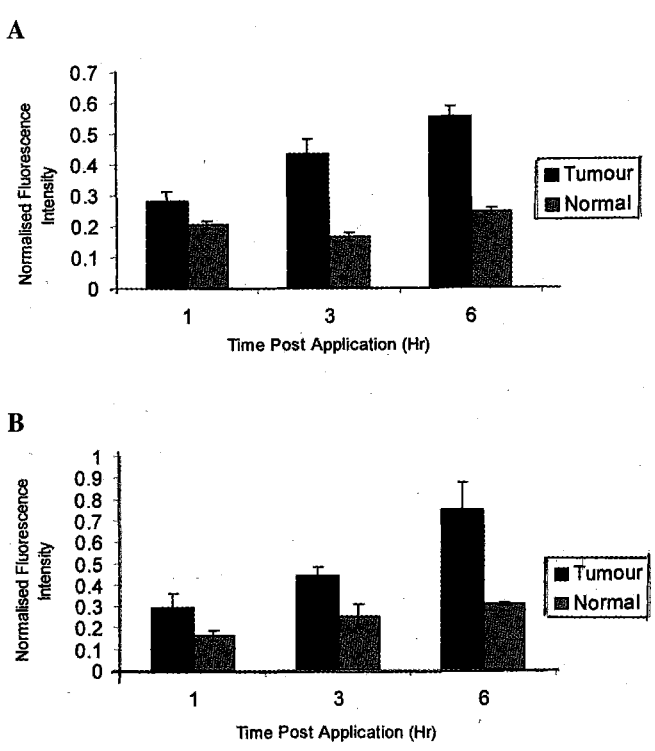

Figure 4. (A), Normalised fluorescence intensity of tumour versus normal, as a function of time for 1 mg/cm^2 GP application; (B), Normalised fluorescence intensity of tumour versus normal as a function of time for 2.5 mg/cm^2 GP application.

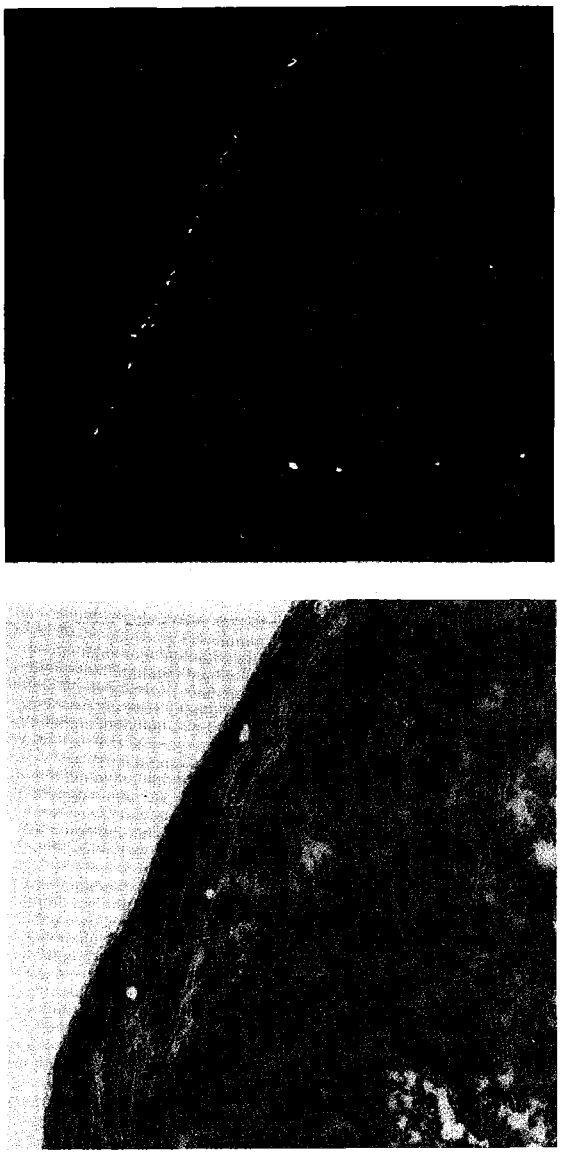

Figure 5. (A), PpIX microscopic fluorescence of NPC/CNE-2 xenograft (x20). (B), Haematoxylin and eosin staining of NPC/CNE-2 xenograft (x20).

1-3 h (Fig. 2). Hence the tumour to normal (T/N) ratio was not found to be statistically significant. However, although the overall fluorescence intensity for both tumour and normal tissue was high for the earlier time points there was still discrimination of the tumour. Cutaneous fluorescence in remote distal regions was also found to be high indicating that the 5-ALA that is still circulating is continuing to induce localised PpIX production in different parts of the anatomy. Microscopic confocal images were obtained using the laser scanning confocal microscope (LSM510). Only tumours with and without the 5-ALA treatment were compared, as this is a xenograft. Fig. 5A shows the PpIX micro-fluorescence in a NPC/CNE-2 tumour. Fig. 5B is the H&E counter-staining of the same section. It was found that the 5-ALA is well taken up by the NPC/CNE-2 tumour.

Discussion

Human tumour xenografts on Balb/c nude mice were used as a model to investigate the efficacy of the topical application in terms of PpIX production of tumour cells in an *in vivo* system. Intravenous administration of 5-ALA for endogenous porphyrin based PDD and PDT, has been the normal mode of administration. The main drawback of intravenous administration is that the circulating 5-ALA causes extensive cutaneous PpIX accumulation, and makes it necessary for the patients to avoid light, as they would still be photosensitive. Thus, there are merits for local 5-ALA delivery via topical applications. This delivery system can take many forms such as rinses, gels or creams, which have to be constituted extemporaneously when, needed due to instability of 5-ALA. Patches prepared for this study can be made and stored in an airtight container at room temperature for three to six months. These patches also have muco-adhesive properties and can be good candidates for use in the oral cavity PDD and PDT. Oral rinses require the patient to continuously rinse for 20 min. Hence oral rinses are not practical for routine clinical use. Also the contact time for 5-ALA on oral tissue is short with the use of an oral rinse.

In our investigations, it was detected that the intravenous 5-ALA administration not only gives high fluorescence of tumour but also that of normal tissues at the early time points both 1 and 3 h post 5-ALA administration. This is due to considerable amounts of circulating 5-ALA inducing local PpIX (and not circulating PpIX). In most cases normal liver and skin appeared to have high production levels of PpIX. The T/N fluorescence ratio was found to be not significant between 1.5-2 h. The fluorescence intensity pattern observed when both the GP and PP patches applied were found to

be superior to the intravenous administration. The T/N fluorescence ratio is significantly high at 3 h following topical PP or GP muco-adhesive patch application. Despite the expected differences in the availability of 5-ALA in the GP and PP patches, their *in vivo* performances did not show significant differences. This indicates that the rate-limiting step to 5-ALA delivery to the tumour tissue was the transdermal transport of the hydrophilic 5-ALA compound. In conclusion, there was no difference between the two polymer based patches. Apart from the difference in the intensity of the fluorescence amongst the different concentrations, the T/N fluorescence ratio was consistent in both the low and high 5-ALA concentration patches at both 3 and 6 h. Between the intravenous administration and the topical application, the difference lies in the fact that there is low systemic uptake in the topical application and therefore T/N fluorescence ratio is larger and found to be statistically significant with the topical patches.

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