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Aspects of photodetection in cervical and ovarian neoplasia

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Chapter 4

Photodetection with 5-aminolevulinic acid induced protoporphyrin IX in the rat abdominal cavity: drug dose dependent fluorescence kinetics

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Abstract

In 75% of cases, ovarian carcinoma has already metastasized in the abdominal cavity at the time of diagnosis. For determination of the necessity for a supplementary therapy, in addition to surgical resection, it is important to localize and stage microscopical intra-peritoneal metastases of the tumor. Intra-peritoneal photodetection of tumor metastases is based on preferential tumor distribution of a fluorescent tumor marker. The time dependent differences in drug concentration between tumor and normal tissue can be used to visualize small tumors. We performed fluorescence measurements on abdominal organs and tumor in the peritoneal cavity of rats. ALA induced PpIX was used as the fluorescent marker. Three different drug doses (100, 25 and 5 mg/kg) were used and PpIX fluorescence profiles were followed up to 24 hours after i.v. administration. Maximum tumor to normal ratios were found two to three hours after administration of ALA with all drug doses. A significant tumor to normal tissue contrast was obtained for all abdominal organs tested after administration of 5 mg/kg.

Introduction

The majority of patients with ovarian carcinoma is diagnosed at an advanced stage when tumor spread has already occurred into the abdominal cavity. This delayed diagnosis is due to late onset of complaints and the absence of appropriate screening or early detection methods¹⁻⁴. Metastatic spread can be obvious macroscopically and easy to recognize but it can also be silent, in which case only microscopic examination of tissue samples will indicate the presence of tumor. Treatment is dependent on the spread of the disease and normally consists of surgery alone (early stage) or surgery combined with chemotherapy or radiotherapy (in more advanced stages). To gain insight in the spread of the disease a staging procedure is required. In cases without overt spread of tumor, a standard staging procedure is currently performed by taking multiple random tissue biopsies from predilection areas, which are sent for microscopic examination. This method is not very sensitive and carries a high risk of understaging with subsequent undertreatment of disease.

Photodetection is a new diagnostic modality in cancer using fluorescent dyes for the detection of small tumors^{1, 5-20}. In general, the technique of photodetection of malignancies is based on differential concentration of fluorescent dyes/fluorophores between normal and tumor tissue; the higher the differential, the greater the sensitivity and specificity of photodetection. Insight in fluorescence pharmacokinetics is required in order to gain optimal photodetection conditions such as optimal application period and dose of dye used. Pharmacokinetics in photodetection are determined by tissue properties and metabolism, dye properties, and dose and route of dye administration.

Photodetection is already being clinically used for the detection and localization of bladder, skin and esophageal tumors by using locally applied fluorescent contrast agents^{7,8,16,20}. The most commonly used fluorescence contrast agent is 5-Aminolevulinic acid (ALA), a precursor of the fluorescent protoporphyrin IX (PpIX). Photodetection using ALA may also be used for abdominally disseminated tumors. Recently Hornung *et al.* published the results of a feasibility study for photodetection of ovarian tumor metastases in the rat abdominal cavity using ALA¹. He showed that by using fluorescence guided inspection of the abdominal cavity it is possible to obtain a good tumor to normal contrast for visualization of small peritoneal tumors in rats. In Hornung's study the combination of a high ALA dose (100 mg/kg) and a short time interval (3 hours) after administration was considered to be optimal. However, we subsequently showed that such high dosages of ALA carry a potential risk of phototoxic damage to healthy tissues like bowel and liver²¹ which are exposed to high intensity operating room lights

for several hours. For this reason we performed the current study on fluorescence pharmacokinetics in order to evaluate the possibility to use safe doses of ALA in photodetection of intra-abdominally disseminated tumors.

The present study was performed in a tumor bearing rat model, preceding clinical studies in patients with ovarian cancer. The study was approved by the institute's local animal ethical committee conformed to national guidelines.

Materials and methods

Tumor model and experimental procedure.

Female Wistar rats of approximately 200 g. were injected intra-peritoneally with 10^6 cells of the tumor cell line CC531, which resulted in peritoneal carcinosis with tumor nodule diameters of 1-3 mm after four weeks. At that time ALA dissolved in Phosphate Buffered Saline (PBS) was administered through the tail vein in different dosages. At various time intervals (0, 4, 16 hours) after administration of ALA the animals were anesthetized (a mixture of Dormicum 0.01ml /kg, Hypnorm 0.01 ml/kg and sterilized water, i.p.) after which a laparotomy was performed. To maintain a stable body temperature of 37°C, continuously recorded by rectal measurement, animals were kept on a heated underlay. If necessary, additional anesthesia was given by intra-peritoneal instillation of the above described mixture of Dormicum and Hypnorm.

A midline incision was made from xiphoid to symphysis. A surgical retractor was used during the measurements to expose the intra-abdominal organs. Throughout the experimental period fluorescence measurements were performed on the liver, cecum, bladder, small and large intestines and on tumor nodules. Two animals were treated simultaneously. During the surgical experimental procedure, which was carried out during 4-6 hours, the abdominal cavity was kept moist using a 37°C warmed saline solution. Ambient light was dimmed to avoid photobleaching or phototoxic damage to the tissue. At the end of experiments the animals were sacrificed.

Fluorescence measurements.

The fluorescence measurement setup consists of a mercury lamp with a computer controlled filter wheel for selection of the excitation wavelength. Optical fibers are used to guide the excitation light to the tissue and collect the fluorescence light from the tissue. Spectral information is extracted from the measured light with an Optical Multichannel Analyzer (Oriel Instaspec V). In order to obtain unbiased tumor to normal ratios of the

concentration of the fluorophores, we performed both Double Ratio²² and Normalized Fluorescence Ratio²³ calculations on the spectra. In short, PpIX was excited with 405 and 435 nm and the resulting fluorescence spectra in the range from 550 to 800 nm were stored. For the calculations, the PpIX specific part of the spectrum (610-650 nm) and the autofluorescence specific part of the spectrum (500-550 nm) were used. The mathematics behind these methods are briefly described in Appendix A. The Double Ratio (DR) method corrects for differences in optical properties of the different tissue types and variations in the excitation light. This method can only be used at low drug doses (5 mg/kg) as the DR saturates at higher drug doses. The reason to use this technique for the 5 mg/kg dose is the higher sensitivity. The normalized fluorescence ratio (NFR) was used for the measurements after administration of 25 or 100 mg/kg ALA.

The statistical analyses were performed using the Student's t-test. A P value of less than 0.05 (double sided) was considered to be a statistically significant difference.

Results

Figure 1 shows the PpIX fluorescence in tumor tissue as a function of time after administration of 5, 25 and 100 mg/kg. Each point represents the mean tumor fluorescence of 12 measurements (4 rats). The measurements were performed arbitrarily on tumor nodules

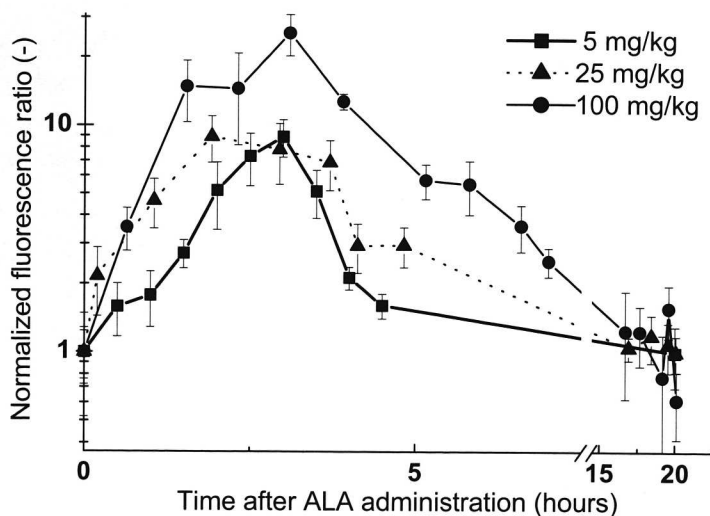


Figure 1: tumor fluorescence as function of time after administration of 100, 25 and 5 mg/kg ALA. The errorbars represent 1 standard deviation.(67 % confidence interval) Each point represents the mean tumor fluorescence of 12 measurements (4 rats).

that exceeded 2 mm in diameter. The porphyrin concentration in tumor increases until the time of maximum fluorescence is reached at 2 (25 mg/kg) and 3 (5 and 100 mg/kg) hours after administration. Maximum fluorescence levels after administration of 5 and 25 mg/kg were alike, while 100 mg/kg induced a 2.5 times higher fluorescence level.

Figures 2a and 2b illustrate the PpIX concentration ratio between tumor and normal tissue as a function of time after administration of 100 mg/kg ALA. The porphyrin concentration ratios from tumor to intestine or cecum increased from the time of administration and reached maximum levels of >10 at 2 and 3 hours respectively. Thereafter the concentration ratios gradually declined. From 3 to 4 and from 1.5 to 4 hours there was a significantly higher fluorescence in peritoneal tumor than intestines and cecum respectively. Fluorescence ratios from tumor to peritoneum varied from 0.3 to 2 throughout the observation period with no clear pattern. Fluorescence in the tumor was never higher than in the liver but significantly lower tumor fluorescence was seen at 0.5 and 4 hours after administration.

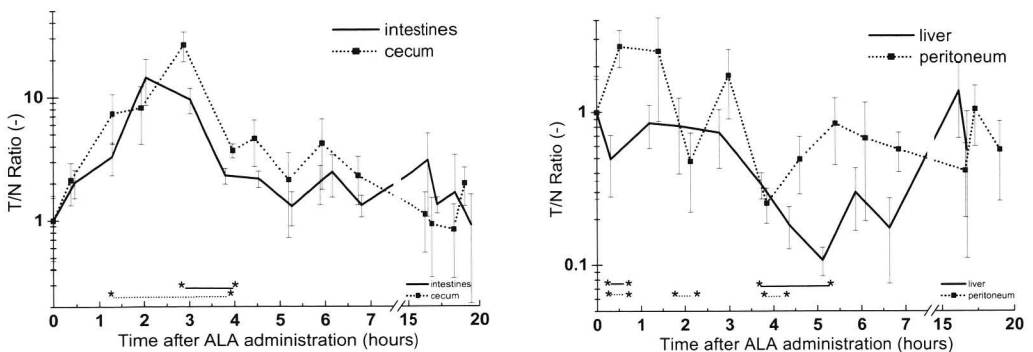


Figure 2a, 2b: tumor/normal fluorescence contrast as function of time after administration of 100 mg/kg ALA. The errorbars represent 1 standard deviation.(67 % confidence interval). The lines under the experimental curves indicate statistically significant T/N ratios.

Similar results were obtained for fluorescence ratios after 25 mg/kg ALA (figures 3) T/N ratios for intestines and cecum increased to a maximum of four and six respectively at 1.5 to 2.5 hours after ALA. T/N ratios for the peritoneum remained about one and tumors were significantly less fluorescent than liver at 4.5 hours after ALA.

For the lowest dose of ALA tested (5 mg/kg, figures 4), the T/N fluorescence ratios were significantly >1 at 2.5 to 3 hours for all tissues, although the contrast was modest

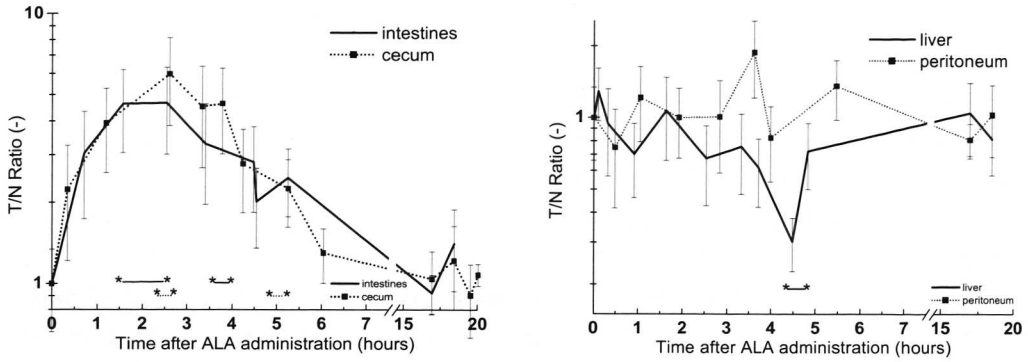


Figure 3a, 3b: tumor/normal fluorescence contrast as function of time after administration of 25 mg/kg ALA. The errorbars represent 1 standard deviation.(67 % confidence interval). The lines under the experimental curves indicate statistically significant T/N ratios.

for tissues other than the cecum. A high concentration contrast was seen for the intestines at 3 hours after administration. All organs showed some tumor to normal contrast after 1.5-3 hours. Maximum tumor to normal contrasts were seen in the cecum at three

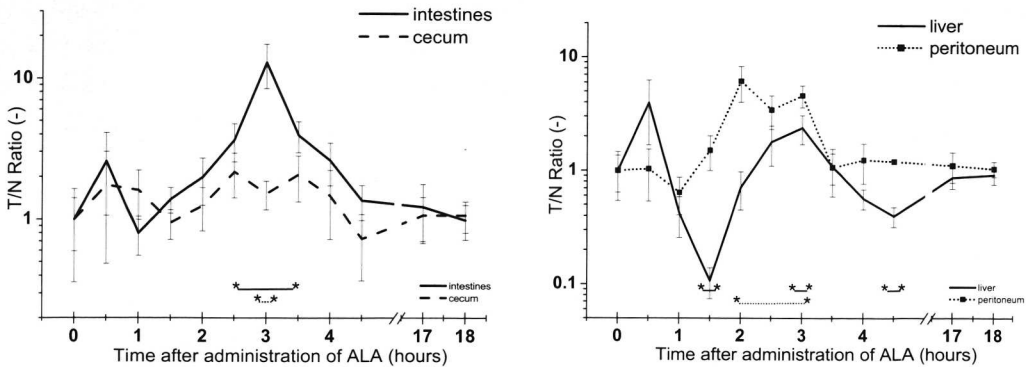


Figure 4a, 4b: tumor/normal fluorescence contrast as function of time after administration of 5 mg/kg ALA. The errorbars represent 1 standard deviation.(67 % confidence interval). The lines under the experimental curves indicate statistically significant T/N ratios.

hours, in the intestines after 2.5 hours and in the peritoneum after 2-3 hours and in the liver at 1.5, 3 and 4.5 hours.

Discussion

This study aims to find the optimum conditions for photodetection of intra-abdominal tumors using ALA. The optimal conditions are determined by tissue properties, the type and dose of fluorescent dye, the route of administration and the time interval between

administration of fluorescent dye and illumination.

ALA induced Protoporphyrin IX (PpIX) is an effective fluorescence contrast agent, which has been successfully used for the detection and localization of various malignancies⁵⁻¹⁰. After administration ALA is metabolized to PpIX, a precursor of heme. Small volume intra-abdominal malignant lesions can be detected after administration of a high dose of ALA, as shown by Horning in a tumor bearing rat model¹. In addition to being fluorescent, PpIX is, however, also a potent photosensitizer.

The most attractive dose of ALA for clinical tumor detection is that dose which induces maximal fluorescence contrast between healthy tissue and tumor without inducing significant phototoxic damage. Considering the results of tumor/normal fluorescence ratios the lowest dose of 5 mg/kg ALA appeared to be the best choice as a significant contrast was found for all types of abdominal tissue at the same time (3 hours after administration of ALA). An even higher T/N ratio was seen for intestines and cecum at 3 hours after higher ALA doses but contrast for other tissues (liver/peritoneum) was less good. Another disadvantage at high doses is the potential to induce normal tissue damage, which could result from photosensitization during the laparotomy²¹. Hornung showed the feasibility of photodetection of intra-abdominal ovarian tumors. However the ALA dose of 100 mg/kg ALA used in his study carries a risk of normal tissue damage, while our results now indicate that low dosages of ALA are good for photodetection and safe as shown by our previous study on white light phototoxicity. The contrast seen between tumors and peritoneal structures at 3 hours after 5 mg/kg ALA is an encouraging finding since abdominal disseminated ovarian cancer often expresses itself as carcinosis peritonei.

The conclusions from this study are based on relationships between type of tissue and amount of measured fluorescence. Fluorescence measurements on tissue can be influenced by artifacts relating to the optical properties of tissue, the measurement setup and the tissue architecture. The first two artifacts are mainly eliminated by using multiple wavelengths for excitation and detection and mathematical algorithms (double ratio²², normalized fluorescence ratio²³). The interpretation of the measurements is, however, difficult if the protoporphyrin is distributed according to a layered structure of the tissue e.g. intestine. In the mathematical interpretation of the measurements, a semi-infinite tumor is assumed. The problem emerges when the tumor is thin compared to the penetration depth of the excitation light. The calculations will then underestimate the actual

concentration in the tumor. In chapter 9, the dependency of the DR on layer (tumor) thickness is mathematically evaluated.

Our findings indicate clearly that, given detection equipment of sufficient sensitivity, a low dose of ALA (5 mg/kg) is sufficient for photodetection of intra-abdominal disseminated tumors. The optimal time for photodetection at this dose was at three hours after administration of ALA. At this low dose of 5 mg/kg ALA the risk of damage to healthy tissue due to photosensitization is minimal.

Acknowledgements

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Appendix A

Fluorescence ratios.

Discrimination between fluorescence from tumor localizing compounds and intrinsic tissue fluorescence is necessary for the interpretation of data obtained by fluorescence spectroscopy or fluorescence imaging. Furthermore, differences in absorption of excitation light and differences in emission of fluorescence light also influence the measurements and decrease the specificity of the fluorescence detection technique. There are several ways to correct fluorescence measurements for the tissue artifacts. This appendix gives a brief introduction of the two mathematical techniques (NFR and DR) that were used in our study.

The Normalized Fluorescence Ratio (NFR) as described by Saarnak *et al*²³ is defined as the ratio between fluorophore fluorescence and autofluorescence from one excitation wavelength, normalized by this ratio without any fluorophore present.

$$NFR = \frac{F_{\lambda_{excit405}, Fluorescencepeak}}{F_{\lambda_{excit405}, Autofluorescence}} \bigg/ \left(\frac{F_{\lambda_{excit405}, Fluorescencepeak}}{F_{\lambda_{excit405}, Autofluorescence}} \right)_{noPpIX} = 1 + \alpha_{\lambda_{exc}, \lambda_{em}} C_p \quad (1)$$

With: $F_{\lambda_{exc}XX, Fluorescencepeak}$: Fluorophore fluorescence excited with XX nm
 $F_{\lambda_{exc}XX, Autofluorescence}$: Intrinsic tissue fluorescence excited with XX nm
 $\alpha_{\lambda_{exc}, \lambda_{em}}$: a factor that contains the fluorescence yield of the tumor localizer and the autofluorescence and the concentration autofluorophores.
 C_p : Concentration fluorophores.

The NFR is proportional to the amount of fluorophore in the tissue and can be used for semi- quantitative measurements of fluorophore concentration. The tumor/normal ratio can be calculated as:

$$T/N \text{ Ratio} = \frac{NFR_{tumor}}{NFR_{control}} = \frac{C_{p,tumor}}{C_{p,control}} \quad (2)$$

The Double Ratio (DR) as described by Sinaasappel *et al*²² uses two excitation wavelengths. One excitation wavelength is chosen to be at a fluorescence excitation peak (405 nm) of PpIX, the second excitation wavelength is chosen to excite intrinsic tissue fluorescence while being less efficient in exciting porphyrins(435 nm). The DR is

obtained by dividing the fluorescence ratios of both excitation wavelengths.

$$DR = \frac{\left(\frac{F_{\lambda_{excit}405, fluorescencepeak}}{F_{\lambda_{excit}405, autofluorescence}} \right)}{\left(\frac{F_{\lambda_{excit}365, fluorescencepeak}}{F_{\lambda_{excit}365, autofluorescence}} \right)} \quad (3)$$

The DR is, in theory, independent of the autofluorescence and measurement geometry and can also be written as:

$$DR = \frac{1 + aC_p}{1 + bC_p}, \text{ where } a > b \quad (4)$$

Where a and b are functions of the fluorescence yield of the tumor localizer and the autofluorescence and the concentration autofluorophores.

The DR is sensitive at low PpIX concentrations but reaches an asymptote (a/b) at high PpIX concentrations where, the DR will asymptotically approach (a/b).

Equation 4 can be rewritten to equation 5 to extract the porphyrin concentration

$$C_p = \left(\frac{1 - DR}{DR - \left(\frac{a}{b} \right)} \right) \cdot \frac{1}{b} \quad (5)$$

With C_p : the concentration fluorophore

The tumor/control fluorophore concentration ratio can now be written as:

$$Ratio = \frac{C_{tumor}}{C_{control}} = \frac{1 - DR_{tumor}}{1 - DR_{control}} \cdot \frac{DR_{control} - a/b}{DR_{tumor} - a/b} \quad (6)$$

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