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Research Note

Photodynamic Treatment with Fractionated Light Decreases Production of Reactive Oxygen Species and Cytotoxicity *In Vitro via* Regeneration of Glutathione¶

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ABSTRACT

Photodynamic therapy removes unwanted or harmful cells by overproduction of reactive oxygen species (ROS). Fractionated light delivery in photodynamic therapy may enhance the photodynamic effect in tumor areas with insufficient blood supply by enabling the reoxygenation of the treated area. This study addresses the outcome of fractionated irradiation in an *in vitro* photodynamic treatment (PDT) system, where deoxygenation can be neglected. Our results show that fractionated irradiation with light/dark intervals of 45/60 s decreases ROS production and cytotoxicity of PDT. This effect can be reversed by addition of 1,3-bis-(2-chlorethyl)-1-nitrosurea (BCNU), an inhibitor of the glutathione reductase. We suggest that the dark intervals during irradiation allow the glutathione reductase to regenerate reduced glutathione (GSH), thereby rendering cells less susceptible to ROS produced by PDT compared with continuous irradiation. Our results could be of particular clinical importance for photodynamic therapy applied to well-oxygenated tumors.

INTRODUCTION

Photodynamic treatment (PDT) is based on the administration of a photosensitizing agent followed by irradiation with light of an appropriate wavelength. This activates the photosensitizer, which is further able to react with molecular oxygen ($^3O_2$), generating either singlet oxygen ($^1O_2$) or other reactive oxygen species (1,2), eventually leading to necrotic and apoptotic cell death (3,4).

As demonstrated in several studies, photodynamic therapy effects may be augmented *in vivo* by modifying the irradiation regime without increasing the total light energy (J/cm^2) by either modulating the fluence rate (5,6) or by introducing dark intervals during light application (fractionated irradiation) (6–8).

Under conventional clinical photodynamic-therapy conditions, the amount of molecular oxygen in the treated area is rapidly depleted during the photochemical processes, leading to a diminished capacity for further production of reactive oxygen species (ROS) (8–10). This effect is especially important in antitumor applications of photodynamic therapy where the tumor areas are characterized by poor vascularization or where photodynamic therapy causes damage to afferent blood vessels, both leading to insufficient oxygen supply during treatment (11,12). Deoxygenation may be reduced by fractionated light delivery by providing sufficient time for reoxygenation of the treated tissue during the dark periods (6,8). Additional antitumor effects of fractionated irradiation are ROS damage produced during reperfusion of the area, which occurs after a transient period of ischemia (reperfusion injury) (13) and relocalization of the sensitizer during the dark interval(s) (14).

Apparently, the success of fractionated photodynamic therapy is dependent on several factors, with the light-delivery regime as the one probably most important (9,10,15). Several theoretical and practical approaches were able to demonstrate that the most effective light fraction schedules consist of equal light on and off periods, with intervals between 15 and 60 s (9,10). Shorter intervals have been shown to cause other mechanisms to become dominant, as for intervals in the nanosecond levels an enhanced photodynamic effect may occur (16).

However, the literature on fractionated light delivery in photodynamic-therapy applications is still contradictory and incomplete, especially for *in vitro* systems. Some investigators did not find any positive antitumor action of fractionated photodynamic therapy *in vivo*, which may be explained by the influence of cellular repair mechanisms becoming active in the dark intervals to compensate for the initial damage (6,17).

In the present study, we addressed the question whether regeneration of ROS quenching mechanisms, namely the glu-

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Abbreviations: AlPcS4, aluminum (III) phthalocyanine tetrasulfonate; BCNU, 1,3-bis-(2-chlorethyl)-1-nitrosurea; DCF, 2′-7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PDT, photodynamic treatment; ROS, reactive oxygen species.

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tathione system, influences the outcome of fractionated PDT in an *in vitro* system, where oxygen supply during PDT is granted. We, therefore, monitored ROS formation and the cytotoxic effect of fractionated PDT, using hypericin and aluminum (III) phthalocyanine tetrasulfonate ($AlPcS_4$) as sensitizers, with and without pharmacological inhibition of glutathione regeneration.

MATERIAL AND METHODS

Cell culture and photodynamic treatment. A431 human epidermoid carcinoma cells (ATCC CRL-1555) were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 4.5 g/L glucose supplemented with 10 mM 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethanesulfonic acid (HEPES), 4 mM L-glutamine, 1 mM Na-pyruvate, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 5% fetal calf serum (FCS) (all from PAA-laboratories, Linz, Austria), in a humidified atmosphere at 37°C and 7.5% CO_2. For measurement of ROS production, 3×10^5 cells in 1.5 mL 5% FCS DMEM were seeded into 30 mm Petri dishes (Greiner Bio-One, Kremsmuenster, Austria); for measurement of cytotoxicity, 7500 cells in 100 µL 5% FCS DMEM were seeded into 96-well microplates (black walls, clear bottom; Greiner Bio-One). In a first step, cells were incubated for 20 h and 18 h in 0% FCS DMEM containing 1.5 µg/mL hypericin (Fluka BioChemika, Buchs, Switzerland) and 10 µM $AlPcS_4$ (Porphyrin Products, Logan, UT), respectively. For inhibition of the glutathione reductase, supernatants were aspirated and fresh, sensitizer-free medium containing 500 µM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; Sigma-Aldrich, Vienna, Austria) (18) was added for an additional hour.

Immediately before irradiation, the culture supernatants of all samples were replaced with fresh media. Irradiation was performed using a red-light irradiation diode array with λ_{max}, $AlPcS_4 = 660 \pm 20$ nm (cat. no. L53SRC-F) and a light power of 10 mW/cm^2 and λ_{max}, hypericin = 610 ± 20 nm (cat. no. L7113SEC), 0.9 mW/cm^2, respectively (both superbright diodes, Kingbright Electronics, Issum, Germany). Fractionated light was delivered in a discontinuous irradiation regime, consisting of 45 s irradiation and 60 s dark intervals.

Samples were protected from ambient light to avoid unspecific sensitizer activation; for all experiments, cells from passages 5–15 were used.

Measurement of cytotoxicity. Metabolic/mitochondrial activity was assessed as before (19,20) 24 h post irradiation by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT; Sigma-Aldrich) to the insoluble blue formazan catalyzed by mitochondrial dehydrogenases (21).

Measurement of intracellular ROS. Intracellular ROS were measured by the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes Europe, Leiden, Netherlands) and quantified by flow cytometry (FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ) (22,23).

Thirty minutes prior to irradiation, cells were incubated with 50 µM DCFH-DA and PDT-treated as described above. Ten minutes after irradiation, cells were harvested together with the supernatants using Accutase (PAA-laboratories); all subsequent steps were performed on ice. The cells were spun down at 840 *g* at 4°C, washed with 1 mL phosphate-buffered saline (PBS), the pellets were resuspended in 500 µl PBS, the resulting green fluorescent DCF (2′-7′-dichlorofluorescein) was analyzed by flow cytometry (Fl-1 channel; $\lambda_{EX} = 488$ nm, $\lambda_{EM} = 530 \pm 30$ nm). For each sample, 10 000 cells were analyzed and the fluorescence signals were evaluated as described in the *Results* section.

Statistical evaluation. Data represent mean of three independent experiments ± SEM. Statistical significance was evaluated using Student's *t-test*.

RESULTS

Cytotoxicity after PDT with continuous and fractionated irradiation

In order to determine the viability of A431 cells following PDT with $AlPcS_4$ and hypericin under either continuous or fractionated irradiation conditions, we used the MTT assay to measure the metabolic/mitochondrial activity. To specifically investigate the impact of the reduced form of glutathione (GSH) recycling system on the viability of the treated cells, we added BCNU, an inhibitor of the glutathione reductase, interfering with the cells' capacity to reduce the oxidized form of glutathione (GSSG) to its reduced form, GSH. In control experiments, we could show that BCNU application for 1 h *per se* was not toxic within the first 24 h after addition (data not shown) and, furthermore, did not affect the intracellular GSH levels (see [24]). Additionally, no cytotoxic effect was found for dark controls (only photosensitizer without irradiation) and light-only controls (irradiated without sensitizer) (data not shown).

Figure 1 shows the percentage of MTT signal under several conditions after $AlPcS_4$-PDT (A) and hypericin-PDT (B), related to the respective controls (not illuminated but identically treated samples without sensitizer).

The MTT signal for $AlPcS_4$-sensitized and continuously irradiated cells (filled squares) was reduced quite rapidly to <5% in a fluence range from 1.5 to 3.5 J/cm^2. The curve of fractionatedly irradiated samples (filled triangles) is similarly shaped, but the cells were far less sensitive to photodynamic treatment. The MTT signal remained at the control level up to a light dose of approximately 2 J/cm^2 and eventually decreased to about 15% at a light dose of 4 J/cm^2.

Expectedly, BCNU-treated cells appeared to be more sensitive to PDT-mediated cell killing than those without BCNU. Cells treated with continuous PDT and BCNU (open squares) showed a rapid decline of the MTT signal, starting at a light fluence of about 1 J/cm^2 and reaching <5% at approximately 3 J/cm^2. Addition of BCNU (open triangles) seemed to compensate for the effect of fractionated irradiation at least at fluences >2.25 J/cm^2; the survival curve of cells treated with fractionated irradiation and BCNU and the curve of continuously irradiated cells appear similar, both reaching the base level at about 3.5 J/cm^2.

The results of hypericin experiments in principle match those of $AlPcS_4$-PDT; the particular curves are similarly shaped, albeit different fluences were applied.

ROS production after PDT with continuous and fractionated irradiation

In order to explore whether the decreased cytotoxicity of fractionated light application was caused by lower ROS formation, we determined intracellular ROS levels in A431 cells after PDT under the conditions mentioned above. In control experiments, no increase of the DCF signal was found in dark controls (only photosensitizer without irradiation) as well as in light-only controls (irradiated without sensitizer) (data not shown).

Figure 2 shows the percentage of the DCF signal after $AlPcS_4$-PDT (A) and hypericin-PDT (B) with varying light doses, related to respective control cells (not illuminated, without $AlPcS_4$ and either with or without BCNU); addition of BCNU increased the ROS level of controls by a factor of about 2.5 due to a reduced capacity of GSH to detoxify endogenously generated ROS (24).

In $AlPcS_4$-PDT, the dynamics of ROS levels generated by continuously irradiated cells were characterized by an increase of the ROS content from about three-fold of control levels at a light fluence of 0.9 J/cm^2 to the 6.5-fold at a light fluence of 1.8 J/cm^2. In contrast, light delivery in fractions caused a lower oxidation of DCFH-DA, starting from approximately two-fold (0.9 J/cm^2) to four-fold (1.8 J/cm^2) of control.

The addition of BCNU provoked corresponding effects to those seen in the cytotoxicity experiments (Fig. 1). Continuous irra-

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diation together with addition of BCNU caused a very rapid increase of intracellular ROS from approximately 5.5-fold at a light dose of 0.9 J/cm^2 to eight-fold at 1.8 J/cm^2. Treatment with BCNU reversed the effect of fractionated light application; the relative ROS levels increased to about five-fold and 7.5-fold with light doses of 0.9 and 1.8 J/cm^2, respectively.

In hypericin-PDT, the results fully confirm those of $AlPcS_4$-PDT at slightly lower overall levels of ROS compared with $AlPcS_4$-PDT experiments.

DISCUSSION

In vivo fractionated light delivery in photodynamic therapy causes, in many cases, increased cytotoxicity, which can mainly be explained by reoxygenation effects during the dark periods (6,8,9,25). Additionally, relocalization of the particular photosensitizing agent (14) and induction of apoptosis by reperfusion injury (13) have been reported to occur and support the success of photodynamic therapy. Effective light fraction schedules have been shown to consist of equal light and dark periods with intervals between 15 s and 1 min (9,10).

However, this time frame is sufficient to restore the ROS quenching capacity of the glutathione system by the fast-acting, reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme glutathione reductase (18,26,27). The latter antagonizes the PDT-induced damage to a certain degree, depending on several parameters, such as the dominant reactive oxygen species or the localization of the photosensitizer, both of which might have a negative impact on the glutathione reductase itself.

Within this study, we could show that, under *in vitro* PDT conditions, where sufficient oxygen supply due to the high amount of oxygen in the supernatant medium and continuous gas exchange with the outside is granted, fractionated light application using light/dark intervals of 45/60 s was found to reduce intracellular ROS levels, compared with continuous irradiation, by a factor ranging from 1.5 and 1.1 at low fluence rates to 1.7 and 1.2 at high fluences, for $AlPcS_4$- and hypericin-PDT, respectively. Accordingly, the cytotoxic effect decreased when irradiation was performed in fractions. Addition of BCNU, an inhibitor of the glutathione reductase (18), reversed the effect of fractionated irradiation to a large extent. Both effects, lower ROS levels and decreased cytotoxicity, and their reversibility by BCNU could be observed for two photosensitizing agents with different chemical properties, *i.e.* the hydrophilic $AlPcS_4$ (mainly localizing in lysosomes [28,29]), and the lipophilic hypericin (localizing in mitochondria and membrane systems [30,31]). These different chemical properties ultimately reflect the ROS production induced by PDT with the respective sensitizer: $AlPcS_4$-mediated PDT is known to preferentially produce hydroxyl radicals (32) whereas hypericin-PDT more likely induces the formation of singlet oxygen (33,34). This is of particular importance for the interpretation of the ROS measurements because DCFH-DA is specific for hydroxyl radicals and, only to limited degree, for other ROS (35,36).

Because GSH is mainly effective in quenching hydroxyl radicals (26), the effects of fractionated irradiation and BCNU addition on the production of ROS and cytotoxicity are more profound with $AlPcS_4$-PDT compared with hypericin-PDT.

Under conditions of continuous irradiation, samples incubated with BCNU produced more ROS than those without BCNU. This might be due to some regeneration of GSH (and subsequent decomposition of ROS) in samples without BCNU under continuous irradiation, at least unless glutathione reductase is damaged by ROS, a possible occurrence at higher fluences. This effect is also reflected in the cytotoxicity measurements. The difference between continuous and fractionated irradiation is the (more or less) complete regeneration of GSH during the dark intervals in the latter.

We noticed a discrepancy in the ROS level and cytotoxicity for the fractionated and continuous irradiation with BCNU: the (statistically nonsignificant) differences in the DCF signal result in a significant increase in cytotoxicity (at least for some doses, for $AlPcS_4$; results of statistical analysis not shown in Fig. 1). We suggest two hypotheses to explain this effect: (i) singlet oxygen (or other ROS not detected by DCFH-DA) is decomposed during the dark intervals of fractionated irradiation, thus leading to less cytotoxicity at similar DCF-signal levels; and (ii) cellular repair mechanisms restore the cellular protein/lipid ensemble during the dark intervals and thus cause less cytotoxicity.

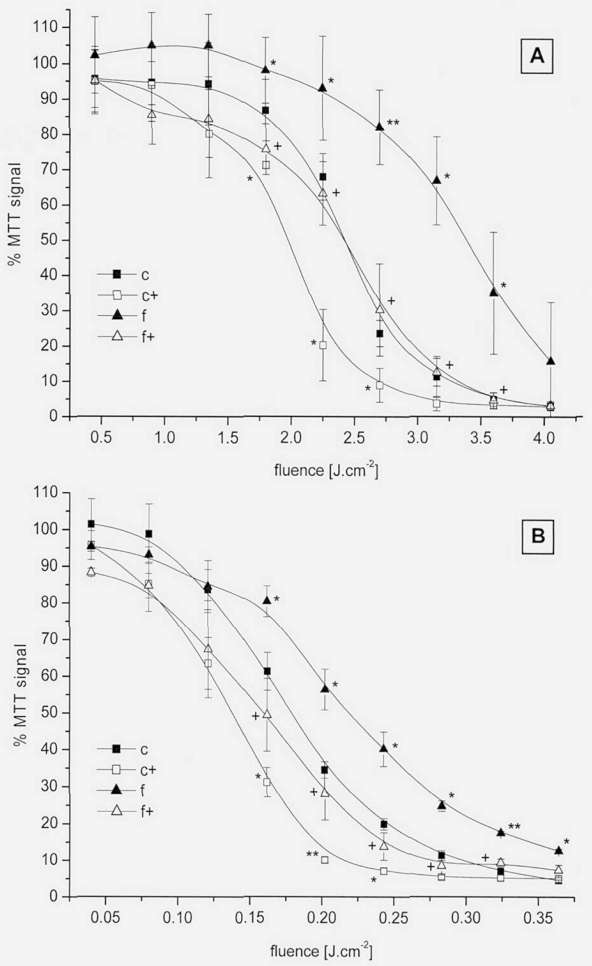

Figure 1. Metabolic/mitochondrial activity of $AlPcS_4$- (A) and hypericin- (B) photosensitized A431 cells after fractionated or continuous irradiation and/or under conditions of impaired glutathione recycling. Results are expressed as relative MTT activity related to control samples (without sensitizer and nonirradiated). Each point represents the mean of at least three independent experiments ± SEM. Data were statistically compared with continuously irradiated cells (**: $P < 0.01$, *: $P < 0.05$) and to fractionatedly irradiated samples (++: $P < 0.01$, +: $P < 0.05$), respectively. A: $AlPcS_4$; B: hypericin; (c) continuous, (c+) continuous + BCNU, (f) fractionated, (f+) fractionated + BCNU.

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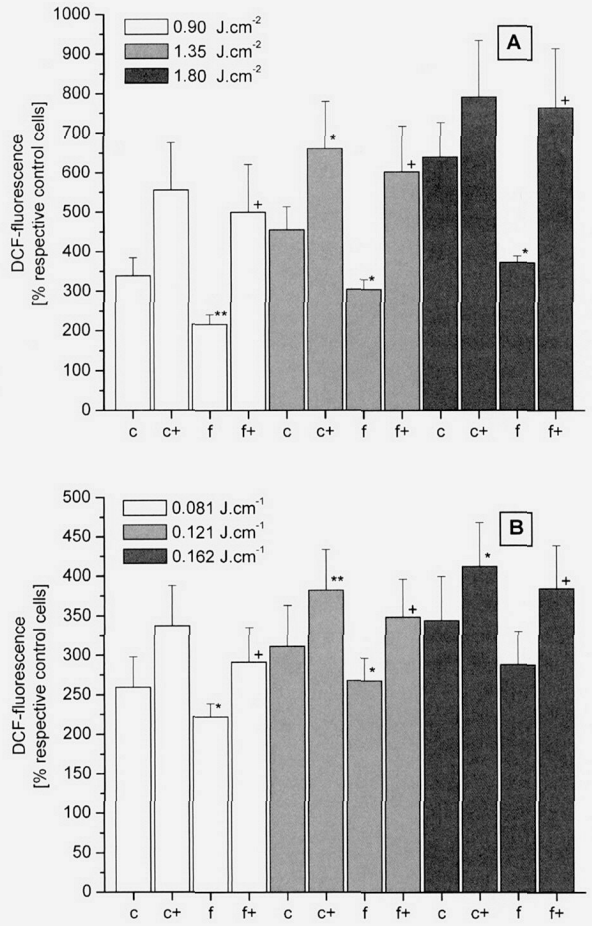

Figure 2. ROS production after $AlPcS_4$-PDT (a) or hypericin-PDT (b) for fractionatedly and continuously irradiated A431 cells and/or under conditions of impaired glutathione recycling. Samples to which BCNU was added are related to the respective BCNU-containing control, whereas BCNU-free samples are presented in relation to untreated control samples (both controls without sensitizer and nonirradiated). Data represent mean values of three independent experiments ± SEM. Data were statistically compared with continuously irradiated cells (**: $P < 0.01$, *: $P < 0.05$) and to fractionatedly irradiated samples (++: $P < 0.01$, +: $P < 0.05$), respectively. A: $AlPcS_4$; B: hypericin; (c) continuous, (c+) continuous + BCNU, (f) fractionated, (f+) fractionated + BCNU.

Taken together, our data suggest that, with fractionated PDT, the glutathione reductase is able to recycle a significant portion of GSH during the dark intervals. This counteracts the photodynamic effect and, thus, reduces the efficiency of *in vitro* PDT. In good agreement with our results, Perotti and coworkers (37) could show that PDT on the LM2 cell line was less cytotoxic when GSH was added to the incubation medium. The scavenging effect of GSH during PDT was described as the one most important observed in this study.

However, the fractionation scheme represents an important factor because the high activity of glutathione reductase (38) could allow almost complete GSH regeneration during the dark periods of sufficient duration (*e.g.* 60 s, as used in the present study).

In conclusion, our results may be of interest for photodynamic-therapy applications on any well vascularized tumor/tissue in which oxygen deprivation is not to be expected. In clinical applications of photodynamic therapy, it has to be considered that GSH recycling may antagonize the therapeutic efficiency.

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