Photodiagnosis and Photodynamic Therapy 13 (2016) 130–138

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Assessment of the specificity of a new folate-targeted photosensitizer for peritoneal metastasis of epithelial ovarian cancer to enable intraperitoneal photodynamic therapy. A preclinical study

Henri Azaïs[a,b,*], Caroline Schmitt[c], Meryem Tardivel[d], Olivier Kerdraon[e], Aurélie Stallivieri[f], Céline Frochot[f], Nacim Betrouni[a], Pierre Collinet[a,b], Serge Mordon[a]

[a] Univ. Lille, Inserm, CHU Lille, U1189 – ONCO-THAI – Image Assisted Laser Therapy for Oncology, F-59000 Lille, France
[b] Department of Gynecology, University of Lille, 59000 Lille, France
[c] Centre Français des Porphyries, Assistance Publique – Hôpitaux de Paris, Hôpital Louis Mourier, Colombes, France and INSERM U1149, Paris Diderot University, 75018 Paris, France
[d] Plate-Forme D'imagerie Cellulaire BICEL – IFR 114, Pôle Recherche, University of Lille, 59000 Lille, France
[e] Centre de Biologie-Pathologie, University of Lille, 59000 Lille, France
[f] Laboratoire Réactions et Génie des Procédés, UMR 7274CNRS – University of Lorraine, France

ARTICLE INFO

Article history:
Received 5 March 2015
Received in revised form 29 May 2015
Accepted 14 July 2015
Available online 19 July 2015

Keywords:
Folate receptor
Targeted-therapy
Photodynamic therapy
Ovarian peritoneal carcinomatosis
Photosensitizer

ABSTRACT

Background: Ovarian cancer's prognosis remains dire after primary therapy. Recurrence rate is disappointingly high as 60% of women with epithelial ovarian cancer considered in remission will develop recurrent disease within 5 years. Special attention to undetected peritoneal metastasis during surgery is necessary as they are the main predictive factors of recurrences. Folate Receptor α (FRα) shows promising prospects in targeting ovarian cancerous cells and intraperitoneal photodynamic therapy (PDT) could be a solution in addition to macroscopic cytoreductive surgery to treat peritoneal micrometastasis. The aim of this preclinical study is to assess the specificity of a folate-targeted photosensitizer for ovarian peritoneal micrometastasis.
Methods: We used the NuTu-19 epithelial ovarian cancer cell line to induce peritoneal carcinomatosis in female Fischer 344 rats. Three groups of 6 rats were studied (Control (no photosensitizer)/Non-conjugated photosensitizer (Porph)/Folate-conjugated photosensitizer (Porph-s-FA)). Four hours after the administration of the photosensitizer, animals were sacrificed and intraperitoneal organs tissues were sampled. FRα tissue expression was evaluated by immunohistochemistry. Tissue incorporation of photosensitizers was assessed by confocal microscopy and tissue quantification.
Results: FRα is overexpressed in tumor, ovary, and liver whereas, peritoneum, colon, small intestine, and kidney do not express it. Cytoplasmic red endocytosis vesicles observed by confocal microscopy are well correlated to FRα tissue expression. Photosensitizer tissue quantification shows a mean tumor-to-normal tissue ratio of 9.6.
Conclusion: We demonstrated that this new generation folate-targeted photosensitizer is specific of epithelial ovarian peritoneal metastasis and may allow the development of efficient and safe intraperitoneal PDT procedure.

1. Introduction

The prognosis of advanced epithelial ovarian cancer remains poor. The current treatment is the association of complete macroscopic cytoreductive surgery and platinum-based chemotherapy. It is accepted that the absence of residual disease after surgery is a key to improve prognosis [1,2]. 60% of patients treated by complete cytoreductive surgery and chemotherapy will develop peritoneal recurrence [3].

Peritoneal cavity must be oncologically considered as an organ in its own right [4] and the surgeon must treat peritoneal metastasis with a curative intent. Our aim is to provide another option to improve the completion of cytoreductive surgery by inducing necrosis of micrometastasis by targeted photodynamic therapy (PDT) and so to decrease the incidence of recurrence. PDT is a non-

* Corresponding author at: INSERM U1189 – ONCO-THAI, University of Lille 1, Avenue Oscar Lambret, 59037 Lille, France.
E-mail address: henriazais@gmail.com (H. Azaïs).

http://dx.doi.org/10.1016/j.pdpdt.2015.07.005
1572-1000/

invasive ways of treating malignant tumors that is clinically applied in wide range of medical conditions. In the photodynamic reaction, after ligth absorption, the photosensitizer (PS) is promoted to an excited singlet state and then to a triplet state that transfers its energy to oxygen leading to the production of highly toxic reactive oxygen species, such as singlet oxygen. The selectivity of the treatment is brought by light, the photosensizer being active only after irradiation. A limit of this technique is the toxicity induced by the low PS specificity for tumor tissue if the light cannot be specifically applied. This would be the case in peritoneal metastasis in advanced ovarian cancer. To solve this problem, a solution is the design of selective PS, that is to say PS coupled to an unit that target over-expressed receptors on tumoral cells.

Folate receptor alpha (FRα) is a promising target as it is highly specific of epithelial ovarian cancer [5,6]. It is a well-known tumor associated protein that can actively internalize bound folates *via* endocytosis allowing delivering high concentrations of cytotoxic agent to tumor expressing high levels of the folate receptor [7]. In previous studies we already demonstrated the potential of folate-targeted porphyrin or chlorin *in vitro* and *in vivo* to improve the selectivity of the PS and the improvement of PDT efficiency [8,9]. Our objective is to assess the specificity of a new generation photosensitizer (*N*-{2-[2-(2-aminoethoxy)ethoxy]ethyl}folic acid}-4-carboxyphenylporphyrin) targeted by folate (called **Porph-s-FA**) for ovarian peritoneal metastasis in a preclinical study.

2. Material and methods

2.1. Preclinical model of peritoneal carcinomatosis (ovarian cancer)

Pathogen-free Fischer 344 female rats (140–160 g) were obtained from HARLAN Laboratories (Gannat, France). They were housed in a pathogen-free animal facility and given commercial basal diet and water *ad libitum*. The protocol was approved by our animal use and ethic committee (DHURE—Departement Hospitalo-Universitaire de Recherche Expérimentale), University of Lille, France (n° = 59-350120).

NuTu-19 is a syngeneic adenocarcinoma used to develop ovarian cancer in an immunocompetent rat model [10]. It is a poorly differentiated adenocarcinoma originally derived from a female athymic mouse after injection of Fischer 344 ovarian surface epithelial cells that spontaneously underwent malignant transformation *in vitro*.

NuTu-19 cells were cultured in DMEM (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin 1% and streptomycin 1%. Cell culture plates were incubated under standardized conditions (5% carbon dioxide, 100% humidity, 37 °C). The cell line was cryopreserved in liquid nitrogen (10^7 cells/vial). Each experiment was performed by thawing a vial of cells and expanding them biweekly to provide the appropriate cell number. After harvesting the NuTu-19 cells with 0.25% trypsin (Gibco Life Technologies™, NY, USA), they were washed with Dulbecco's phosphate-buffered saline (PBS, Gibco Life Technologies™, NY, USA) and counted. Viability was testing using trypan blue exclusion. With a minimum of 90% survival, 10^6 cells ml^{-1} PBS were injected intraperitoneally in the Fischer rats. Small nodules up to 2 mm form along the peritoneal lining of the abdominal wall, pelvis, and omentum with haemorrhagic ascites. This preclinical model enables to study folate-targeted therapy [11].

2.2. Photosensitizers

5-(4-Carboxyphenyl)-10,15,20-triphenylporphyrin (**Porph**) and {*N*-{2-[2-(2-aminoethoxy)ethoxy]ethyl}folic acid}-4-carboxyphenylporphyrin called **Porph-s-FA** (Fig. 1) were provided by a French research laboratory (Laboratoire Réactions et Génie des Procédés, UMR 7274CNRS—University of Lorraine, France) [9] and were stored in powder form and kept refrigerated. Synthesis of this compounds have been described previously [9]. Samples were prepared immediately prior to use by dissolution of the powder in 200 μl of DMSO first and then in phosphate buffered saline (PBS), and were protected from light exposure.

2.3. Tissue sampling protocol

18 Rats were divided into three groups:

(1) Control. No photosensitizer injected (n = 6).
(2) Injection of 5-(4-carboxyphenyl)-10,15,20-triphenylporphyrin (**Porph**) (n = 6).
(3) Injection of {*N*-{2-[2-(2-aminoethoxy) ethoxy]ethyl}folic acid}-4-carboxyphenylporphyrin (**Porph-s-FA**) (n = 6).

6–8 Weeks, after peritoneal injection of NuTu-19 cells, the PS was administered. Each animal received an intraperitoneal injection of 3×10^{-6} mol/kg of **Porph** (Group 2, 1.8 mg/kg) or **Porph-s-FA** (Group 3, 3.6 mg/kg). Animals from Group 1 did not receive any injection. Animals were sheltered from light prior tissue sampling. 4 h after administration, according to Gravier et al. [8], they were anesthetized with isofluran and placed in the supine position to perform a precise surgical examination of the peritoneal cavity. Then, they were sacrificed by blood depletion, and sampling of the tissues (liver, kidney, colon, small intestine, ovary, peritoneum, and tumor) was immediately performed under dim light. Peritoneal fluid was carefully wiped off the tissues and feces were evacuated from the intestine by gentle pressure.

Samples for PS tissue quantification were dropped into liquid nitrogen immediately after removal and then conserved at −80 °C. Samples for histology analysis and confocal microscopy were dropped into formaldehyde solution for 24 h and then into ethanol 70% before paraffin inclusion. All tissue samples were individually covered in aluminum to protect them from light exposure.

2.4. FRα tissue expression by immunohistochemistry

Tissue expression of FRα was determined for several organs of the peritoneal cavity. Two primary antibodies (Santa Cruz Biotechnology™ and Antibodies-online™) were previously tested and adjusted on human high grade serous ovarian carcinoma used as a positive control. Optimal staining was obtained with Santa Cruz Antibody™.

Tissue sections were fixed on glass slides. They were deparaffinised with xylene, rehydrated through a graded series of ethanol and rinsed in distilled water. Antigen retrieval was achieved by heating in EDTA buffer solution pH8 for 9 min at 98 °C. Slides were cooled down to room temperature and rinsed with PBS. After blocking endogenous peroxidase in 3% H_2O_2 in PBS for 15 min, sections were pre-treated by avidin/biotin reagents to reduce non-specific background. Sections were incubated with the primary antibody FL-257 (Santa Cruz Antibody™) for 60 min in a 1:1000 dilution at room temperature. After washing with PBS, sections were incubated with secondary biotinylated anti-rabbit antibody. Peroxidase activity was visualized with 3,3-diaminobenzidine and slides were counterstained with haematoxylin, mounted with mounting medium and coverslips were added. All histology has been assessed by a senior pathologist.