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5-aminolevulinic acid as a potential contrast agent for image-guided surgery in pancreatic cancer

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ABSTRACT

Introduction: Pancreatic cancer often recurs following surgery suggesting new operative approaches are required. Fluorescence-guided surgery aims to assist surgeons in identifying tumour intraoperatively to facilitate complete resection. However, the ideal contrast agent for this purpose is not yet determined. The Rose criterion states that accurate image-guided surgery requires a Tumour-to-Background Ratio of contrast agent greater than 5. We investigated the potential of 5-aminolevulinic acid (ALA) for this purpose.

Methods: Pancreatic cancer cell lines CFPAC-1 and PANC-1 were compared with the control pancreatic ductal cell line H6c7. Cells were seeded on day 1 and fluorescence measured on day 4 following 4, 8, 24 or 48 hours incubation with 0.25, 0.50, 0.75 or 1.00mM ALA. Fluorescence was measured using a plate reader and microscopy.

Results: The maximum ALA-induced fluorescence for CFPAC-1 and PANC-1 was achieved after 48 hours incubation with 0.50mM ALA. Compared to cells incubated without ALA, a relative fluorescence increase of 39.4-fold in CFPAC-1 and 2.7-fold in PANC-1 was seen. ALA concentrations above 0.50mM did not result in higher fluorescence. In contrast, the control cell line H6c7 showed progressively increasing fluorescence with increasing ALA concentrations. The highest cancer/control cell fluorescence ratios for ALA were after 48 hours incubation with 0.25mM ALA; 122.9 in CFPAC-1 and 9.7 in PANC-1.

Conclusion: ALA-induced fluorescence in CFPAC-1 is significantly higher than the control cell line H6c7. PANC-1 achieved only mildly increased fluorescence compared to H6c7. ALA has the potential to provide an adequate level of fluorescence for image-guided pancreatic surgery in ALA-susceptible cancers.

Keywords: 5-aminolevulinic acid, ALA, pancreatic cancer, photodiagnostics, image-guided surgery

1. INTRODUCTION

Pancreatic cancer

Despite advances in oncological treatments over the last few decades, five-year survival from pancreatic cancer remains extremely low (2.9% in 1980-1981 and 3.3% in 2010-2011, data for England and Wales)¹. The majority of patients (60%) are diagnosed with stage 3 or 4 disease due to aggressive tumor biology and the late onset of symptoms². As a result, only 10% of patients will undergo curative-intent surgery and only 28% will receive chemotherapy^{2,3}. Even for those undergoing curative-intent resection, local disease recurrence is common (24%-40%)⁴⁻⁶. Reasons for this include anatomical restrictions to more radical surgery and early vascular and perineural invasion with micrometastases⁷.

Fluorescence-guided surgery

Fluorescence-guided surgery (FGS) aims to assist in the intraoperative identification of tumour to aid resection. At present,

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surgeons rely on inspection and palpation of tissues to determine the location and extent of tumour, with preoperative information usually provided by non-invasive imaging such as Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI). Intraoperative imaging techniques (such as ultrasound) are only helpful at the beginning of the procedure before the tissue planes become distorted once resection begins. In addition, laparoscopy has removed the tactile element of surgery, making the visual identification of residual tumour the most significant (and often only) intraoperative real time diagnostic tool. For oncological FGS, fluorescent contrast agents are needed that are taken up avidly by tumour cells but not by surrounding non-neoplastic tissue. The Rose criterion states that accurate image-guided surgery requires a signal-to-noise ratio (i.e. tumour-to-background tissue ratio) of greater than 5.⁸ A suitable fluorescent contrast agent for pancreatic cancer resection has not yet been identified.

5-aminolevulinic acid

5-aminolevulinic acid (ALA) is a naturally occurring amino acid that forms an early substrate in the haem biosynthesis pathway. Protoporphyrin IX (PpIX), the penultimate molecule in the pathway, fluoresces red when excited with blue/violet light with a peak emission at 635 nm⁹. The addition of exogenous ALA leads to an accumulation of PpIX that can be exploited for the purpose of FGS. Although not specifically targeted to pancreatic cancer, there is preferential accumulation of ALA-induced PpIX fluorescence in many neoplastic tissues when compared to surrounding non-neoplastic tissues^{10,11}.

ALA has clinical approval for use in FGS in grade III and IV malignant gliomas¹². There is also evidence that ALA FGS could be used in a number of other malignancies, such as ovarian and renal cell cancers^{13,14}. However, research on the use of ALA in pancreatic cancer is limited. A recent prospective study investigated the use of ALA in the detection of peritoneal metastases compared to standard surgery from 138 patients with known peritoneal disease from a variety of cancers (appendiceal, ovarian, mesothelial, colorectal, gastric and pancreatic)¹⁵. Although only 44.2% of the cohort had their peritoneal metastases identified using ALA, three out of the four patients with metastatic pancreatic cancer had their metastases identified.

Aim

Our aim was to investigate the degree of ALA-induced PpIX fluorescence in the two pancreatic cancer cell lines CFPAC-1 and PANC-1 compared to the control cell line H6c7 (an immortalized non-tumorigenic pancreatic ductal cell line with near-normal genotype) to assess if ALA had potential as a fluorescent contrast agent for FGS in pancreatic cancer.

2. Methods

Cell culture

CFPAC-1 was purchased from ECACC (European Collection of Authenticated Cell Cultures, Salisbury, United Kingdom), PANC-1 was purchased from RIKEN BioResource Centre (RIKEN BRC, Tsukuba, Japan), and H6c7 from Kerafast (Kerfast, Inc., Boston, USA). PANC-1 was cultured in Dulbecco's modified Eagle's medium containing 4.5g L⁻¹ glucose and 0.58 g L⁻¹ L-glutamine (Lonza®) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco™). CFPAC-1 was cultured in Iscove's modified Dulbecco's medium containing 4.5g L⁻¹ glucose, 4.0mM L-glutamine and HEPES buffer (Gibco™) supplemented with 10% FBS. H6c7 was cultured in Keratinocyte serum free media with L-glutamine, epidermal growth factor, bovine pituitary extract (Gibco™) and 1% antibiotic/antimycotic. Cells were cultured in 75cm² tissue culture flasks (Techno Plastic Products®) in a humidified incubator at 37.0°C with 5% CO₂. Cells were trypsinized when ~80% confluent with 0.5% trypsin-EDTA (Gibco™) and passaged at a split ratio of 1:4.

Fluorescence microscopy

Cells were seeded on Fluorodishes on day 1 and had fluorescence microscopy performed on day 3. Prior to imaging, cells were incubated for either 4 or 24 hours with 0.0, 0.25, 0.5, 0.75 or 1.0mM ALA (Sigma-Aldrich®) in serum-free culture media. After the appropriate exposure time, cells were washed three times in Phosphate Buffered Saline (PBS), fixed in 4% formaldehyde for 20 minutes, and washed again three times in PBS before having images taken on an Olympus® BX63 fluorescence microscope.

Quantitative fluorescence

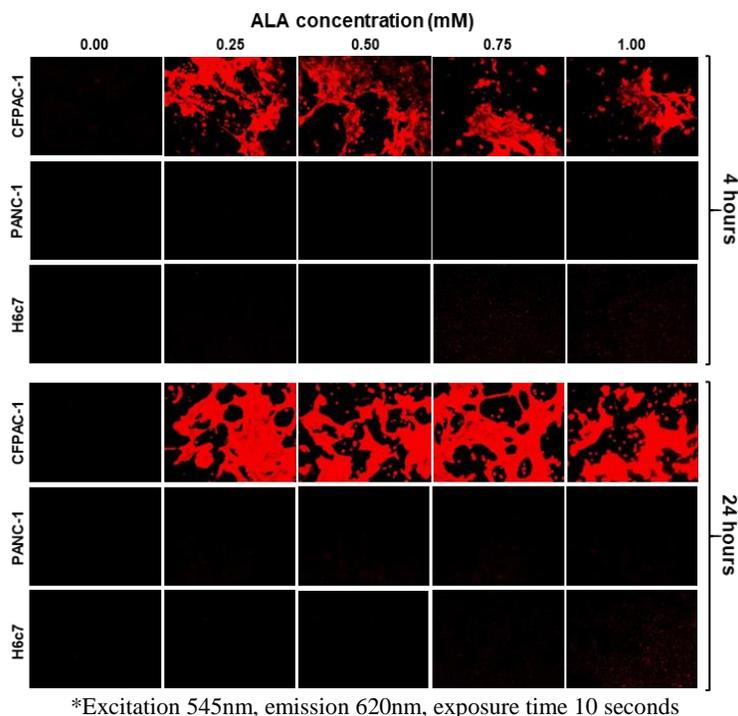
Cells were seeded onto 96 well plates (Techno Plastic Products®) on day 1 and had fluorescence measured on day 4 using an Infinite® 200 PRO plate reader (excitation wavelength 420nm, emission wavelength 635nm). Prior to fluorescence measurement, cells were incubated with the aforementioned ALA hydrochloride concentrations in serum-free media for 4, 8, 24 or 48 hours and washed twice in PBS immediately prior to reading.

3. Results

Fluorescence microscopy

CFPAC-1 readily converted ALA into PpIX resulting in strong red fluorescence at both 4 and 24 hours (**Figure 1**). In contrast, PANC-1 showed almost no red fluorescence at any ALA concentration at either time point. H6c7 showed minimal PpIX fluorescence at lower concentrations and time points but started to show some fluorescence at 1.0mM ALA at both 4 and 24 hours.

Figure 1 Fluorescence of pancreatic cell lines CFPAC-1, PANC-1 and H6c7 following 4 and 24 hours incubation with ALA*



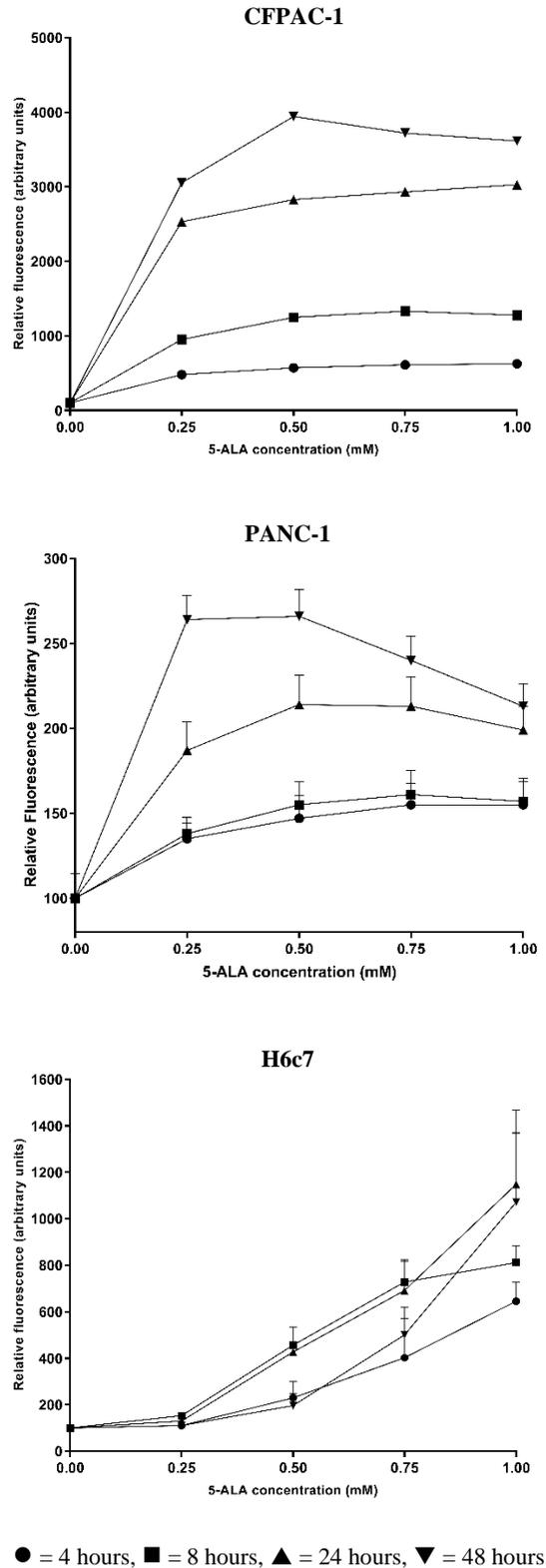
Quantitative fluorescence

The maximum ALA-induced fluorescence for CFPAC-1 and PANC-1 was achieved after 48 hours incubation with 0.50mM ALA (**Figure 2**). Compared to cells incubated without ALA, a relative fluorescence increase of 39.4-fold in CFPAC-1 and 2.7-fold in PANC-1 was seen. ALA concentrations above 0.50mM did not result in higher fluorescence. In contrast, the control cell line H6c7 showed progressively increasing fluorescence with increasing ALA concentrations. The highest cancer/control cell fluorescence ratios (a surrogate for the Rose criterion) for ALA were after 48 hours incubation with 0.25mM ALA; 122.9 in CFPAC-1 and 9.7 in PANC-1. At the more clinically relevant time point of 4 hours (current oral preparations of ALA are administered 3-6 hours pre-surgery) the highest cancer/control cell fluorescence ratios were also at 0.25mM; 21.3 in CFPAC-1 and 5.2 in PANC-1.

4. Conclusion

CFPAC-1 displayed very high ALA-induced PpIX fluorescence (maximum increase >120-fold) compared to the control cell line H6c7. In contrast, PANC-1 showed only mildly increased fluorescence compared to H6c7, with a maximum fluorescence fold increase of 9.7. This difference is most likely due to variations in the relative up- or downregulation of the enzymes and transporters involved in the haem biosynthesis pathway secondary to ALA exposure (see abstract 11070-210). Using the Rose criterion as a standard cut-off for effective FGS, ALA has the potential to play a role in FGS in pancreatic cancer in ALA-susceptible cancers. An early phase clinical trial is warranted to investigate the effect of ALA FGS in pancreatic cancer surgery as a photodiagnostic tool.

Figure 2 Relative fluorescence of pancreatic cancer cell lines CFPAC-1 and PANC-1 and pancreatic ductal cell line H6c7 following 4, 8, 24 or 48 hours incubation with increasing doses of ALA (normalized to 100). Error bars on CFPAC-1 smaller than data points.



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