Adjuvant intraoperative photodynamic therapy diminishes the rate of local recurrence in a rat mammary tumour model

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Summary The use of photodynamic therapy (PDT) as an adjunct to curative tumour resection was investigated in a tumour recurrence model, using rat mammary adenocarcinoma BN472. Tumours were inoculated subcutaneously in 60 animals and resected after 21 days of growth. Immediately after removal, the operation site was exposed to 320–450 nm light of $0.1\ W\ cm^{-2}$ and $60\ J\ cm^{-2}$ after photosensitisation with either Photofrin ($5\ mg\ kg^{-1}$ i.v. 48 h before illumination) or 5-aminolaevulinic acid (ALA) ($2\ mg\ ml^{-1}$ in drinking water for 9 days). Porphyrin concentrations were measured in tissue samples. After 28 days, animals treated with adjunctive PDT had a significantly longer tumour-free interval than controls ($P<0.01$; median 25 days (Photofrin), 18 days (ALA), 14 days (controls). Moreover, in the PDT groups significantly fewer rats had lymph node metastasis. A porphyrin concentration ratio between tumour and mammary tissue of 2:1 was found after Photofrin and 4:1 after ALA. The results indicate that adjuvant intraoperative PDT may be a safe and effective method of destroying residual tumour, thereby preventing locoregional tumour recurrence.

Keywords: intraoperative photodynamic therapy; rat; recurrence; cancer; mammary tumour

Despite radical surgery and perioperative chemo- or radiotherapy, local regional recurrence remains one of the major problems in cancer treatment. Most disappointing are the results of resection of pancreatic adenocarcinoma, in which local recurrence has been found in more than 80% at autopsy (Kayahara et al., 1993; Westerdahl et al., 1993). In colorectal cancers (Dukes' B_2–C), isolated locoregional disease accounts for 15–34% of the 50% of patients that can be expected to experience recurrence (Moertel et al., 1990; Galandiuk et al., 1992). For breast cancer, local recurrence rates of 5–30% have been reported after radical mastectomy and 2–21% after breast-conserving surgery combined with post-operative radiotherapy (Fisher et al., 1989; Ames and Balch, 1990; MacMillan et al., 1994). These recurrences are thought to arise from microscopic residual tumour, owing to incomplete removal or contamination by the surgical manipulation (Holland et al., 1985; Buyse et al., 1988; Frazier et al., 1989). Thus, there is clearly a need for an adjuvant treatment that would sterilise the tumour bed intraoperatively with minimal or no side-effects.

Photodynamic therapy (PDT) could be especially suitable for that purpose. PDT is a relatively new local cancer treatment that has been used successfully in patients with bronchus, bladder, skin and gastrointestinal tumours (Dougherty, 1993). The therapy is based on the accumulation of a photosensitiser in malignant tissues after local or systemic administration. Subsequent illumination with light of the appropriate wavelength creates a photochemical reaction resulting in tissue destruction (Gomer, 1989).

The most commonly used photosensitiser is porfimer sodium, an aggregated mixture of trimer/oligomer porphyrin molecules marketed as Photofrin (QLT, Vancouver, Canada, and Cyanamid, Pearl River, NY, USA). This substance is an effective photosensitiser, however accumulation in normal organs and prolonged skin photosensitivity have limited its application (Razum et al., 1987; Bellnier et al., 1989). Recently, the use of 5-aminolaevulinic acid (ALA) as a means of endogenous photosensitisation has received much interest. We found that oral administration of ALA causes

selective accumulation of protoporphyrin IX (PROTO) in a transplantable rat colon carcinoma (Van Hillegersberg et al., 1992). With ALA, the photosensitivity is limited to a period less than 24 h (Pottier et al., 1986; Loh et al., 1993). The reason for selective porphyrin accumulation in malignant tissue after ALA may be an altered activity of the haem biosynthetic pathway enzymes. Several studies have reported a higher activity of porphobilinogen deaminase (PBGD) in malignant and regenerating cells, whereas the opposite was found for ferrochelatase (Smith, 1987; Schoenfeld et al., 1988). PBGD catalyses the formation of uroporphyrinogen (URO) from four molecules of porphobilinogen and ferrochelatase converts protoporphyrin to haem.

The aim of the present study was to investigate the effect of adjuvant intraoperative PDT on tumour recurrence as well as lymph node and lung metastases, following curative tumour resection. An aggressively growing rat mammary tumour was used for the experiments with either Photofrin or ALA as photosensitisers. Additionally, porphyrin concentration, ferrochelatase and PBGD activity were measured in tumour and surrounding tissue to study the accumulation of photoactive agents.

Materials and methods

Animals and tumour model

Sixty female BN rats (Harlan CPB, Zeist, The Netherlands), weighing 200–225 g, were used for the experiments. They had free access to tap water (acidified, pH 3.0) and rat chow (AM II, Hope Farms, Woerden, The Netherlands).

Mammary tumour BN472 was chosen for this particular study, as it grows invasively from the inoculation site, metastasises to regional lymph nodes and lungs, and mostly recurs at the operation site despite complete macroscopic excision (Kort et al., 1986a). The tumour is a moderately differentiated adenocarcinoma, originating from a BN female rat in one of our studies on spontaneous tumour incidence (Kort et al., 1986b).

From tumour maintained in syngeneic animals, pieces of approximately 1 mm³ were inoculated subcutaneously in the right flank (abdominal lateral) of each animal. Twenty-one days later, the tumours had macroscopically not invaded the surrounding tissues and were dissected carefully from the abdominal wall and overlying skin. Tumour diameter was

measured using sliding callipers. Hypnorm (Janssen Pharmaceutica, Tilburg, The Netherlands) 0.1 ml i.m. was given to relieve post-operative pain. All procedures were performed under ether anaesthesia.

Photosensitisation

The animals were randomly allocated to three groups of 20 each. In two experimental groups the animals received either Photofrin in a single intravenous dose of 5 mg kg^{-1} body weight on day 19 post tumour inoculation or a solution of ALA-HCl (Sigma, St Louis, MO, USA), 2 mg ml^{-1} in drinking water, from days 12 to 21. As the water intake of rats of this species and weight is approximately 10 ml day^{-1}, the daily ALA dosage corresponds to 100 mg kg^{-1} body weight. The control group received intravenous saline on day 19. After and during drug administration the animals were kept in subdued light, with preservation of night/day rhythm.

Light delivery

Immediately after tumour resection on day 21 post inoculation, in all groups the tumour bed was illuminated with a 'black-body' light source, emitting 320–450 nm (peak at 380 nm) with a power of 0.1 W cm^{-2}. The lamp was kept at a distance of 10 mm from the tissue surface for 10 min, delivering a total energy of 60 J cm^{-2}. Surrounding skin was covered with black gauze to avoid normal tissue-damage and the wound bed was irrigated during exposure to protect the tissue from dehydration. The black-body light source was chosen, as it is cheap, readily available and has proved to be effective in destroying erythroleukaemic cells photosensitised by incubation with ALA *in vitro* (Malik and Lugaci, 1987).

Score of recurrence, lymph node and lung metastasis

After treatment, the operation site was palpated daily to identify tumour recurrence. Recurrence was considered when regrowth to 10 mm in diameter had occurred. The day of recurrence was then scored and the animal sacrificed. At autopsy, lymph nodes (axillar, inguinal and para-aortal) were examined bilaterally and the lungs were exposed to determine metastases. When lymph nodes and lungs were macroscopically involved, they were scored positive. At day 28 post treatment, the animals without signs of recurrence were also killed and autopsied. A post-operative period of 28 days was chosen, as in previous experiments all animals died within 20 days from disseminated disease.

Tissue porphyrin, ferrochelatase and PBGD measurements

On the day of primary tumour resection, in five animals samples were taken from the periphery of the tumour without central necrosis and from normal mammary tissue on the contralateral site. The porphyrin analysis was carried out according to Bayley and Needham (1986) with modifications as described previously (Van Hillegersberg *et al.*, 1992). The tumour or mammary tissue was suspended in saline (1:10, w/v) and homogenised in a tissue grinder. Each specimen was analysed separately by reversed-phase high-performance liquid chromatography (HPLC) and porphyrin fluorescence detection with excitation and emission wavelengths of 405 and 625 nm respectively.

Ferrochelatase was measured by a modification of the method of Li *et al.* (1987), using zinc and protoporphyrin as substrates. Individual samples were homogenised in water (1:10, w/w) and treated as described previously (Van Hillegersberg *et al.*, 1992). Zinc protoporphyrin was detected using HPLC with fluorimetric detection (excitation 415 nm, emission 580 nm). For comparison, the ferrochelatase was also measured in rat fibrosarcoma BN175, inoculated subcutaneously in four animals.

PBGD was measured as described previously (Wilson *et al.*, 1986), following an initial incubation at 55°C for 60 min

and cooling to room temperature to destroy the activity of uroporphyrinogen decarboxylase and to prevent further metabolism of uroporphyrinogen.

Results are expressed per g of tissue and per mg of tissue protein, measured by the method of Lowry *et al.* (1951). The presentation per mg of tissue protein is generally used to correct for the differences in tissue water content between tissue samples.

Statistics

The values are expressed as mean ± standard error of the mean (s.e.m.). Comparisons were made using the Mann–Whitney *U*-test for day of recurrence, the Fisher exact probability test for lymph node metastasis and lung metastases, and ANOVA for porphyrin concentrations. Differences were considered significant when *P*-values were <0.05.

Results

Of the 60 animals entering the study, three had to be killed early because of extensive tumour growth. In one animal the tumour did not take, and three animals had performed autotomy at the operation site. In the remaining animals no signs of discomfort or skin lesions could be observed. The PDT did not cause any visible tissue changes to the exposed abdominal wall tissue. Overall, 15 animals in the Photofrin group, 19 in the ALA group and 19 in the control group could be evaluated.

The mean ± s.e.m. tumour diameter at the day of treatment was 25.9 ± 2.0, 25.9 ± 1.5 and 27.4 ± 1.0 mm in the Photofrin, ALA and control group respectively. These differences were not statistically significant.

Tumour-free interval

Figure 1 shows the tumour-free interval after surgical excision and intraoperative PDT for the various groups. A significant longer tumour-free interval was found after surgical excision combined with adjuvant PDT *vs* surgery alone (*P*<0.01). Median values were 25 days for Photofrin, 18 days for ALA and 14 days for controls. The difference between photosensitisation with Photofrin or ALA was not statistically significant. However, in the group treated with Photofrin, 6 out of 15 (40%) animals were still without recurrence 28 days post treatment, whereas this ratio was only 3/19 (16%) after photosensitisation with ALA and 0/19 (0%) in controls.

Lymph node and lung metastasis

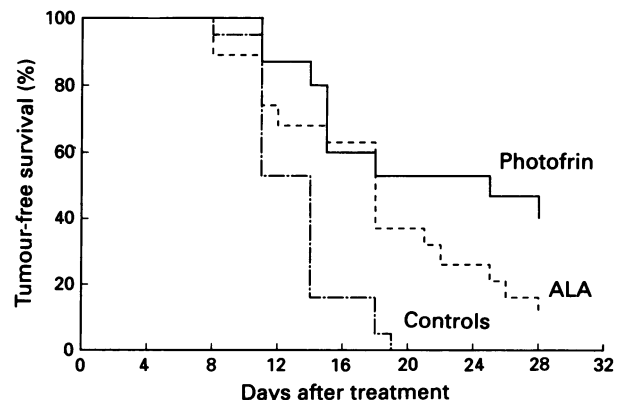Table I shows the scores on tumour spread to lymph nodes and lungs. When metastases had occurred, they were always

Figure 1 Tumour-free interval after local resection followed by adjunctive PDT with either Photofrin (5 mg kg^{-1} i.v., 48 h before PDT), ALA (2 mg ml^{-1} in drinking water for 9 days) or saline i.v. (controls).

located ipsilaterally in the axillary lymph nodes. Occasionally positive para-aortal nodes were found (two in the ALA group and four in controls). The involvement of lymph nodes was significantly related to the type of adjuvant intraoperative PDT applied ($P < 0.05$). Remarkably, none of the animals treated with Photofrin had positive lymph nodes, even though a substantial part of the animals in this group lived longer than the animals in the other two groups. After ALA, around 30% of the animals had positive lymph nodes, compared with 60% in the control group.

Differences in metastases to the lungs were statistically non-significant, although a similar trend as with the lymph nodes was found: Photofrin, 53%; ALA, 63%; controls, 74%.

Tissue porphyrin concentration, ferrochelatase and PBGD activity

Figure 2 shows the total porphyrin accumulation in tumour and normal breast tissue. In the control group, no significant difference was found between the porphyrin concentration in tumour (0.315 ± 0.05 nmol g^{-1} tissue) and normal tissue (0.233 ± 0.02 nmol g^{-1} tissue). However, after photosensitisation with either Photofrin or ALA, porphyrins had accumulated in both tissues, with higher values in tumour
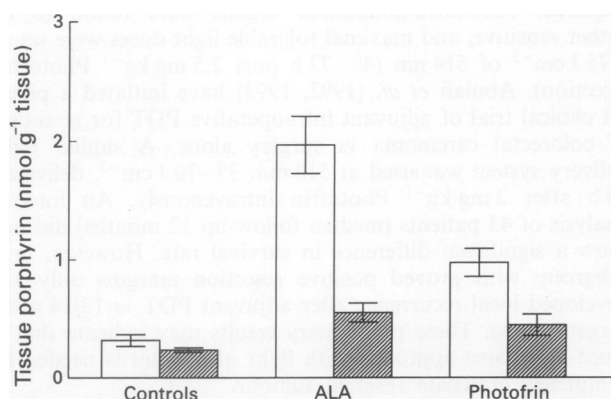
($P < 0.01$). The chromatogram showed mainly protoporphyrin in the group treated with ALA, while in the other group the typical peaks of the Photofrin components were found as described previously (Van Hillegersberg et al., 1992). After Photofrin, the mean $\pm$ s.e.m. porphyrin concentration was 0.974 ± 0.12 nmol g^{-1} in tumour and 0.444 ± 0.09 nmol g^{-1} in normal tissue, leading to a ratio of 2:1. After ALA, these values were 1.969 ± 0.24 nmol g^{-1} in tumour compared with 0.549 ± 0.08 nmol g^{-1} in normal tissue, leading to a porphyrin concentration ratio of 4:1.

The ferrochelatase activity was 0.62 ± 0.04 nmol h^{-1} of zinc-PROTO per mg of protein in mammary tumour and 0.22 ± 0.01 nmol h^{-1} mg^{-1} protein in normal breast tissue (Table II). The tumour value is in the same range as that measured for rat fibrosarcoma (0.49 ± 0.02 nmol h^{-1} mg^{-1} protein) and rat colon carcinoma CC531 (0.84 ± 0.10 nmol h^{-1} mg^{-1} protein) which showed a 3-fold lower activity than normal liver (2.47 ± 0.26 nmol h^{-1} mg^{-1} protein) (Van Hillegersberg et al., 1992). PBGD showed an activity in tumour ($34 \pm 2 \times 10^{-3}$ nmol h^{-1} of URO per mg of protein) similar to that in normal breast tissue ($30 \pm 2 \times 10^{-3}$ nmol h^{-1} mg^{-1} protein).

Figure 2 Total $\pm$ s.e.m. porphyrin concentration in tumour (□) and normal mammary tissue (▨) after photosensitisation with either Photofrin (single 5 mg kg^{-1} i.v. dose, 48 h before), ALA (2 mg ml^{-1} in drinking water for 9 days) or saline i.v. (controls).

Table I Number of animals with metastases in lymph nodes and lungs after adjunctive PDT following tumour resection[a]

	No. of animals	Lymph nodes + No.	Lymph nodes + %	Lungs + No.	Lungs + %
Photofrin[b]	15	0	0	8	53
ALA[c]	19	6	32	12	63
Controls[d]	19	12	63	14	74

[a]Illumination with 320–450 nm light of 0.1 W cm^{-2} and 60 J cm^{-2}; 28 days' follow-up. [b]5 mg kg^{-1} i.v. 48 h before illumination. [c]2 mg ml^{-1} in drinking water for 9 days. [d]Saline i.v. Statistics – lymph nodes: photofrin vs ALA, $P < 0.05$; ALA vs controls, $P < 0.05$; photofrin vs controls $P < 0.01$; lungs: non-significant.

Discussion

In this study adjuvant intraoperative PDT caused a substantial prolonged recurrence-free interval in rats, following surgical excision of the subcutaneously implanted mammary tumour. The treatment did not cause complications or any visible damage to the exposed normal tissue, suggesting that intraoperative PDT can be applied safely. Remarkably, although no effect on metastases to the lungs was found, lymph node metastasis was significantly reduced in the groups treated with PDT. In the Photofrin group none of the animals had positive nodes, compared with 6/19 rats in the ALA group and 12/19 controls. The mechanism of this additional PDT effect is not clear, but it might be caused by a direct effect on the lymphatic vessels in the illuminated area. There was no statistically significant relation between the method of photosensitisation and the rate of tumour recurrence, although 28 days post treatment 40% of the animals were still without recurrence after Photofrin, compared with only 16% after ALA. This may suggest that Photofrin is a better photosensitiser than protoporphyrin IX induced by administration of ALA. However, the results may not be completely comparable, as the optimal light delivery regimen could be different for photosensitisation with endogenously produced porphyrins. The protoporphyrin molecule may be more susceptible to reaction with singlet oxygen or tissue components during illumination (photobleaching) (Kennedy and Pottier, 1992). Photobleaching competes with the photodynamic reaction and the porphyrin products do not contribute to further photodynamic activity (Mang et al., 1987). Therefore, periodic illumination or prolonged exposure with lower output power may improve efficacy by allowing regeneration of sufficient levels of unbleached protoporphyrin (Joseph et al., 1993). Furthermore, a different intracellular localisation of the endogenous porphyrin may have its consequences for the PDT effect.

After ALA, a higher and more favourable tumour to normal tissue porphyrin concentration was found (4:1), com-

Table II Ferrochelatase and porphobilinogen deaminase activity in rat tumours, normal liver and breast

Tissue type	Ferrochelatase nmol h^{-1} of zinc-PROTO per g tissue	Ferrochelatase per mg protein	Porphobilinogen deaminase nmol h^{-1} of URO per g tissue	Porphobilinogen deaminase per mg protein ($\times 10^{-3}$)
Mammary carcinoma BN472[a]	66 ± 5	0.62 ± 0.04	3.68 ± 0.18	34 ± 2
Fibrosarcoma BN175[b]	52 ± 4	0.49 ± 0.02	3.50 ± 0.14	34 ± 2
Colon carcinoma CC531[c]	100 ± 12	0.84 ± 0.10	5.93 ± 0.60	49 ± 5
Liver[c]	541 ± 58	2.47 ± 0.26	17.0 ± 0.5	79 ± 2
Breast[a]	6.2 ± 0.6	0.22 ± 0.01	0.87 ± 0.14	30 ± 2

Data represent the mean $\pm$ s.e.m. of [a]5, [b]4 and [c]11 samples; [c]from Van Hillegersberg et al. (1992).

pared with Photofrin (2:1). In a previous study in intra-hepatic rat colon carcinoma, we found a similar ratio of tumour to normal liver after ALA, whereas for Photofrin the ratio was almost reversed (1:3) (Van Hillegersberg et al., 1992). Remarkably, in that study higher tissue porphyrin concentrations (up to a factor of 20) were found. As the activity of ferrochelatase is in the same range for both tumours (Table II), this might be caused by a higher uptake of ALA in liver as a result of (1) a better vascular supply of hepatic tissue or (2) preferential targeting to the liver, which is one of the main sites of haem biosynthesis in humans (Bottomley and Muller-Ebenhard, 1988). The low activity of ferrochelatase found in normal breast tissue can be explained by the high fat content with little porphyrin metabolism. Therefore, the values given in Table II are probably not representative for the real enzyme activity in mammary gland tissue. As fat cells contain proteins as well, the representation per mg of protein leads to an additional unclear transforma-tion of these values. Actually, as both PBGD and ferro-chelatase are located in mitochondria, a representation per number of tissue mitochondria would have been a better option. However, other mitochondrial enzymes that could be used as a marker for the number of mitochondria may have altered activities in tumour as well. The reason for low ferrochelatase activity in tumours may be the use of glycolysis rather than oxidative phosphorylation for their metabolism. In particular, rapdily growing tumours contain lower activities of mitochondrial cytochrome oxidase (Smith, 1987). Mitochondrial ferrochelatase would therefore be deficient secondary to deficiencies in mitochondrial cytoch-rome oxidase (Rimington and Riley, 1993). A promising option to increase endogenous porphyrin accumulation is the modification of the enzymes responsible for porphyrin biosynthesis. Substances such as griseofulvin and hexach-lorobenzene have already been used to induce porphyrin accumulation in hepatic cells in vitro (Visser et al., 1991). More recently, 1,10-phenanthroline has been shown to dou-ble the amount of ALA-induced protoporphyrin accumula-tion in rapidly proliferating cells (Rebeiz et al., 1992).

In this study, we used a light source emitting 320–450 nm (peak at 380 nm) to activate the accumulated porphyrins at their highest absorption peak at 400 nm. Mostly the weaker peak at 625 nm is used, as light of this wavelength penetrates deeper into the tissue (Star et al., 1990). However, in the intraoperative adjuvant application of PDT, tissue penetra-tion may not be crucial since the tumour bulk has been removed surgically and only residual tissue of not more than millimetres in thickness has to be eliminated. To determine the influence of wavelength, Lantz et al. (1992) compared the effect of the copper metal vapour laser (mainly 510.5 nm) and the rhodamine argon-pumped dye laser (630 nm) at 150 J cm^{-2} in a mouse colon carcinoma photosensitised with Photofrin. They found a similar depth of necrosis (4–5 mm) with both lasers. However, it has to be noted that the copper vapour laser also induced hyperthermia of $>40°C$, which has

been shown to potentiate PDT (Waldow et al., 1987). In another study by Davis et al. (1990), surgical resection and intraoperative PDT at 630 nm resulted in 60% tumour recur-rence of a mouse neuroblastoma, whereas the same treatment at 488–514 nm (argon laser) resulted in only 20% recurrence. In that study, the additional effect of hyperthermia was clearly demonstrated, as laser illumination alone substantially reduced the recurrence rate. The advantage of the copper metal vapour laser would be the much higher power output (25 W), producing a 30–60 cm^2 beam of 0.5–1 W cm^{-2}. The dye lasers currently available deliver only 2–4W at 625 nm, requiring exposure times of several hours to treat clinically relevant tissue areas.

There are only few other studies on the use of PDT as an adjunct to curative surgery. Herrera-Ornelas et al. (1986) and Nambisan et al. (1988) demonstrated the feasibility of this approach in patients undergoing resection of recurrent col-orectal carcinoma and retroperitoneal sarcoma. Delaney et al. (1993) completed a phase I feasibility study of PDT following debulking surgery for disseminated intraperitoneal tumours. In an attempt to illuminate the tissue surfaces equally, special light delivery devices were constructed consisting of a single fibre embedded in a balloon filled with diffusing Intralipid. To apply sufficient light energy to the large surface ares, two argon dye laser systems were used simultaneously at 514 nm. Light of 630 nm, which could only be delivered at a lower power output, was used when deeper tissue penetration was required. The intra-abdominal organs were found to be rather sensitive, and maximal tolerable light doses were set at 3.75 J cm^{-2} of 514 nm (48–72 h post 2.5 mg kg^{-1} Photofrin injection). Abulafi et al. (1992, 1993) have initiated a phase III clinical trial of adjuvant intraoperative PDT for resection of colorectal carcinoma vs surgery alone. A similar light delivery system was used at 510 nm, 35–70 J cm^{-2}, delivered 48 h after 2 mg kg^{-1} Photofrin intravenously. An interim analysis of 43 patients (median follow-up 12 months) did not show a significant difference in survival rate. However, in a subgroup with proved positive resection margins only 1/8 developed local recurrence after adjuvant PDT vs 12/14 after surgery alone. These preliminary results may indicate that a more aggressive approach with light of 630 nm is needed to completely eradicate residual tumour.

In conclusion, adjuvant intraoperative PDT seems to be a promising approach to sterilise the tumour bed after tumour debulking surgery. Clinical applications have mainly focused on the intra-abdominal use, in which sufficient light delivery and dosimetry have been a major drawback (Evrard et al., 1993). However, the method is potentially applicable to various other locations, including breast cancers requiring a mastectomy.

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