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Photofrin as a radiosensitizer in an in vitro cell survival assay

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Abstract

Chemical modifiers (radiosensitizers) are used in order to increase the efficacy of radiotherapy. The use of Photodynamic Therapy for tumor treatment, especially with Photofrin II, is also known. At present, no chemical modifier has been found to act as a selective radiosensitizer. Experiments using several series of cell lines were performed; human bladder cancer cell line (RT4), colon adenocarcinoma cells (HT-29), and the glioblastoma cells (U-373 MG) were investigated, with and without incubation with Photofrin II, before irradiation. The irradiation was performed using doses ranging from 0 to 8 Gy. Colony forming tests were applied to determine the efficiency of Photofrin II as a radiation sensitizer in comparison to irradiation alone. Two of the cell lines tested, cultures of the RT4 and U-373 MG, treated with Photofrin II prior to radiation, showed cell survival lower than cultures untreated with Photofrin II but irradiated under identical conditions. For the HT-29 cells, the results did not differ between the two groups (with and without Photofrin). The results of this study showed that Photofrin II can act, under certain conditions as a tumor radiosensitizer.

Keywords: Radiation therapy; Ionizing irradiation; Photofrin II; Radiosensitizer; Radioresistant

Photodynamic therapy (PDT) is presently established as widely accepted modality for treatment of a variety of solid tumors [1,2]. This technique involves the topical or systemic administration of a photosensitizing agent, which is preferentially accumulated or retained by the tumor tissue, followed by illumination of the neoplastic area with light wavelengths specifically absorbed by the photosensitizer. In this connection, porphyrins and their analogues are most frequently adopted as photosensitizers: their tetrapyrrolic macrocycle exhibits absorption bands in the red region (600–800 nm) of the visible spectrum which is endowed with an especially high penetration power into human tissue [3] and is not absorbed by the endogenous constituents of cells, thereby minimizing the risk of general photodamaging effects. In particular, Photofrin II, a complex mixture of porphyrins originated from chemical modification of hematoporphyrin (Hp) [4], has been recently approved for the PDT of specific tumors at a clinical level in several countries [1,2]. At present, a few thousand patients have been treated by PDT with Photofrin worldwide with objectively positive results [1,2].

Nevertheless, Photofrin-associated PDT exhibits some important limitations, including the low molar extinction coefficient in the clinically useful red spectral region, which limits the efficiency of its activation by light. Additionally, the red absorption maximum of Photofrin corresponds to the 630 nm wavelength which has reduced penetration power within most human tissues. For these reasons, repeated PDT treatments are often necessary [1,2]. As a consequence, the use of PDT in combination with other therapeutic modalities, such as radiation therapy, is being investigated [1,5,7].

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In general, the biological effects of radiation, which concerns both tumor and normal tissues, are modifiable and selectivity can be improved by sophisticated computerized dosimetry. A parallel approach is based on the introduction of radiation reaction effect which depends on cellular biology (i.e., oxygenation, cell cycle, etc.) and can be modified by chemicals (sensitizers, protectors, and chemotherapy) acting as radiosensitizers [6]. This modification is important to achieve the maximum effect on tumor tissue, while at the same time minimizing the effect on normal tissue.

Two main factors must always be considered, when using a radiosensitizing agent—the local tumor control probability (TCP) and normal tissue complication rate (NTCP), which both result in the therapeutic ratio (TR).

Most of the presently known and routinely used radiosensitizing agents have a poor selectivity and are not tumor specific. The use of these compounds may also result in severe side effects due to their inherent toxicity [6].

The observations published in the 1950s and 1960s by Schwartz and Cohen [7–9] and later by others [8,10] showed that hematoporphyrin derivative (HpD), a highly heterogeneous chemical derivative of Hp, of which Photofrin II represents a partially purified form [4], can act as a radiosensitizer for tumors. Moreover, the enhancement of radiation-induced cell killing by synthetic metalloporphyrins has been subsequently reported [11].

Recently published papers [12,13] showed the advantage of using photosensitizers such as gadolinium texaphyrin (Gd-Tex) in the treatment of brain metastases.

Several other published studies on murine tumor models have demonstrated the in vivo efficacy of Photofrin II as both a specific and a selective radiosensitizing agent [14–16]. These animal studies assessed the efficacy of the radiosensitizing action in terms of tumor doubling time.

The aim of this paper was to report on the radiosensitizing effect of Photofrin II on three different cell lines in vitro, two of them (human bladder cancer and glioblastoma cells) known to be radioresistant in vivo.

Materials and methods

Chemicals. Photofrin was purchased from Axcan Pharma (Mont-Saint-Hilaire, Canada) as freeze-dried porfimer. It was stored as a stock solution in phosphate-buffered saline (PBS, Invitrogene) with a concentration of 2.5 mg/ml and kept at −20 °C until use. Storage, dilution steps, and incubation period were performed under experimental conditions avoiding the exposure of Photofrin to light.

All chemicals and additives for the cell culture were purchased from Gibco Invitrogen (products for cell culture, Karlsruhe, Germany), unless otherwise specified.

Cell lines and culture. Three different human tumor cell lines were used in this study.

The well-differentiated papillary human bladder carcinoma cell line RT4 and human glioblastoma cell line U-373 MG. Both cell lines are known to be radioresistant cells in vivo [17–21]. The third cell line was represented by human colon adeno-carcinoma HT-29 known to be radiosensitive cells [22].

Stock cultures of each cell line were maintained in 80 cm^3 flasks (Nalge Nunc, Wiesbaden, Germany). RT4 cells were grown in chemically defined RPMI 1640 medium containing Glutamax, supplemented with 10% fetal bovine serum, 1% sodium pyruvate (100 mM, Sigma–Aldrich), and 1% Eagle's minimal essential medium with Earle's salts. U-373 MG cells were kept in Dulbecco's MEM/F12 (1:1) medium with Glutamax, containing 15% fetal bovine serum and HT-29 cells were kept in McCoy's 5A medium with Glutamax, 20% fetal bovine serum. Then 100 U/ml penicillin and 100 μg/ml streptomycin was added to each medium. All cell lines were maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells were passaged in the exponential growing phase once per week, using 0.05% trypsin plus 0.02% EDTA in PBS at 37 °C.

Treatment of cells. The experiments were carried out in four-well culture dishes. RT4 cells were seeded at a density of 200 cells per well (these wells were irradiated with 0–6 Gy). A different group of cell dishes containing 1000 cells per well was irradiated with 8 Gy. The higher cell number in the second group was to compensate for the low cell survival rate at high irradiation doses. The U-373 MG cells (glioblastoma cells) and HT-29 cells (colon adenocarcinoma cells) were seeded at a density of 100 cells (0, 2 Gy), 200 cells (4 Gy), 600 cells (6 Gy), and 1000 cells (8 Gy) per well, based on preliminary measurements that showed optimal cell concentration for each irradiation dose. The culture dishes were kept in a light-protected humidified chamber to avoid any activation of Photofrin II. After 24 h the medium was removed and 3 ml of a Photofrin-PBS solution at a final concentration of 1 μg/ml was added to each well. This concentration was chosen after toxicity tests with the different concentrations of Photofrin (Table 1). Cell incubation with PBS without Photofrin served as a control. Following exposure of the cells to the test compound for 1 h in the incubator, the Photofrin-containing supernatant was discarded and replaced with 3 ml of fresh culture medium. Then, the Photofrin loaded cells were irradiated with ionizing radiation (Müller RT 250 X-ray device, 225 kV, 15 mA, 0.35 Cu-filter and a dose-rate of 0.9 Gy/min) with a dose of 2, 4, 6, and 8 Gy, respectively. During irradiation, cells were kept at 37 °C. Control cells underwent the same procedure as irradiated cells but without irradiation.

Colony preparation and staining. The response of the cells to irradiation was evaluated by determining the cell survival; colonies with more than 50 cells were scored at 13 days postseeding. Colony prep-

Table 1
Influence of different Photofrin concentrations on cell survival without irradiation

Cell line	Photofrin (μg/ml)	Colonies (means ± SD)[a]
RT4	0	100 ± 12.2
RT4	1	97.5 ± 13.5
RT4	2	82.5 ± 16.6[b]
U-373 MG	0	100 ± 6.6
U-373 MG	1	93.0 ± 7.0
U-373 MG	2	72.2 ± 18.7[b]
HT-29	0	100 ± 11.3
HT-29	1	102.8 ± 27.9
HT-29	2	89.7 ± 15.4[b]

[a] Mean values (mean) and standard deviations (SD) are calculated for at least 24 single data sets.

[b] Significant difference for $p < 0.05$ in a Student's t test.

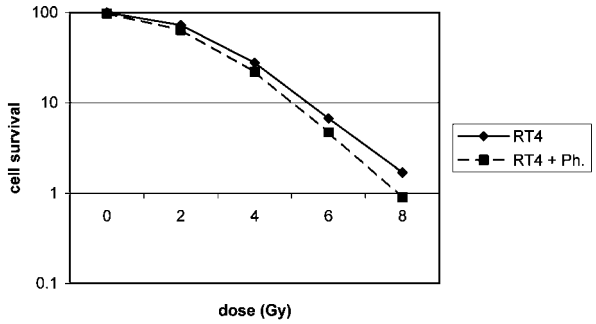

Fig. 1. Cell survival curve for RT4 cells with and without Photofrin-incubation (cells were incubated with 1 μg/ml Photofrin II prior to X-ray irradiation).

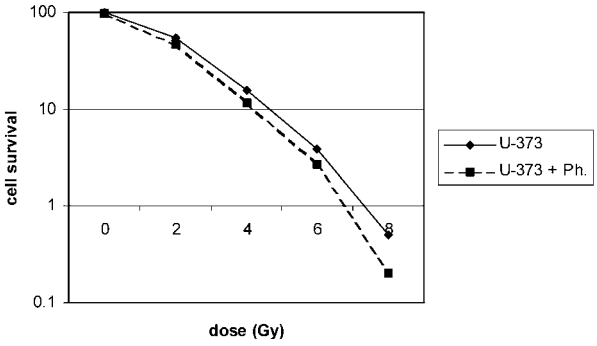

Fig. 2. Cell survival curve for U-373 cells with and without Photofrin-incubation (cells were incubated with 1 μg/ml Photofrin II prior to X-ray irradiation).

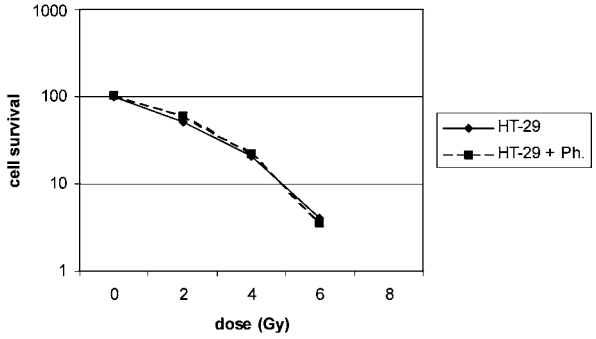

Fig. 3. Cell survival curve for HT-29 cells with and without Photofrin-incubation (cells were incubated with 1 μg/ml Photofrin II prior to X-ray irradiation).

aration and staining were carried out identically for all three cell lines. For colony preparation, the culture medium was removed and a 3 ml aliquot of fixative (2 vol of ethanol plus 1 vol of acetic acid) was added per well. After incubation for 5 min at room temperature, the fixative was replaced by a staining solution (20% Gram's crystal violet solution for microscopy, Merck, Darmstadt, Germany) for additional 30 min. Subsequently, the staining solution was discarded and cells were washed thoroughly under carefully running tap water and allowed to air-dry.

Cell survival analysis. The toxicity test of the different concentrations of Photofrin (1 and 2 μg/ml) was tested in two separate experiments with duplicate four-well dishes.

Each examined combination between Photofrin exposure dose and irradiation was examined in separate experiments repeated three times with duplicate four-well cultures, resulting in a minimum of 24 single data sets.

Analyses of colonies were carried out under a stereomicroscope (Bausch and Lomb, New York). Surviving cells were those which gave rise to colonies composed of 50 or more cells. A Student's t test with $p \leqslant 0.05$ was adopted to evaluate the statistic significance of our results. To avoid the influence of different plating efficiencies between replicate experiments the number of scored colonies was adjusted to 100. This was calculated by multiplication of the scored number of colonies by a factor, specifically determined for each independent experiment and subsequently the per cent value was calculated. The factor was ascertained by division of the seeded cell number through the actually scored cell number of the control experiment, means without Photofrin incubation and without irradiation. Thus, the individual plating efficiency of each experiment was included in each calculation.

Dose modifying factor. Survival curves with and without Photofrin II were generated for all three cell lines (Figs. 1–3) and the dose modifying factor (DMF) was measured by dividing the radiation dose without the porphyrin over the radiation dose with the porphyrin for the same survival level.

Results

Table 1 describes the influence of two different concentrations of Photofrin II on cell cultures. Apparently there was no appreciable difference, indicating that toxicity was not detected for all cell lines using a concentration of 1 μg/ml.

In comparison to cells irradiated without Photofrin II, a significant decrease in the survival rate ($p \leqslant 0.05$ in a Student's t test) of cells was observed for RT4 cells and U373 cells, which were irradiated after incubation with Photofrin II (Table 2). This effect is not observed for HT-29 cells (Table 2). The survival curves for the three cell lines are shown in Figs. 1–3. The DMF for RT4 cells ranges between 1.1 at a survival level of 50% and 1.2 at a survival level of 5% and between 1.2 and 1.3 at a survival level of 50% and 5%, respectively, for U-373 cells.

HT-29 cells showed no difference between the two treatment modalities. Only after irradiating with 6 Gy a slightly enhanced effect was seen. No increase of DMF could be observed for HT-29 cells.

Discussion

The present findings demonstrate that Photofrin II, under appropriate experimental conditions, can act as a radiosensitizing agent for tumors, especially in cell lines known to be highly radioresistant in vivo, such as RT4 and U-373 cells [17–21]. Such an effect was not observed in HT-29 cells which are known to be radiosensitive [22]. The decrease of the cell survival after incubation with Photofrin II and ionizing irradiation was statistically significant in a Student's t test of cells for the two cell

Table 2
Influence of X-ray irradiation on RT4, U-373, and HT-29 cell survival, with and without of Photofrin II

Dose (Gy)	Photofrin	RT4 colonies (mean ± SD)[a]	U-373 colonies (mean ± SD)[a]	HT-29 colonies (mean ± SD)[a]
0	–	100 ± 12.2	99.1 ± 12.9	99.3 ± 25.5
0	+	97.7 ± 13.5	96.5 ± 13.4	102.8 ± 27.9
2	–	72.8 ± 7.8	54.4 ± 10.9	51.0 ± 13.5
2	+	64.1 ± 11.4[b]	46.4 ± 7.8[b]	61.2 ± 18.3
4	–	27.7 ± 8.1	15.7 ± 5.1	20.6 ± 7.1
4	+	22.0 ± 4.1[b]	11.7 ± 3.4[b]	22.2 ± 8.8
6	–	6.7 ± 2.2	3.9 ± 1.1	4.1 ± 2.5
6	+	4.7 ± 2.3[b]	2.7 ± 1.1[b]	3.6 ± 2.1
8	–	1.7 ± 0.7	0.5 ± 0.2	n.c.
8	+	0.9 ± 0.5[b]	0.2 ± 0.1[b]	

[+]Cells were incubated with 1 μg/ml Photofrin II prior to irradiation.
[a] Mean values (mean) and standard deviations (SD).
[b] Significant difference for $p < 0.05$ in a Student's t test.

lines: RT4 and U-373 (not distributed randomly). Our findings are in agreement with recently published papers [14–16] which showed in vivo the radiosensitizing activity of Photofrin on bladder carcinoma and Lewis sarcoma tumors, that are also known as hypoxic and radioresistant tumors [18,20,21,23]. However, the radiosensitizing effect shown in the in vitro study was modest as shown by the DMF of 1.3. In vivo experiments with and without Photofrin during irradiation showed enhancement of tumor doubling time (ca. ×2) using the combination modality Radiation + Photofrin. When we used a higher concentration of Photofrin II (2 and 4 μg/ml) the DMF values after irradiation increased, too (data not shown). However, we did not continue the experiments with this concentration because the increase of cell death (radiosensitizing effect) can be caused by the combination of radiation effect and toxic effect of the porphyrin (Table 1). The different degrees of efficiency that occurred between the radiosensitizing effect in vitro and in vivo experiments may be caused by the different surrounding conditions of the cells in tissue culture and in vivo. For instance, there are no hypoxic conditions during our in vitro experiments. It could be hypothesized that the radiosensitizing effect of Photofrin is most efficient in cells with a low requirement of oxygen for their metabolism.

However, a hypoxic effect cannot be assumed for the current in vitro experiments. In our observation, only the two cell lines, known to be radioresistant in vivo, showed a radiosensitizing effect in vitro, in the presence of Photofrin II. A complete understanding of this different reaction between the two cell lines RT4 and U-373 and the HT-29 cells, respectively, is not clear to us. Recent information has shown that overexpression of the growth factor receptor EGFR is associated with radioresistance [27] may be this can explain this difference. The radiosensitizing effect of porphyrins and their derivatives received little attention, so far, in spite of reports pointing out that their efficacy on hypoxic cells in vitro is better than that of etanidazole [6]. In this connection, HpD appeared to be particularly effective. Most of the publications reported the efficacy of this mode of treatment by using HpD [5,7,9,10,24]. A detailed understanding of the mechanism involved in the radiosensitization of tumors by Photofrin is hampered, at least in part, by the highly heterogeneous chemical composition of such porphyrins. Since a number of monomer porphyrins appear to be devoid of any significant radiosensitizing activity towards cells, it has been proposed [14] that the especially high radiosensitivity exhibited by Photofrin is connected with the oligomeric porphyrin species, which are particularly abundant in Photofrin [3,4]. Such oligomers can efficiently interact [3] with some cytotoxic transient intermediates such as hydroxyl radicals, which are known to be generated as a result of the primary interaction of X-ray with water [25]. As a consequence, a large number of radical species are formed through a chain reaction within the Photofrin aggregates [3]. Thus, Photofrin would act as a radiation amplification factor. This mechanism of action has already been confirmed in the case of another porphyrin, namely, Gd-Tex, which is used as a radiosensitizer [13,26]. It can also be possible that in the presence of Photofrin the repair process of sub-lethal cell damage, after ionizing irradiation, is inhibited, thus helping in the tumor control. Further investigations have to be carried out to understand the mechanisms of the process, leading to radiosensitization effect of Photofrin.

In general, under anaerobic conditions, the radiation dose must be increased by a factor of 2.5–3 to achieve the same degree of cytotoxicity that occurs under oxygenated conditions [26]. Since hypoxic cells are substantially more resistant to radiation compared to oxygenated cells, even a small hypoxic fraction in a tumor will dominate the overall response to radiation by

increasing the probability that some viable tumor cells will survive the treatment.

The property of porphyrins and their derivatives, to be accumulated by tumor tissues in larger amounts than normal tissues—three to seven times more than normal tissues [1–4], the results of the in vivo studies [14,15] and our observed results reported above, suggest the use of Photofrin II as a tumor selective radiosensitizer, even though a direct extrapolation from in vivo experiments with animals cannot be directly extrapolated to the human situation. This choice is endowed with specific advantageous: First, Photofrin is already approved for clinical use in PDT of tumors and has no detectable toxicity in humans at the radiotherapeutically useful doses [1–4]. In contrast, it is known that several clinically utilized radiosensitizing agents show a very poor tumor selectivity and can produce severe side effects in vivo [6]. Second, and more importantly, the possibility of using the same chemical compound both as a photo- and radiosensitizing agent offers the possibility of combining the two therapeutic modalities in order to achieve more efficient tumor control.

In fact, the first clinical application of Photofrin as a tumor radiosensitizer is presently ongoing with promising results [8]. The observed side effects of the therapeutic regimen did not differ from the side effects routinely encountered with sole radiation therapy [8]. The skin photosensitization effect caused by Photofrin II may limit the use of this compound in a routine clinical application in radiation therapy. The metabolism of Photofrin in the body differs from patient to patient and depends upon a variety of factors [28].

At present, we are actively pursuing our investigations with an aim of obtaining a more precise definition of the role performed by the individual parameters in enhancing or reducing the radiosensitizing efficiency of Photofrin II, as well as in identifying specific clinical situations where the radiotherapeutic treatment with Photofrin is particularly useful.

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