



Introduction to 5-Aminolevulinic Acid-Protoporphyrin IX-Mediated Radiodynamic Therapy (RDT)

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Abstract

Photodynamic Therapy (PDT) is a light-based method that uses photo-reactive molecules, such as protoporphyrin IX (PpIX), to ablate tumors. Recently, PpIX was shown to act as a radio-reactive molecule by enhancing generation of Reactive Oxygen Species (ROS) upon X-ray irradiation. This characteristic enables radiodynamic Therapy (RDT), which uses radiation as a physical stimulus instead of light used in photodynamic therapy. The merit of RDT over PDT is the X-ray's penetrability through tissues, which will find many applications for treatment of deep cancers. In this mini-review, we discuss the potential applications of radiodynamic therapy.

Keywords: Radiotherapy; Radiodynamic therapy (RDT); 5-aminolevulinic acid (5-ALA); Protoporphyrin IX (PpIX); Photodynamic therapy (PDT)

Introduction

Surgical resection, when feasible and safe, should be performed and in many cancers stands as the first line treatment. Depending on cancer location, the role of radiotherapy is underestimated but cannot replace existing therapies. However, acute toxicity and potential long-term adverse effects often limit the dose of radiation to levels that are insufficient for controlling tumors. Radiosensitizers have the ability to specifically increase tumor radiosensitivity [1-5], and the radiosensitizing effects of *cis*-platinum and other chemotherapeutic agents have been previously reported [6-9]. Although some clinical benefits have been achieved, the use of these compounds did not significantly improve outcomes compared to radiation treatment alone.

Photodynamic therapy (PDT) is becoming a more widely accepted modality for treating solid tumors [10-11]. Topical or systemic administration of a photosensitizer leads to its accumulation and retention in tumors, and accumulated photosensitizers can be subsequently activated by a specific wavelength of light. Upon light activation, an excited photosensitizer undergoes type I (electron transfer) and/or type II (energy transfer) reactions to produce highly Reactive Oxygen Species (ROS), resulting in apoptosis and/or necrosis of exposed cells [12-13]. The most important pathways for clinical PDT are the generation of type II photochemical reactions. In these, a photosensitizer interacts with oxygen to generate ¹O₂, which is considered to be essential for PDT's ability to ablate tumors [11,13].

Since the 1950s, *in vivo* studies have shown that porphyrins can modify the effects of ionizing radiation [14,15]. Photofrin and hematoporphyrin derivatives are known to be a mixture of porphyrins formed by acetic acid-sulfuric acid treatment of hematoporphyrin. In 1981 Dougherty et al. [16] described a gel filtration procedure to isolate hematoporphyrin derivatives in relatively pure form. But being insufficiently purified, Photofrin and hematoporphyrin derivatives were chemically heterogeneous. Although porphyrins, under certain conditions, seemed to be act as radiosensitizers, the observed radiosensitizing effects were considered not due to the main components but rather to minor ones [17]. There is some contradicting evidence in the literature, because both protective and sensitizing effects have been reported for porphyrins [18]. Until recently, the radiosensitizing effects of the porphyrins have been studied under biological settings, and these studies were inconclusive.

Recently, protoporphyrin IX (PpIX) was characterized as a radio-responsive compound and shown to produce ROS upon X-ray irradiation [19]. This discovery demonstrated the use of radiation as a physical stimulus for radiodynamic therapy (RDT), instead of laser light used for photodynamic therapy (Scheme 1). RDT has already been tested, both *in vitro* and *in vivo*, and

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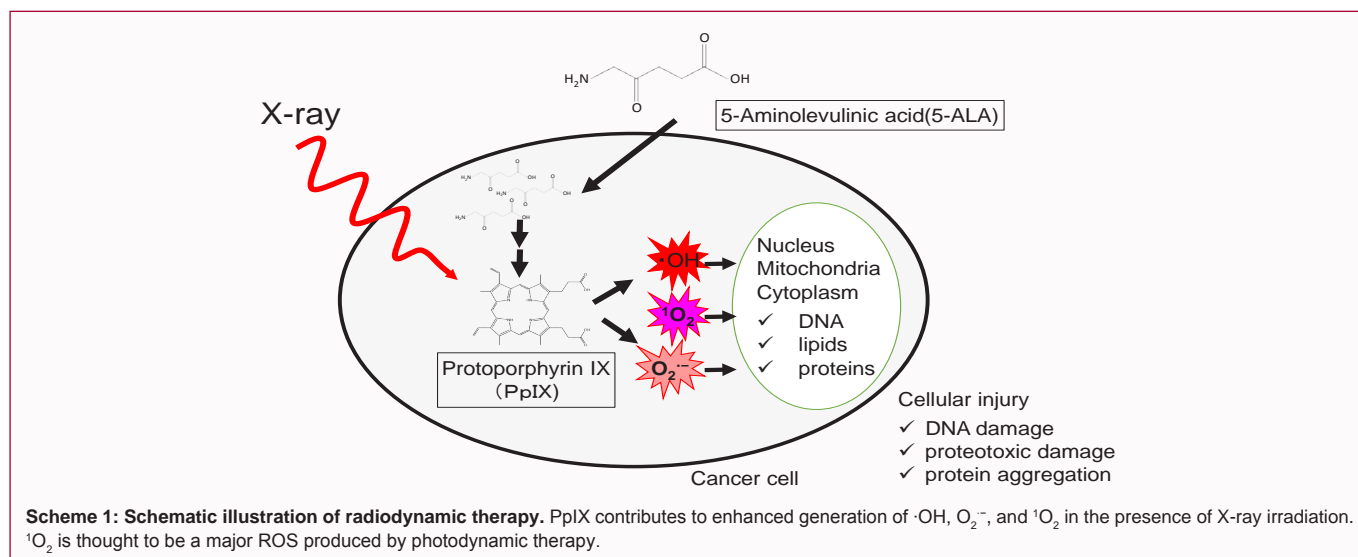
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it is currently being tested in clinical trials [20]. The merit of RDT over PDT is the X-ray's penetrability through tissues, which will find many applications for treatment of deep cancers. This review aims to describe the mechanisms which could be assumed as general for the radiosensitizers, those attributable to porphyrin-type, underlying RDT.

PpIX Produces ROS Upon X-ray Irradiation by Physicochemical Reactions

PpIX generates ROS effectively upon X-ray irradiation [19]. ROS generation was monitored using two ROS detection reagents: 2-[6-(4-amino)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) [21], and dihydroethidium (DHE) [22], with ethanol as a quencher of $\cdot\text{OH}$ in solutions containing different concentrations of PpIX. Upon X-ray irradiation, $\cdot\text{OH}$ and $^1\text{O}_2$ were detected by APF, while the generation of $\text{O}_2^{\cdot-}$ and/or $^1\text{O}_2$ was detected by DHE. While the APF reaction was quenched by the addition of ethanol, the DHE reaction was not. Upon UV irradiation, $\text{O}_2^{\cdot-}$ and/or $^1\text{O}_2$ were generated, and these results were consistent with those of previous reports [11]. These results confirmed that X-ray irradiation with PpIX enhanced generation of $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, and $^1\text{O}_2$ for potential use in RDT, as well as UV irradiation with PpIX generated $^1\text{O}_2$ and/or $\text{O}_2^{\cdot-}$ for PDT applications [19]. To study the role of PpIX in mitochondrial metabolism of H_2O_2 , Zeng et al. [23] analyzed catalytic conversion of H_2O_2 into $^1\text{O}_2$ by PpIX. In their experiment, no $^1\text{O}_2$ was produced in the presence of only PpIX without X-ray irradiation, but $^1\text{O}_2$ was produced with irradiation. The $^1\text{O}_2$ formation appeared to increase with an increase in the concentration of H_2O_2 . They might observe the reactions of PpIX and X-ray irradiation unintentionally.

Intracellular ROS Generation by X-ray Irradiation of PpIX

The effects of ROS generated by X-ray irradiation of PpIX were evaluated in cancer cells *in vitro*. Intracellular ROS production by PpIX was assessed by pre-incubating the HeLa cells with cell-permeable fluorescent probes (APF or DHE) with different sensitivities to specific ROS species ($\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, and $^1\text{O}_2$). ROS levels increased with increasing X-ray doses and intracellular PpIX concentrations, according to both APF and DHE detection systems [24]. Gene expression profiling was performed using DNA microarray. Differences in gene expression were observed in HeLa cells treated with PpIX or X-rays only, and

cells treated with PpIX and X-ray radiation together. PpIX and X-ray irradiation treatment together induced systematic changes in the expression of genes related to cell-cycle arrest and inhibition of DNA replication. Treatment PpIX and X-ray irradiation together resulted in higher numbers of genes with altered expression profiles relative to control than treatment with X-ray irradiation only; however, the qualitative gene expression remained the same between the two conditions. These results suggest a complex mechanism by which PpIX enhances ROS generation during application of X-rays. As a result, cell viability decreased by inducing DNA damage and cell-cycle arrest.

Yamamoto et al. [25] showed that intracellular PpIX induced by 5-aminolevulinic acid (5-ALA) plays an important role in radiosensitization upon ionizing irradiation. Intracellular ROS generated by ionizing radiation or heme content in glioma 9L cells was detected by dichlorofluorescein (DCF) fluorescence using the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (DCFDA). Oxidation of DCFH is known to be not always related the generation of ROS but may be related heme content in cells [26,27]. Pretreating cells with 5-ALA before irradiation resulted in increased DCF fluorescence. Interestingly, the DCF fluorescence generated by ionizing irradiation predominantly coincided with 5-ALA-induced PpIX accumulation in the cytoplasm of 9L cells [25]. Intracellular ROS levels were significantly higher in 9L cells 12 h after applying ionizing irradiation than in those measured immediately after applying ionizing irradiation, and 5-ALA pretreatment strongly enhanced ROS levels [28].

PpIX Enhances Cancer Radiotherapy in a Tumor Model

To evaluate the tumor-suppressive effects of PpIX as a radiosensitizer, a B16-BL6 tumor model was established in C57BL/6 J mice [29]. Porphyrin accumulation in implanted B16-BL6 tumors 24 h after a topical dose of 50 mg/kg 5-ALA administration was 6.2 times higher than with systematic, local administration providing efficiencies equal or surpassing those of PDT ($3.0 \pm 1.4 \mu\text{g}$ per gram wet weight). Using a protocol in which ALA was administered immediately after X-ray irradiation for convenient preparation for the next irradiation, tumor suppression significantly improved in animals treated with 5-ALA and fractionated doses of X-ray irradiation ($3\text{Gy} \times 10$; total, 30 Gy). Microarray analyses of tumor tissues collected

after 10 sessions of fractionated irradiation co-treated with or without 5-ALA revealed that the majority of dysregulated genes were related to cell-cycle arrest. Co-treatment with 5-ALA affected the level of expression but not the pattern of gene expression [30]. These results suggest that irradiation treatment leads to PpIX-mediated ROS generation. Yamamoto et al. [31] also showed that 5-ALA-induced generation of PpIX upon fractionated X-ray dosing (2 Gy \times 5; total, 10 Gy) enhances the antitumor response and strongly inhibits growth of 9L gliomas in rats.

Other Mechanisms Underlying Radiosensitization by Porphyrins

Lemay et al. showed substituting cationic, N-propyl porphyrins with bromines onto the propyl side-chains substantially enhances the radiosensitizing potency of the parent compound. As a similar radiosensitizing effect was detected for different radiation energies, they thought that the high energy photons could be used to treat tumors in conjunction with the radiosensitizer [32].

Porfimer sodium, sold as Photofrin, and hematoporphyrin are used as photosensitizers in PDT, and also thought to be radiosensitizers [17,33-36]. Schaffer et al. [33] showed that Photofrin significantly improved the tumor responses to 5 Gy and 15 Gy radiation doses in mice implanted subcutaneously with human bladder cancer RT4 cells. Two cell lines, RT4 and U-373 MG, treated with Photofrin before radiation had lower survival than untreated cells, which were treated with irradiation only under identical conditions [34]. Luksiene et al. [17] examined the radiosensitizing properties of three porphyrin-type compounds: hematoporphyrin dimethyl ether, Photofrin, and hematoporphyrin derivatives, and found that hematoporphyrin dimethyl ether was the most effective of the three (hematoporphyrin dimethyl ether > Photofrin > hematoporphyrin derivatives). The authors showed that only aggressive Ehrlich Ascites Carcinomas (EAC) were radiosensitized by porphyrins, and ligands of peripheral benzodiazepine receptors (PBR) may diminish cell growth in aggressive EAC. They thought that porphyrins, being ligands of PBR, which are highly expressed in aggressive tumors only, can inhibit tumor cell proliferation and act in concert with ionizing radiation [17]. Benayoun et al. [35] showed that Photofrin treatment radiosensitizes the tumor-initiating U-87MG cells derived from human glioblastoma and improves outcomes when combined with radiotherapy *in vitro* and *in vivo*. However, the corresponding mechanisms of action of Photofrin or its derivatives remain unknown. Lam et al. [36] found that PDT treatment before external-beam radiotherapy improved survival in a randomized comparative analysis of the safety and efficacy of PDT, using Photofrin combined with palliative radiotherapy in patients with obstructive endobronchial tumors. In this case, Photofrin was used as a photosensitizer, not as a radiosensitizer, trial studies combining photosensitizers and radiotherapy in patients are underway. If X-ray irradiation were done during photosensitizer accumulated in tumor, it might act as radiosensitizer unintentionally.

In the aspect of radiosensitizing effects to cells, the complexity of the radiosensitizing activity of PpIX to the essential biological factors must be considered including conditions occurring in each systems. Sailer et al. [37] showed that the different PDT efficacy of 5-ALA induced PpIX was related to the different intercellular location of glioblastoma, breast cancer and ovarian cancer cells. It is known that the cytotoxic ROS, singlet oxygen or superoxide radicals, react with various biomolecules (e.g. proteins) and caused cell damages in PDT.

Maitra et al. [38] showed that external and internal porphyrinogenic stress caused proteotoxic damage and protein aggregation. On the other hand, X-ray induced $\cdot\text{OH}$ caused DNA degradation, and PpIX enhance generation of $\cdot\text{OH}$ in addition to $^1\text{O}_2$, O_2^- , cytotoxic effect of RDT might be DNA and protein damage.

The modification of spectral properties of PpIX were monitored as the specific indicators of the PDT effect [39,40]. Dysart and Patterson showed that fluorescence photobleaching of ALA induced PpIX exhibited complex photobleaching kinetics *in vitro*, likely resulting from differential binding of the sensitizer within the cell [39]. In RDT, the changes in absorption spectrum and fluorescence emission spectrum of PpIX were observed with the increase of X-ray irradiation dose [30].

The essential questions concerning to the enhanced ROS generation still remained. What is the mechanism of the enhanced production of ROS in the case of PpIX exposed to X-rays? How efficient is the enhancement in comparison with the standard PDT procedure? Those studies will be essential along with that PDT is more accepted as the cancer therapy.

Conclusion

PDT, which uses porphyrins as tumor-targeting and photosensitizing drugs, has emerged as a promising alternative therapeutic modality for treating early and localized tumors. Thick tumors cannot yet be treated by this method because of limited radiation penetration into the tissue. Recently, PpIX was characterized as a radio-responsive compound and was shown to enhance generation of ROS, including $^1\text{O}_2$, O_2^- , and $\cdot\text{OH}$, by X-ray irradiation. These findings suggest that RDT can be used instead of chemotherapy or surgery. Further studies and clinical trials are needed to establish the proper and practicable procedures for optimizing RDT.

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