Increased efficacy of photodynamic therapy of R3230AC mammary adenocarcinoma by intratumoral injection of Photofrin II

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Summary Photodynamic therapy consists of the systemic administration of a derivative of haematoporphyrin (Photofrin II) followed 24–72 h later by exposure of malignant lesions to photoradiation. We investigated the efficacy of this treatment after direct intratumoral injection of Photofrin II. This direct treatment regimen resulted in higher rates of inhibition of mitochondrial cytochrome c oxidase (5.13% J−1 cm−2×10−2) and succinate dehydrogenase (3.14% J−1 cm−2×10−2) in vitro at 2 h after intratumoral injection compared to rates of inhibition obtained after intraarterial drug administration: 0.51 and 0.42% J−1 cm−2×10−2, respectively. A significant delay in tumour growth in vivo was observed in animals that received intratumoral injections 2 h before photoradiation compared to animals injected intraperitoneally at either 2 or 24 h before photoradiation. The treatment protocols were compared with control groups, consisting of Photofrin II administration intratumorally or intraperitoneally without photoradiation, or photoradiation in the absence of Photofrin II. These data indicate that the intratumoral injection regimen with Photofrin II enhanced the efficacy of photodynamic therapy. The greater delay in tumour growth observed after intratumoral administration of Photofrin II suggests a mechanism favouring direct cell damage.

Materials and methods

Materials

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise noted. Photofrin II, generously provided by Quadra Logic Technologies Inc. (Vancouver, British Columbia, Canada), was received frozen, thawed at room temperature in the dark, divided into 1 ml aliquots, and stored at −70°C until used.

Animals and tumours

The R3230AC mammary adenocarcinoma was maintained by transplantation into the axillary region of 80–100 g female Fischer rats, using the sterile trocar method described earlier (Hilf et al., 1965).

In vivo—in vitro protocol

Photofrin II was administered to tumour-bearing rats, either systemically by intraperitoneal (i.p.) injection or by intratumoral (i.t.) inoculation. Each injection was followed by an equilibration period of either 2 or 24 h, during which time tumour-bearing animals were housed in the dark. Intratumoral injections employed in the in vivo—in vitro protocol were, depending on the required injection volume, performed with either a 1 ml disposable syringe (>50 μl) or a Hamilton syringe (<50 μl), each fitted with a 27 gauge 5/8 inch needle. The needle was inserted laterally at the tumour midline and positioned approximately at the centre of the tumour, where the Photofrin II was injected. Initial tumour volumes before administration of Photofrin II i.t. or i.p. ranged from 0.79 to 2.56 cm3 allowing for a sufficient amount of tissue for preparation of mitochondria. Animals bearing tumours were selected randomly for either i.t. or i.p. administration of Photofrin II, each treatment group consisting of animals with...
tumours having volumes spanning the above stated range. Photofrin II concentrations administered i.t. were adjusted to attain equivalent body weight (b.w.) doses of 0.25, 0.5, 2.5 or 5.0 mg kg$^{-1}$ for the dose studies and 5.0 mg kg$^{-1}$ for the i.t. versus i.p. comparative studies. The upper limit of tumour volume, 2.56 cm$^3$, used in these studies represents tumours that measured less than 1.5 cm maximum diameter. Based on photon microscopy and magnetic resonance imaging studies of this mammary tumour model, the extent of necrosis in this range is estimated to be less than 10% of tumour volume and is usually focal in nature; it was not thought to alter significantly either the distribution or clearance of the injected Photofrin II. The animals were killed at selected times, tumours and livers were surgically excised in dimmed room light, and suspensions of mitochondria were prepared from whole tissues and stored in 1 ml aliquots at $-70^\circ$C until assayed (Gibson & Hill, 1983).

Photoradiation of mitochondrial suspensions in vitro

One ml aliquots of tumour or liver mitochondrial suspensions were removed from storage, thawed at room temperature and adjusted to the desired initial enzyme activity by dilution with preparation buffer (see below) before photoradiation of the suspensions in vitro. One ml aliquots of these suspensions were exposed to photoradiation emitted from a filtered (570–700 nm) focused quartz halogen light source. The samples placed in 3 ml quartz cuvettes were positioned in the 1 cm diameter focussed beam and irradiated with a power density of 150 mW cm$^{-2}$, measured by a power radiometer (Model Rk 5200, Laser Precision, Utica, NY, USA) connected to an Rk 545 radiometer probe. The suspensions were stirred magnetically and, at selected times, samples (10–40 µl) were removed for analysis of enzyme activity.

Temperature of the suspensions, which was monitored during the irradiation protocol (1 h, 540 J cm$^{-2}$ total fluence), did not rise above ambient (25°C).

Enzyme activity analysis

The activities of cytochrome c oxidase and succinate dehydrogenase were analysed at various intervals during the in vitro exposure of the mitochondrial suspensions to photoradiation. Before photoradiation, liver or tumour suspensions were adjusted to selected initial enzyme activities by dilution with the preparation buffer (0.33 M sucrose, 1 mM dithiothreitol, 1 mM EGTA, 0.03% bovine serum albumin and 100 mM KCl); these activities were 0.4–0.6 µmol cytochrome c oxidised per min per mg protein for cytochrome c oxidase and 1.3 x 10$^{-3}$ mmol p-iodonitrotetrazolium blue (INT) oxidised per min per mg protein for succinate dehydrogenase.


described using a power radiometer (RK5200, Laser Precision, Utica, NY, USA).

Tumour volume determinations and examination of treatment efficacy

Tumour volume was calculated according to $V = \pi r^2 H$, where the width, r, and the length, H, were obtained with calipers. The actual tumour volume was assessed by measuring the water displacement for the whole tumour, and comparing such values with volumes calculated by caliper measurements on the same tumour before its removal from the host. Estimation of volume by use of the equation yielded an average over-estimate of 25% for a cohort of six representative tumours whose volume was obtained by displacement measurements. Nevertheless, growth of each tumour was followed by caliper measurement and the increase in calculated tumour volume is presented as the number of days required for each tumour to reach 2, 5 or 10 times its initial volume. Analyses of the data using designated increments in tumour volume provide a more consistent basis for comparison among groups, particularly when initial tumour volumes, i.e. start of treatment, could vary (usually ±20%) and the initial treatment may have begun at different days after tumour implantation.

Statistical analysis

Tukey’s multiple comparison procedure (Snedecor & Cochran, 1967) was used to assess significant differences in tumour volume; changes from initial to 2 times initial volume, and from 2 times initial to 10 times initial volume were compared. A value of $P<0.05$ was considered to be significant.

Results

Effects of Photofrin II induced photosensitisation on mitochondrial enzyme activities in vitro following intratumoral drug administration

Photofrin II, administered i.t., results in a dose dependent inhibition of tumour mitochondrial cytochrome c oxidase and succinate dehydrogenase (SDH) during in vitro exposure of mitochondrial suspensions to visible irradiation (Gibson et al., 1989). Here we examined whether such a dose relationship existed after Photofrin II was administered intratumorally (i.t.). Two hours before killing, Photofrin II was administered i.t. at doses equivalent to 0.25, 0.5, 2.5 or 5.0 mg kg$^{-1}$ and mitochondria prepared from tumour and liver were exposed to photoradiation (see experimental methods). The data for cytochrome c oxidase (Figure 1) are presented to demonstrate that both a drug-dose and light-dose relationship existed for the inhibition of this enzyme and for SDH (not shown), in mitochondria prepared from tumours that were injected i.t. with Photofrin II 2 h before killing. Liver mitochondria prepared from the same animals also displayed dose-related inhibitions of both of these enzymes, but at this 2 h time point the extent of inhibition of liver enzymes was considerably less than that observed in tumours (data not shown). The rates of inhibition, calculated as per cent enzyme inhibition per joule per cm$^2$, which were derived from the linear initial portion of the inhibition curves as in Figure 1, are compiled in Table I. The increases in the enzyme inhibition rates were drug-dose dependent, displaying linearity in tumours for i.t. doses up to 2.5 mg kg$^{-1}$ b.w. and for liver up to 5.0 mg kg$^{-1}$ b.w., results suggesting that a maximum tumour porphyrin level was reached by direct injection. A comparison between tumour and liver mitochondrial preparations, at 2 h after i.t. injection, demonstrated that liver was 5–10-fold less susceptible to photosensitisation for each dose of Photofrin II administered. We interpret these results to indicate that higher concentrations of porphyrin were present in tumour tissue at this time, rather than inherent differences in enzyme sensitivity in these two tissues.
Figure 1 Effects of intratumoral injection of Photofrin II on inhibition of mitochondrial enzymes in vitro. Tumour mitochondria were prepared 2 h after i.t. administration of Photofrin II at 0.25 (□), 0.5 (▲), 2.5 (○) or 5.0 (■) mg kg⁻¹ b.w. and photoradiated in vitro with broad band light (570–700 nm) at a power dose of 150 mW cm⁻². The data are expressed as per cent of initial enzyme activity (zero time before photoradiation) for cytochrome c oxidase in the tumour. Each data point represents the mean of four separate experiments performed in duplicate; error bars are the s.e.m.

Liver and tumour mitochondria from untreated animals were prepared, incubated with Photofrin II in vitro, resuspended in buffer after removal of Photofrin II solution and photoradiated. No difference was observed in the photo-induced rate of inhibition of either cytochrome c oxidase or SDH present in either preparation, demonstrating that there were no inherent differences in enzyme sensitivity attributable to tissue source.

Comparison of intraperitoneal versus intratumoral administration of Photofrin II on the activities of mitochondrial enzymes in vitro

Photofrin II (5 mg kg⁻¹ b.w.) was administered either i.p. or i.t. at 2 or 24 h before killing of tumour-bearing animals and preparation of mitochondria. The data obtained for the photoradiation-induced inhibition of cytochrome c oxidase and SDH in tumour and liver preparations in vitro, presented as the calculated rates of enzyme inhibition, are compiled in Table II. The data clearly demonstrate that photosensitised inhibition of tumour mitochondrial enzymes in vitro 2 h after administration of Photofrin II i.t. was much greater than that observed after i.p. administration (5.45 vs. 0.51% and 3.98 vs. 0.45% inhibition J⁻¹ cm⁻² x 10⁻⁴ for cytochrome c oxidase and succinate dehydrogenase, respectively). At 2 h, the liver mitochondrial enzymes also demonstrated a greater enzyme inhibition rate for i.t. vs. i.p. drug administration. In tumour preparations obtained at 24 h post-injection, i.t. administration of Photofrin II continued to be more effective in causing photosensitised inhibition of both enzymes compared to i.p. injection. However, for the liver preparations, obtained 24 h after drug administration, a difference in response of cytochrome c oxidase and SDH relative to route of administration of photosensitiser was no longer apparent.

Comparison of effects of intratumoral versus intraperitoneal Photofrin II administration on tumour growth

Tumour growth was assessed in both treated and untreated animals by determination of tumour volume at regular intervals after tumours became palpable. Analyses of these data, presented as time in days necessary to attain 2, 5 or 10 times initial volume, are displayed in Figure 2. Intratumoral Photofrin II was administered at a dose of 0.5 mg cm⁻³, which represented a range of 0.81–1.55 mg kg⁻¹ b.w. Statistical analysis of the data depicted in Figure 2, using Tukey’s multiple comparison procedure, indicates that Photofrin II at 0.5 mg cm⁻³ tumour i.t. or at 10 mg kg⁻¹ i.p. caused a statistically significant delay of tumour growth when compared to tumour growth in animals injected i.t. with Photofrin II or 10 mg kg⁻¹ i.p. but not irradiated (dark controls). This delay in tumour growth in those animals receiving i.t. Photofrin II

Table I Rates of enzyme inhibition in vitro following i.t. administration of Photofrin II in vivo

| Photofrin dose (mg kg⁻¹) | Cytochrome c oxidase | Succinate dehydrogenase |
|-------------------------|----------------------|-------------------------|
|                         | Tumour   | Liver  | Tumour   | Liver  |
| 0.25                    | 1.18±0.06 | 0.30±0.014 | 1.00±0.06 | 0.39±0.035 |
| 0.50                    | 1.45±0.08 | 0.36±0.037 | 1.37±0.13 | 0.42±0.024 |
| 2.50                    | 4.24±0.36 | 0.57±0.05  | 2.70±0.36 | 0.66±0.054 |
| 5.00                    | 5.13±0.37 | 0.94±0.07  | 3.14±0.22 | 0.74±0.091 |

Photofrin II was administered by direct tumour injection at various doses: 0.25, 0.5, 2.5 or 5.0 mg kg⁻¹ b.w. Tumour and liver mitochondria were prepared 2 h after Photofrin II administration and exposed to broad band illumination (570–700 nm) at a power dose of 150 mW cm⁻². Rates of enzyme inhibition were calculated from the linear portion of the inhibition curves displayed in Figure 2. Rates are expressed as per cent enzyme inhibition J⁻¹ cm⁻² x 10⁻⁴ and are presented as means ± s.e.m. Initial activities (0 light) were adjusted by dilution of mitochondria and were: cytochrome c oxidase, 0.4–0.6 μmol cytochrome c oxidised per min per mg protein; succinate dehydrogenase, 4.6–8.3 x 10⁻⁴ μmol p-iodonitrotetrazolium violet oxidised per min per mg protein.

Table II Comparison of rates of mitochondrial enzyme inhibition following either i.p. or i.t. administration of Photofrin II

| Enzyme                  | Tissue  | 2 h         | 24 h         |
|-------------------------|---------|-------------|--------------|
|                         | i.t.    | i.p.        | i.t.         | i.p.  |
| Cytochrome c oxidase    | Tumour  | 1.13±0.07   | 1.11±0.07    | 0.66±0.09 |
|                         | Liver   | 0.94±0.07   | 0.21±0.013   | 0.94±0.11 | 0.72±0.06  |
| Succinate dehydrogenase | Tumour  | 3.14±0.22   | 0.42±0.05    | 2.12±0.09 | 1.09±0.04  |
|                         | Liver   | 0.74±0.09   | 0.29±0.016   | 0.67±0.05 | 0.85±0.084 |

Photofrin II was administered either i.p. or i.t. at 5 mg kg⁻¹ b.w. at 2 or 24 h before preparation and exposure of tumour or liver mitochondria to irradiation. Photoradiation was performed as described in the Methods. Rates of enzyme inhibition were derived from the linear portion of the inhibition curves displayed in Figure 2. Rates are expressed as per cent enzyme inhibition J⁻¹ cm⁻² x 10⁻⁴ and are presented as means ± s.e.m. Initial activities (0 light) were the same as listed in Table I.
Although combinations of PDT with other treatment modalities, such as X-irradiation (Bellnier & Dougherty, 1986; Winther et al., 1988; Levendag et al., 1989), chemotherapy using adriamycin (Edell & Cortese, 1988), cisplatin or doxorubicin (Nahabedian et al., 1988), hyperthermia by combining PDT with microwave irradiation (Waldow & Dougherty, 1984; Waldow et al., 1987; Levendag et al., 1989) and the use of hypoxic cell sensitisers, e.g. misonidazole (Gonzalez et al., 1986; Winther et al., 1988), have been reported, the results have been inconsistent. Less attention has been given to exploring modification of the commonly used PDT protocol so as to improve its efficacy. A number of variables can be investigated including: (i) increasing the concentrations of photosensitiser in neoplastic tissue to give a higher tumour/normal tissue ratio; (ii) light delivery, which optimally should provide sufficient and uniform photon flux throughout the neoplastic tissue; and (iii) adequate oxygen concentrations for production of singlet oxygen throughout the irradiation schedule. Our laboratory and others have begun to address the first of these components, delivery of photosensitiser. Zhou et al. (1988) administered haematoporphyrin that was incorporated into liposomes and was bound to lipoproteins of various density to improve the uptake of photosensitiser by tumour tissue after systemic administration. With both modified delivery vehicles, tumour response to PDT was more rapid and cell-directed than seen after conventional injection. In several earlier reports (Kostron et al., 1986; Winther et al., 1988), a direct injection of HpD into tumours was used so as to increase the efficacy of PDT as well as investigate the mechanisms of cell cytotoxicity and tumour regression. Although a transplantable rodent glioma demonstrated a greater responsiveness when HpD was administered i.t. (Kostron et al., 1986), response of the mouse MBT-2 bladder tumour was not enhanced, even though 5–10 times more porphyrin was present in tumours after i.t. injection (Levendag et al., 1988 a, h). Perhaps tumour type and/or host species could account for such apparent differences in response.

In this report, we compared the effects of two routes of drug administration on photosensitisation of mitochondria and on tumour growth after PDT. For study of mitochondrial effects, the in vivo–in vitro protocol was employed, an approach that takes into account any host metabolism and intracellular localisation of the photosensitiser occurring in vivo (Gibson & Hilf, 1983; Gibson et al., 1984b, 1989). From such data, we can derive an estimate of the pharmacokinetics of the administered photosensitiser. Previously, after i.p. administration of either HpD or Photofrin II, a time course of photosensitivity of these organelles in vitro indicated that inhibition of mitochondrial function would be greatest when tumours were exposed to light 24–72 h after drug administration (Gibson et al., 1989). Intratumoral administration of Photofrin II, however, showed a greater time-course. At 2 h after i.t. administration, we observed a 10-fold greater level of photosensitiser-induced inhibition of tumour mitochondrial enzymes than that observed in comparable mitochondrial preparations after animals had received equivalent doses of Photofrin II (5 mg kg\(^{-1}\)) intraperitoneally. By 24 h after Photofrin II administration, such differences in photosensitivity of enzymes narrow considerably, with only 2-fold greater sensitivity for i.t. than for i.p. routes. These results imply that there were higher concentrations of Photofrin II in tumours shortly after i.t. administration, whereas after i.p. administration, the level of porphyrin at 2 or 24 h, based on mitochondrial enzyme inhibition, was largely unchanged.

A different pattern of photosensitisation of liver mitochondria was observed. Surprisingly, liver mitochondria displayed greater photosensitivity at 2 h after i.t. administration than after i.p. injection, as also observed by the determination of cytochrome c oxidase and SDH activities. This finding suggests that after direct injection into the tumour, more effective levels of drug reached the liver via the systemic circulation than after i.p. injection. One possible explanation for this observation might be attributed to the presence of multimeric aggregates versus dimeric and monomeric haematoporphyrin species reaching the liver from the peritoneal injection site. While all of these forms after tissue extraction contribute to the amount of drug measured chemically, not all forms give equal singlet oxygen yields upon photoirradiation (Lambert et al., 1986). In contrast, at 24 h, liver mitochondrial enzymes were more photosensitive than at 2 h after i.p. injection, suggesting a greater accumulation and/or a slower efflux of active forms of the photosensitiser. A more extensive study of distribution of photosensitiser in other normal tissues after i.t. injection is warranted.

The results obtained from examination of mitochondrial enzyme inhibition, using the in vivo–in vitro protocol, were correlative to effects of PDT on delay of tumour growth. No significant effects on tumour growth occurred in animals that received Photofrin II (i.p. at 10 mg kg\(^{-1}\) i.t. at 1 mg kg\(^{-1}\) b.w.) but were light-exposed. Similarly, no differences in animals that received HpD but were light-exposed either at 2 or 24 h later displayed a significant delay in the length of time required to...
doubling their initial tumour volume. Most interesting is the finding that subsequent tumour growth, i.e. the time required to increase from 2 times initial size to 10 times initial size, was significantly longer in animals that received i.t. Photofrin II 2 h before light exposure. Mitochondria from tumours of these animals displayed the greatest photosensitivity, implying that the greatest metabolic damage could occur under these conditions. However, vascular damage cannot be excluded as a factor retarding tumour growth. Photosensitivity of liver mitochondria indicates efflux of some i.t. administered drug from the tumour into the systemic circulation. Because prolonged tumour growth retardation was not evident with the other treatment protocols employed, we suggest that vascular damage alone could not be the cause of the observed persistent tumour growth retardation following i.t. injection 2 h before irradiation. Regardless of the mechanism, the data presented demonstrate that intratumoral administration of Photofrin II provides one approach to enhance the effectiveness of PDT on tumour growth.

Although, clinically, lesions may not be accessible or are too numerous to treat by i.t. injections of Photofrin II, there are instances where superficial lesions could be treated by this method of drug delivery. The possibility also exists that, in such cases, the lower amounts of i.t. Photofrin II would result in less skin photosensitivity. Studies directed towards assessing these possibilities are in progress.

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