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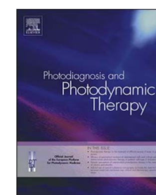

Photodynamic process induced by chloro-aluminum phthalocyanine nanoemulsion in glioblastoma

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A R T I C L E I N F O

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A B S T R A C T

Background: Glioblastoma multiforme (GBM) is a tumor characterized by rapid cell proliferation and migration. GBM constitutes the most aggressive and deadly type of brain tumor and is classified into several subtypes that show high resistance to conventional therapies. There are currently no curative treatments for malignant glioma despite the numerous advances in surgical techniques, radiotherapy, and chemotherapy. Therefore, alternative approaches are required to improve GBM treatment.

Methods: Our study proposes the use of photodynamic therapy (PDT) for GBM treatment, which uses chloro-aluminum phthalocyanine (AlClPc) encapsulated in a new drug delivery system (DDS) and designed as a nanoemulsion (AlClPc/NE). The optimal dark non-cytotoxic AlClPc/NE concentration for the U87 MG glioma cell model and the most suitable laser light intensity for irradiation were determined. Experimental U87 MG cancer cells were analyzed via MTT cell viability assay. Cellular localization of AlClPc, morphological changes, and cell death via the necrotic and apoptotic pathways were also evaluated.

Results: AlClPc remained in the cytoplasmic region at 24 h after administration. Additionally, treatment with 1.0 μmol/L AlClPc under light irradiation at doses lower than 140 mJ/cm resulted in morphological changes with 50 ± 6% cell death ($p < 0.05$). Moreover, 20 ± 2% of U87 MG cells underwent cell death via the necrotic pathway. Measurement of Caspase-9 and -3 activities also suggested that cells underwent apoptosis. Taken together, these results indicate that AlClPc/NE-PDT can be used in the treatment of glioblastoma by inducing necrotic and apoptotic cell death.

Conclusions: Our findings suggest that AlClPc/NE-PDT induces cell death in U87 MG cells in a dose-dependent manner and could thus serve as an effective adjuvant treatment for malignant glioma. AlClPc/NE-PDT utilizes a low dose of visible light and can be used in combination with other classic GBM treatment approaches, such as a combination of chemotherapy and surgery.

1. Introduction

Glial tumors, generally known as glioma, represent the most prevalent neoplastic disease of the central nervous system (CNS) in adults. According to the 2007 report by the World Health Organization (WHO), glial tumors are mainly divided into astrocytic tumors, oligodendroglial tumors, oligoastrocytic tumors, ependymal tumors, and neuronal and mixed neuronal-glial tumors. These groups are classified into four grades, namely, pilocytic astrocytoma (grade I), low-grade astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (GBM) (grade IV). Higher tumor grade is associated with increased malignancy and aggressiveness. GBM tumors arise from astrocytes, oligodendrocytes, and their precursors [1] and can be classified into several groups according to histopathological characteristics [2,3]. Glioblastomas account for 60%–70% of all gliomas [3]. In particular, glioblastoma multiforme (GBM) grade IV (WHO classification) is the most common histological type of brain tumor [4] and is further classified into several other subtypes of primary tumors, including giant cell GBM, gliosarcoma, and the most recently identified variant epithelioid GBM [1]. GBM is characterized by aggressive characteristics, such as high proliferation and migration rates [5,6]. The annual worldwide incidence of malignant glioma is 5 out of 100,000 individuals [7]. Brain cancer is the second most common cause of death in pediatric cancer patients [8].

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The median survival of patients with malignant glioma is only 12–15 months, even after surgery, radiotherapy, and chemotherapy [temozolomide and PCV (procarbazine, lomustine, and vincristine)], with a high index of recurrence a few months after surgical removal. There are several GBM subtypes based on the GBM subclassification that exhibit high resistance to conventional therapies and thus require the development of alternative treatment approaches [1]. At present, there are several effective treatments for newly diagnosed GBM patients, such as maximum safe resection (class 2 evidence) [49,54,55], radiotherapy (typically 60 Gray, given in 30 fractions – class 1 evidence) [50,52,54], temozolomide (class 2 evidence) [51], and combined radiochemotherapy and adjuvant temozolomide (class 2b evidence), which are administered to the patients for at least 6 months [52,53]. However, there is currently no curative treatment for GBM despite numerous advances in surgical techniques, radiotherapy, and chemotherapy. For instance, maximum surgical resection and concurrent chemoradiation increase mean survival by only up to 16 months.

In this regard, there is a high demand for novel, locally targeted therapeutic approaches. Photodynamic therapy (PDT) is a safe and powerful locally targeted technique that has demonstrated potential as an alternative oncological therapy [9]. PDT should be used in combination with classical treatment to increase the security margin after surgery by eradicating any remaining cancer cells present in the area surrounding the excised tumor.

PDT is a promising treatment for cancer and non-oncological diseases [10–13]. PDT works by combining a photosensitizer drug (organic dye) with irradiation using visible light at an appropriate wavelength and molecular oxygen, which are individually harmless. The photodynamic effects that occur after absorption of visible light leads to the production of reactive oxygen species (ROS), which act as cytotoxic agents that can inactivate tumor cells. The photosensitizer drug is preferentially absorbed by diseased tissue, and light irradiation is limited to a specific region. This mechanism confers dual selectivity to the PDT technique, such that tumor cells are selectively destroyed, whereas healthy cells remain intact [14–16]. Hematoporphyrins are the first generation of photosensitizer drugs applied in clinical PDT procedures. To increase PDT efficacy, researchers have developed the second generation of photosensitizer drugs, with phthalocyanine being the most recognized drug, which act by improving the penetration into diseased tissues [15,9]. The past decade has seen a drastic increase in the use of photosensitizer drugs in various research studies and pre-clinical applications.

Chloro-aluminum phthalocyanine (AlClPc) is a chemically stable second-generation photosensitizer that displays photophysical properties that are highly suitable for PDT, such as high triplet quantum yields and long triplet lifetime [17]. Although AlClPc has high molecular weight, its hydrophobic nature facilitates interaction with bilipid layers of cells. AlClPc-PDT has been successfully used in cancer models and has afforded a relevant safety margin in clinical trials [18–20].

To test whether new therapeutic strategies can significantly track and destroy malignant glioma cells *in vitro*, we investigated the photodynamic activity of AlClPc loaded into an oil/water nanoemulsion (AlClPc/NE) and its effect on glioma cell death. The AlClPc/NE combination was selected as loading material because AlClPc is a hydrophobic photosensitizer drug [17] and oil/water NE can potentially deliver poorly soluble drugs to the target site [21]. Results showed that a low AlClPc/NE concentration combined with irradiation with low intensity light can effectively kill U87 MG glioma cells. After 2 h of incubation, AlClPc was observed to remain in the cytoplasmic region of the U87 MG cells. Furthermore, AlClPc/NE-PDT induced cell death via both necrosis and apoptosis. These findings highlight the potential of PDT in combination with classical approaches in the treatment of glioma. Our results showed that AlClPc/NE-PDT treatment promotes cell death in U87 MG cells in a dose-dependent manner. This strategy could thus serve as an effective adjuvant treatment for malignant glioma using low doses of visible light and can be used along with other classical treatment protocols, such as the combination of chemotherapy and surgery.

2. Materials and methods

2.1. Cells and cell culture conditions

The glioblastoma grade IV cell line, U87 MG, was obtained from the American Type Culture Collection (ATCC® HTB-14™). U87 MG cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. Cells in the logarithmic growth phase were cultured in a humidified incubator at 37 °C with 5% CO_2 atmosphere.

2.2. Dark cytotoxicity assay of AlClPc nanoemulsion

AlClPc/NE treatment was carried out based on the drug entrapment in the spontaneous emulsification process, followed by solvent extraction at reduced pressure as previously described by Primo et al. [11]. The AlClPc/NE nanoemulsion is a type of oil-in-water (o/a) mixture prepared via a spontaneous emulsification process. The organic phase containing acetone was prepared using natural soy phospholipids and AlClPc incubated at 55 °C. The resulting organic solution was added to the aqueous phase containing poloxamer 188 as an anionic surfactant under magnetic stirring. In the final step, the organic solvent was removed by evaporation under reduced pressure at 60 °C. To evaluate the intrinsic cytotoxic effects of the AlClPc/NE, U87 MG cells were seeded at 1×10^4 cells/well in a 96-well plate. After 24 h, U87 MG cells were incubated with a mixture of fresh medium and AlClPc/NE to final concentrations of 0.5, 1.0, 5.0, and 10.0 μmol/L and subsequently incubated at 37 °C with 5% CO_2 for 3 h in the dark. After incubation, the medium containing the nanoemulsion dispersion was discarded. Cells were washed twice with sodium phosphate-buffered saline (PBS) and then incubated in fresh medium for an additional 24 h until the cell viability assay (MTT). Control cells were incubated with culture medium alone (untreated cells) or unloaded nanoemulsion mixed with the medium in the dark. Experiments were carried out in triplicate, and sixteen wells were used for each AlClPc/NE concentration.

2.3. Photocytotoxicity assay of the AlClPc nanoemulsion using the human glioblastoma cell line U87 MG

To evaluate the phototoxic effects of the AlClPc/NE, U87 MG cells were seeded at 1×10^5 cells/well in 24-well plates. After 24 h, cells were incubated for 3 h in medium containing 1 μmol/L AlClPc/NE at 37 °C under 5% CO_2 atmosphere. Following incubation, the medium containing the nanoemulsion was removed, and cells were rinsed twice with 1 × PBS. For the photodynamic analysis, fresh medium without phenol red was added, and the cells were irradiated with a diode Eagle laser (Quantum Tech, São Carlos, Brasil). All the laser irradiation procedures were performed under the following conditions: wavelength of 650 nm, average power set to 100 mW and light irradiance, 14 mW/cm^2. Cells were exposed to light doses of 70, 140, and 250 mJ/cm^2. After irradiation, the colorless medium was removed, and cells were incubated at 37 °C under 5% CO_2 in fresh medium for an additional 24 h until the MTT assay. Control cells were incubated with culture medium alone without the nanoemulsion and light irradiation or with AlClPc/NE mixed with medium (nanoemulsion control). Assays were carried out in triplicate, and twelve wells were used for each light dose.

2.4. AlClPc-PDT treatment of U87 MG cells

To investigate the effect of AlClPc/NE-PDT on U87 MG cells *in vitro*, cells were seeded at a density of 5×10^5 cells/well in 6-well plates. U87 MG cells were then treated with medium containing 1 μmol/L

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