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Preclinical report

Determination of the maximal tumor:normal bladder ratio after i.p. or bladder administration of 5-aminolevulinic acid in Fischer 344 rats by fluorescence spectroscopy *in situ*

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The two major steps in our study on the treatment of bladder tumors by photodynamic therapy (PDT) were the development of a new bladder tumor model in Fischer rats by implantation of tumor cells and the use of fluorescence spectroscopy, a semi-quantitative and non-invasive method, in order to determine the time after general or local administration of a photosensitizer when the tumor:normal bladder ratio was at its highest. 5-Aminolevulinic acid (5-ALA) (250 mg/kg body weight) was injected i.p. or instilled directly into the bladder cavity for 1, 2 or 4 h and fluorescence was measured *in situ* on normal and bladder tumor tissues every 30 min for 8–10 h after administration, with a special miniaturized optical-fiber captor. The better tumor:normal bladder ratios were 2.85 ± 1.2 at 3.5 h after i.p. administration and 3.96 ± 1.04 after bladder instillation for 4 h, respectively. These results were confirmed by fluorescence microscopy. PDT with the same dose of 5-ALA as in this pharmacokinetic study must also be carried out in order to compare the toxicity of the two administration routes of the photosensitizer and to determine which one is the better for this bladder tumor model. [© 2002 Lippincott Williams & Wilkins.]

Key words: 5-Aminolevulinic acid, bladder tumor, fluorescence spectroscopy, tumor:normal bladder ratio.

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Introduction

Photodynamic therapy (PDT) is a technique currently under assessment for its possible role in the selective destruction of malignant tumors and premalignant lesions.[1] It involves the activation of a photosensitizer which shows some preferential accumulation in malignant tissue compared with normal tissues with light of a specific wavelength.[2] This causes the photosensitizer to produce cytotoxic oxygen species within the tumor. After a systemic injection, the photosensitizer is located both in the tumor and in the normal surrounding bladder tissue. Therefore, it is necessary to know how it is distributed in these tissues and to specify when the PDT would be the most efficient.

Since cutaneous photosensitivity is a well-known complication following systemic administration, the photosensitizer is now instilled directly into the bladder in order to minimize these adverse reactions.

Preclinical data on the distribution of photosensitizers have been obtained by using animals with transplanted human tumors grown neither in their native connective tissue matrix nor from their normal epithelium. These bladder tumor models do not mimic spontaneous human bladder tumors. It is therefore interesting to evaluate the photosensitizer distribution within the tumor and the normal bladder when the tumor is growing in its own connective tissue matrix. Thus, we developed a new

orthotopic bladder tumor model in the rat by implantation of bladder tumor cells after abrasion of the bladder surface. This provides a suitable system for experimental studies on bladder cancer.[3]

The pharmacokinetic studies were performed on female Fischer rats 344 bearing bladder tumors in order to define the time when the tumor:normal bladder ratio of the photosensitizer was at its highest after systemic (i.p. injection) or local (bladder instillation) administration. This time should then be used in further studies to ensure efficient PDT.

For our experiments, we have chosen 5-aminolevulinic acid (5-ALA), a clinically efficient endogenous photosensitizer precursor that is eliminated from the body within 24–48 h after administration. The administration of 5-ALA causes the tumor-specific accumulation of protoporphyrin IX (PpIX), a fluorescent compound.[4] The distribution of PpIX was monitored by light-induced fluorescence spectroscopy and the tumor:normal bladder ratio was measured after the i.p. injection or the bladder instillation of 5-ALA.[5,6] Fluorescence spectroscopy is a suitable method for monitoring photosensitizer distribution *in vivo*. It is achieved by measuring the fluorescence intensity of the tumor and the surrounding normal tissue at different times after 5-ALA administration. This technique has already been used to characterize the fluorescence distribution of 5-ALA-induced PpIX in human and animal carcinomas. These pharmacokinetic studies have been mainly carried out on early bronchial and esophageal carcinomas, colorectal, mammary, skin, liver or bladder carcinomas.[1,7–12]

Materials and methods

Chemicals

The 5-ALA was supplied as a hydrochloride salt (Sigma, St-Quentin, France) with 98% purity. The 5-ALA powder was dissolved in DPBS (Sigma) at 100 mg/ml and the pH was adjusted with sodium hydroxide to 7.2–7.4 (physiological value) for i.p. injection or to 6.0 (female F344 rat urine physiological pH) for bladder instillation. Each solution was prepared immediately before use.

Tumor cells

The AY–27 bladder tumor cells, originally derived from a *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT)-induced rat bladder transitional cell carcinoma (TCC), were grown as monolayers at 37°C in RPMI 1640 culture medium (Sigma) supplemented with L-glutamine, 10% fetal calf serum (Sigma) and 1% (v/v) of an antibiotic/antimycotic solution (Sigma). The first three passages were only used for tumor implantation. The phenotype of the malignant cells was maintained by serial implantation in syngenic Fischer 344 rats as a solid s.c. tumor.

Induction of bladder tumors on rats

Sixty-nine female Fischer 344 rats (6 weeks old) were obtained from Iffa Credo (L'Arbresles, France) and kept in plastic cages at 25°C with a 12 h light/dark cycle. They were given a standard diet and water *ad libitum*.

To develop the orthotopic model, 45 rats were anesthetized with an i.p. injection of sodium pentobarbital (45 mg/kg body weight) (Centravet, Gondreville, France) and placed in supine position on an animal board to maintain body temperature at 20–25°C. The urethra was catheterized with a polyethylene (PE) catheter (Merck, Clévenot, France) and then the bladder was distended with sterile saline [sodium chloride 0.9% (Cooper, Nancy, France)]. A special abrader was inserted via the PE catheter in the bladder cavity and urothelial denudation of the bottom part of the bladder was performed by rotations of the abrader while exercising pressure on the bladder wall. Once the bladder was rinsed with sterile saline, a suspension containing 10–20 $\times$ 10^6 AY-27 bladder tumor cells was instilled into the bladder and left for 30 min by compression of the urethra to prevent premature bladder voiding of the cell suspension. The catheter was then removed. The 24 control rats received no treatment. The tumor-bearing rats used for pharmacokinetic studies were sacrificed at the end of the experiment to ensure the presence of a bladder tumor and for the histopathological evaluation of the tumors. These tumors were found to be bladder carcinomas (TCC).

Pharmacokinetic studies

Intraperitoneal administration. Six control rats and nine rats with orthotopic bladder tumors large enough to receive the sensor at their surface (5 $\times$ 5 mm) were used to determine the distribution of PpIX in the tumors and normal bladder. Each rat was given an i.p. injection of 5-ALA at 250 mg/kg.

Bladder instillation. Eighteen control rats and 36 rats with orthotopic bladder tumors of the same size (5×5 mm) were used as for the i.p. administration, separated in three groups of six control rats and 12 rats bearing bladder tumors. Each rat of each group was given a bladder instillation of 5-ALA at 250 mg/kg. The instillation in the bladder was maintained for 1, 2 or 4 h for the three groups, respectively, by clamping the urethra. Afterwards the solution was removed and the bladder was rinsed with sterile saline to avoid further exposure of the bladder wall to 5-ALA.

For each administration route, fluorescence spectra of tumors and normal bladder were carried out before administration (autofluorescence) and every 30 min after administration, for 8–10 h. All fluorescence measurements were performed with an excitation wavelength of 410 nm.

Fluorescence was excited by a Kr^+ laser (Spectra-Physics, Paris, France) using the emission line at 410 nm.A miniaturized system for a use *in situ* on the bladder rat was especially created by SEDI Fibres Optiques (Evry, France) for these experiments. It was composed of three optical silica fiber probes (HCN 200, core diameter 200 μm): one for the excitation, one for the collection of fluorescence emission and the last one for backscattered excitation light power measurements.[5,13] Homogenous distribution of the light on the tumor or the normal bladder was obtained by means of a microlens adjusted at the front of the fiber tip. The power of the excitation light was assessed with a calibrated photodiode power meter (Radiometer Labsphere, Newport, USA). The fluorescence was measured in the linear intensity range of the detector at wavelengths ranging from 450 to 750 nm (spatial resolution 2 mm). All the spectra were normalized according to the flavin autofluorescence peak (at 520 nm) obtained before 5-ALA administration.

Statistical analysis

Student's *t*-test was used to determine statistically significant differences among mean porphyrin intensities. The semi-quantitative data were analyzed for each different time point. Results were considered statistically significant at $p < 0.05$.

Results

The distribution of PpIX in the normal bladder and the tumors after 5-ALA i.p. injection and bladder instillation in Fischer 344 rats bearing orthotopic bladder tumors is plotted in Figure 1. The highest tumor:normal bladder ratio was 2.85 ± 1.20 (Figure 1A), 3.5 h after i.p. injection of 5-ALA. After a 1-h bladder instillation, the highest ratio was 1.44 ± 0.75, obtained immediately after the end of the instillation. Similar results were achieved after bladder instillation for 2 and 4 h with ratios of 2.17 ± 1.2 and 3.96 ± 1.04, respectively. Ratios found after i.p. injection and bladder instillation for 2 or 4 h were significantly different ($p < 0.001$) from the highest autofluorescence ratio (1.1 ± 0.21). In addition, the highest ratio obtained after a 4-h bladder instillation presents a significant difference ($p < 0.01$) with the two other ratios.

Standard errors for carcinoma:normal bladder ratios are relatively important and can be explained by the difficulty of placing the sensor exactly at the same spot on the tumor or on the normal bladder wall for each measurement. Consequently, tissue fluorescence measurements at a given time can vary for each animal, but also for the same animal. On the other hand, the animals may not present the same porphyrin endogenous concentration in their tissue. This explains a possible variability of autofluorescence measurements.

However, in spite of these problems, we have noticed that whatever the administration route is, the aspect of the carcinoma:normal bladder ratio curve for each rat is similar (data not shown) with two maxima after i.p. administration and one maximum after bladder instillation, immediately after the end of the instillation, whatever the length of time used.

Figures 2 and 3 depict the spectra of PpIX for the normal and the tumor bladders at the right time for irradiation. For each administration route, the autofluorescence spectra were measured before administration of 5-ALA, with a peak at 635 nm due to endogenous porphyrins (not visible), in comparison with the right time for treatment due to the difference in the fluorescence intensity at the main emission peak of PpIX (630 nm). This difference in fluorescence intensity was particularly marked for the bladder tumors and was directly related to the concentration of PpIX in the tissues. Photobleaching can be neglected in this experiment as the acquisition time was short (0.5 s) and the excitation power was low (1 mW).

Discussion

Before more clinical PDT trials on the bladder are undertaken, further *in vivo* research on

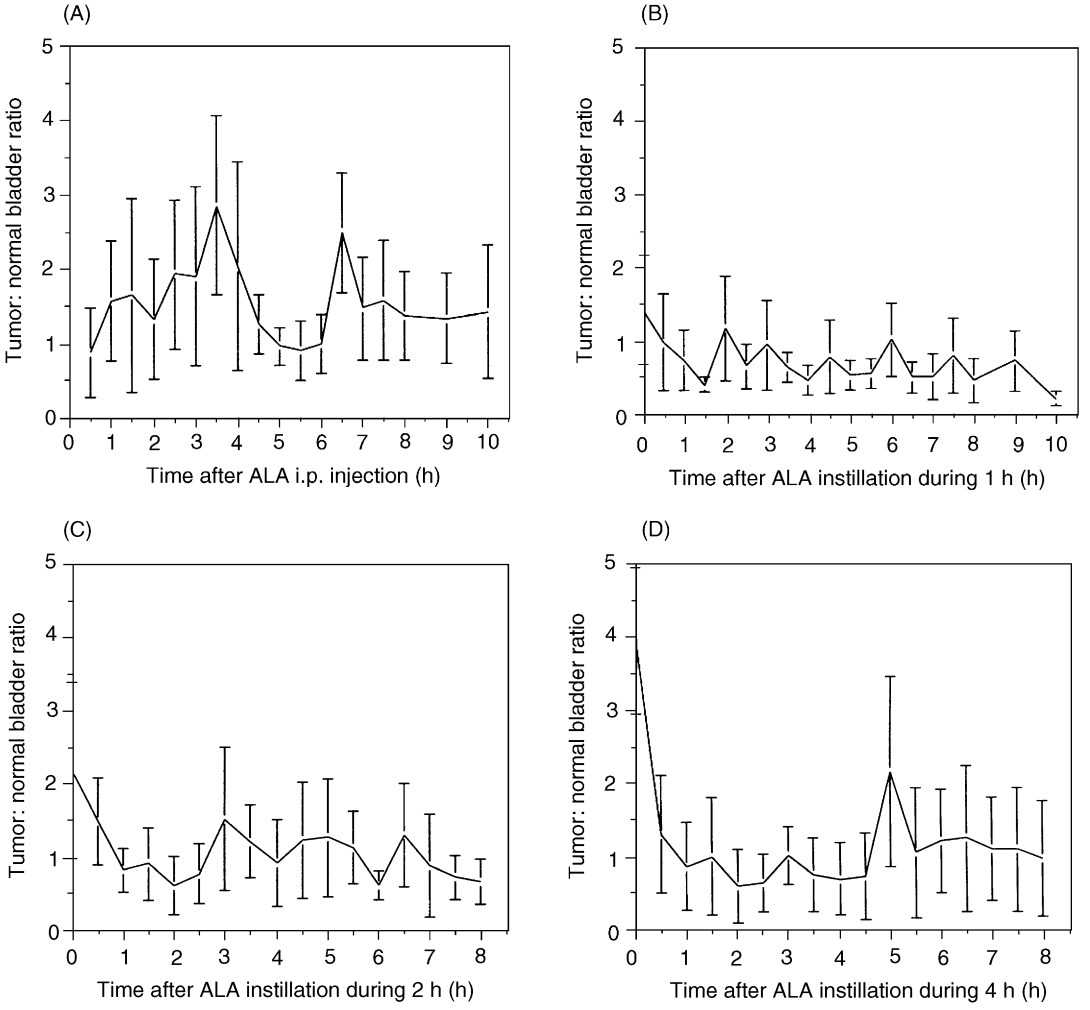

Figure 1. Time course for changes in the tumor:normal bladder ratio after i.p. injection (A) or bladder instillation (B–D) of Fischer 344 rats with 5-ALA (n=12 for each time).

photosensitizer pharmacokinetics, light dosimetry and biological responses under rigorous experimental conditions are necessary. To reduce urological side effects and undesirable cutaneous photosensitization which persists as long as 4–6 weeks after treatment, attempts have been made to deliver the photosensitizing agent by bladder instillation or intratumoral injection.[4,9–11,17–19]

Our motivation for comparing i.p. administration and bladder instillation of 5-ALA was the potential advantage of the instillation of this photosensitizer precursor for PDT of bladder cancer: the PpIX accumulation in organs distant from the bladder should be expected to be minimal.

Fluorescence spectroscopy is a particularly attractive technique because it is non-invasive in comparison with fluorescence microscopy or organic extraction, for example. In addition, rats can be used as their own controls and can be monitored throughout the experiment, and such an experiment requires fewer rats than for microscopic studies.

The results in this study indicate that 5-ALA gives a highest tumor:normal bladder ratio of PpIX 3.5 h after i.p. injection and immediately after the end of the 4-h bladder instillation with 2.85 ± 1.20 and 3.96 ± 1.04, respectively. These results have been confirmed using fluorescence microscopy where the maximum visible fluorescence intensity was observed 4 h after both i.p. administration and bladder instillation (data not shown).

Pharmacokinetic studies have already been carried out on healthy animals, and malignant tumor animal models such as chemical, orthotopic and heterotopic bladder tumor models. Chang *et al.*[16] reported a PpIX biodistribution study by quantitative fluorescence microscopy on the normal bladder wall of

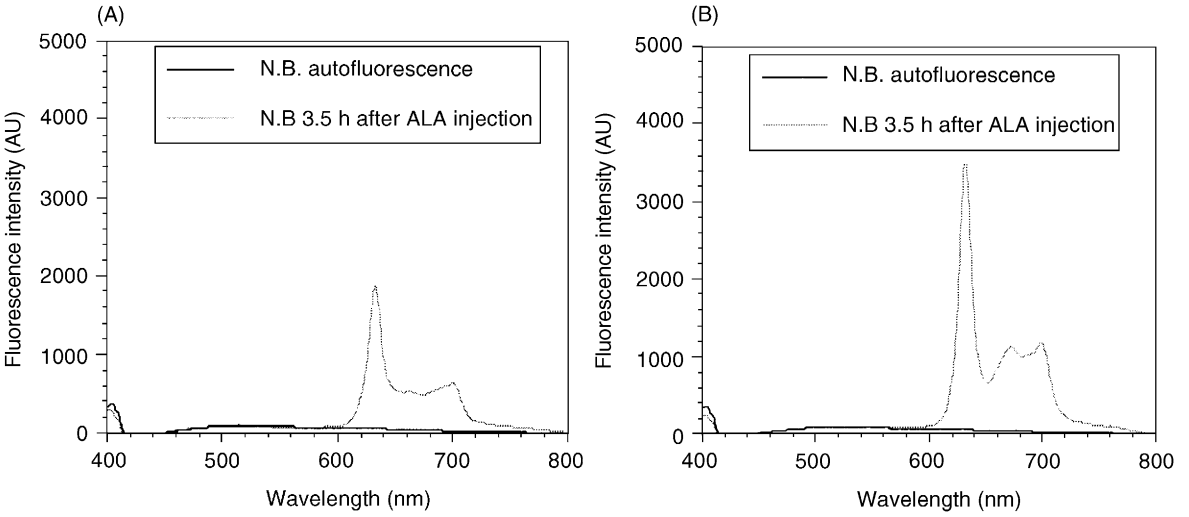

Figure 2. Spectra of the normal bladder (N.B.) (A) and the bladder tumor (B.T.) (B) before and 3.5 h after 5-ALA injection, normalized according to the flavin peak at 520 nm.

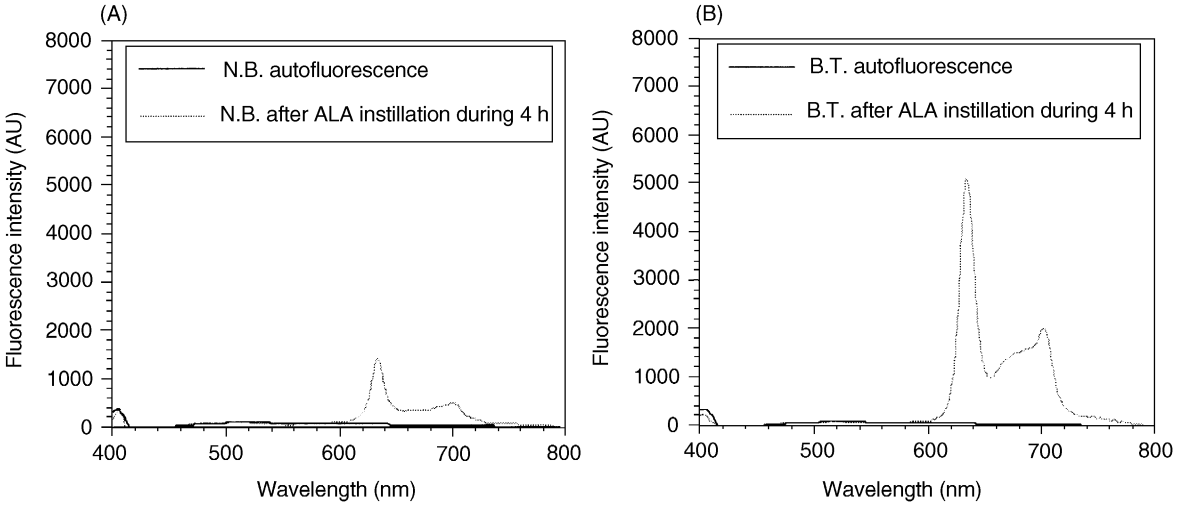

Figure 3. Spectra of the normal bladder (N.B.) (A) and the bladder tumor (B.T.) (B) before and after 5-ALA instillation during 4 h, normalized according to the flavin peak at 520 nm.

female Wistar rats. After the instillation of a 10% 5-ALA solution, PpIX build up in the urothelium peaked from 4 to 7 h and was delayed in comparison with i.v. (3–4 h) and oral administration (4–6 h) as shown by Loh *et al.*[20] These studies also demonstrated that absorption of small molecules like 5-ALA through the tight barrier of the transitional epithelium was possible and that a higher concentration of PpIX accumulated in the urothelium than in the underlying layers. In cutaneous tumors, disturbed *stratum corneum* overlying the tumor area enhanced the diffusion of the 5-ALA in the tumor.[12] Xiao *et al.*[18] reported a pharmacokinetic study by confocal laser scanning microscopy with Photofrin II and 5-ALA after a bladder instillation, and an i.v. injection in female Fischer rats bearing orthotopic and heterotopic bladder tumors. Following the i.v. administration of 5-ALA, the peaks of the PpIX fluorescence in the tumor with doses greater than 100 mg/kg appeared after 4 h and dropped by 6 h. The tumor PpIX intensity rose with increasing 5-ALA doses. The PpIX levels in normal bladder mucosa peaked between 2 and 4 h, and were not dose dependent when the dose was greater than 250 mg/kg. Other

data from Hua *et al.*[21] proved that PpIX levels in s.c. rat mammary tumor were time- and dose-dependent after an i.v. injection of 5-ALA. Heil *et al.*[17] reported fluorescence kinetics of PpIX following the bladder instillation of 5-ALA in a chemically induced rat bladder tumor model using fluorescence spectroscopy. The PpIX fluorescence was detected in the normal and the neoplastic urothelium 30 min after the bladder instillation and a pronounced synthesis of PpIX was observed after a 4-h 5-ALA instillation. The higher PpIX fluorescence intensity in tumor rat bladder revealed an important uptake of 5-ALA which may be explained by the enlarged surface of the neoplastic tissue. The penetration of 5-ALA into the neoplastic urothelium was also favored by the lack of transitional cell-specific rigid plaques at the luminal membrane of benign and cancerous hyperplasias of rat urothelium as reported by Severs *et al.*[22]

The 5-ALA-based fluorescence detection system significantly improved the diagnosis of malignant/dysplastic bladder lesions in patients.[4,9–11] However, determining the optimum drug exposure time requires further investigation using well-characterized instrumentation and study protocols.

Conclusion

The results of the present study pointed out that the bladder instillation of 5-ALA provided a higher tumor labeling than with the i.p. route. Although the mechanism of 5-ALA absorption from the bladder is unknown, the low molecular weight of 5-ALA, a sufficient concentration gradient across the bladder wall and the time of drug retention in the bladder are important factors influencing the PpIX accumulation in the tumor and in the bladder wall. The optimal incubation period for the bladder instillation appeared to be 4 h. At this time, the bladder tumor exhibited its maximal fluorescence ratio with average levels that were approximately 4 times those of the normal bladder wall. Clinical studies proved that the optimum incubation time for the 5-ALA solution was 2–4 h, which is an appropriate time frame for the clinical setting. The tumor tissue is much thicker than the normal bladder wall and, that way, the tumor surface accumulated more fluorescence, but this may partially explain why 5-ALA-based PDT could only achieve incomplete tumor destruction.[19] The complete tumor eradication could potentially be achieved by repeated PDT treatments with bladder instillations of 5-ALA. These experiments suggested that a bladder instillation of 5-ALA is an attractive alternative to other routes because it generated a minor skin photosensitization and kept a considerable PpIX selectivity between the tumor and the normal bladder.

In this *in vivo* study dealing with 5-ALA as a photosensitizer precursor, we conclude that a bladder instillation of 5-ALA solution at an appropriate pH, concentration and time may be a clinically feasible route to achieve a selective accumulation of PpIX in the tumor bladder with reduced photosensitization. Our future investigations will be the application of a PDT treatment on our bladder tumor model involving the best tumor:normal bladder PpIX ratios after a bladder instillation of 5-ALA.

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