Journal of Photochemistry and Photobiology B: Biology 138 (2014) 80–91

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

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

Metformin associated with photodynamic therapy – A novel oncological direction

Iuliana Nenu [a,1], Tiberiu Popescu [a,1], Mihaela D. Aldea [a], Lucian Craciun [a], Diana Olteanu [a], Corina Tatomir [b], Pompei Bolfa [c,d], Rodica M. Ion [e], Adriana Muresan [a], Adriana G. Filip [a,*]

[a] Department of Physiology, "Iuliu Hatieganu" University of Medicine and Pharmacy, 1 Clinicilor Street, 400006 Cluj-Napoca, Romania
[b] Departments of Radiobiology and Tumor Biology, Oncology Institute "Prof. I. Chiricuta", 34-36 Republicii Street, 400015 Cluj-Napoca, Romania
[c] Department of Pathology, Cluj-Napoca, University of Agricultural Sciences and Veterinary Medicine, 3-5 Calea Manastur, 400372 Cluj-Napoca, Romania
[d] Department of Biomedical Sciences, Ross University School of Veterinary Medicine Basseterre, PO Box 334, Saint Kitts and Nevis
[e] National Research & Development Institute for Chemistry and Petrochemistry ICECHIM 202 Splaiul Independentei, 060021 Bucharest, Romania

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 15 April 2014
Accepted 28 April 2014
Available online 21 May 2014

Keywords:
Metformin
Photodynamic therapy
TSPP
Cancer

ABSTRACT

The aim of our study was to assess the effect of the combined treatment of Metformin (Metf) and 5, 10, 15, 20-tetra-sulfophenyl-porphyrin (TSPP)–mediated photodynamic therapy (PDT) on an *in vivo* tumour model. Wistar male rats were divided in 6 groups: group 1, treated with TSPP; groups 2 and 4 treated with TSPP and Metf, respectively, and irradiated 24 h thereafter; group 3 was treated with Metf and the last two groups received the combined treatment, Metf administered prior (group 5) or after (group 6) irradiation. 72 h from the start of the treatment, tumour tissue was sampled for the investigation of oxidative and nitrosative stress. The apoptotic rate, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expressions and matrix metalloproteinases activities were also quantified. Malondialdehyde and glutathione levels were significantly elevated in the groups treated with combined therapy ($p < 0.05$). Metf associated with TSPP–PDT reduced iNOS and COX-2 expressions and enhanced nitrotyrosine levels in both therapeutic regimens. Peroxynitrate formation and its cytotoxic effect on tumour cells were related to an elevated index of apoptosis and necrosis. Moreover, MMP-2 activity reached a minimum in the groups which received combined therapy. Our results confirmed that the association of Metf with PDT might prove a new and promising oncological approach.

1. Introduction

Along with cardiovascular diseases, obesity and HIV infection, cancer has become nowadays a worldwide concern. Because of its increased frequency and high mortality rate, scientists are struggling to find more effective prevention methods and newer therapeutic means. This is the reason why recent research aim at developing various pharmacological schemes, discovering and improving new therapies and also at studying possible immunological and genetic approaches.

Several studies have outlined lately a close connection between type two diabetes mellitus and the incidence of different cancers (e.g. colorectal, breast, pancreatic, hepatic, endometrial and renal cancer) and have linked the incriminated mitogenic effect to obesity, dyslipidemia and hypertension, all hallmarks of the metabolic syndrome [1].

Treating diabetes mellitus seems to have preceded understanding the disease itself, as ancient Egyptians used *Galega officinalis* (also known as the "French lilac") as the source of an active substance called guanidine for ameliorating frequent urination (polyuria) and halitosis (sweet odour on breath) in type two diabetes mellitus (T2DM) [2]. However, it was not until the 1920s that scientists succeeded in linking two guanidine rings and developing the drug Metformin (Metf), which was later approved as an antihyperglycemic drug [3]. Nowadays, Metformin has become the most prescribed oral antidiabetic drug due to its ability to sensitize tissues to insulin and its consequent glucose lowering effect. Moreover, it is one of the most studied drugs in oncological research due to retrospective studies that revealed a decreased cancer incidence and cancer-related mortality in obese and diabetic patients treated with it. Several studies have demonstrated that Metf activates the adenosine monophosphate kinase (AMPK) pathway and induces phosphorylation of different intracellular proteins, fact that further

* Corresponding author. Address: Department of Physiology, 1-3 Clinicilor Street, Cluj-Napoca, Romania. Tel.: +40 745 268704; fax: +40 264 597257.
E-mail addresses: gabriela.filip@umfluj.ro, adrianafilip33@yahoo.com (A.G. Filip).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jphotobiol.2014.04.027
1011-1344/

leads to an increased apoptotic response and a decreased proliferation rate in tumoural cells [4]. In addition, *in vitro* studies revealed a reduced proliferation rate of several tumoural cell-lines after administration of Metf, either alone or as an adjuvant to chemotherapy [5]. The effect is remarkable not only against non-stem tumoural cells but most importantly against tumoural stem cells [6].

As an attempt to improve the outcome of anticancer therapy, our study proposes for the first time the association of Metf with a non-invasive oncological approach – photodynamic therapy. Greeks were among the first to acknowledge the curative effects of light and named this treatment heliotherapy. However, scientists refined and redefined this concept as photodynamic therapy (PDT). Modern PDT is based on three complementary components: visible light of a specific wavelength, an inert photosensitiser (PS) and oxygen, which leads to the formation of reactive oxygen species (ROS) with consequent cytotoxic effects on tumoural cells [7].

Several current clinical trials propose as possible PSs different compounds such as 5-aminolevulinic acid (5-ALA), its methylester or meso-tetra-hydroxylphenyl-chlorin (mTHPC). Although the action of activated porphyrins is important in clinical therapies there are some inconveniences that justify the permanent struggle for searching for an ideal PS. This has to be a PS that must accumulate in the tumour sufficiently enough so that it would generate large quantities of singlet oxygen that will radically cure it and 5, 10, 15, 20-tetra-sulfophenyl-porphyrin (TSPP) might be such a PS. Owing to its aggregation properties, which facilitate the distribution in tissues and raise the quantity of generated ROS [8], this synthetic porphyrin has demonstrated its efficiency on both *in vitro* and *in vivo* tumour models.

In order to enhance the antitumoural response and to target different mechanisms involved in cellular death, our study proposes the association of the antidiabetic drug Metf with TSPP mediated-PDT on an *in vivo* experimental model. The effects of this combined therapeutic regimen were evaluated by determination of oxidative and nitrosative stress parameters, matrix metalloproteinases activities (MMPs), inducible nitric oxide synthase and cyclooxigenase-2 expressions and immunohistochemically quantification of apoptosis in correlation with the histopathological findings in the tumour.

2. Materials and methods

2.1. Reagents

TSPP was obtained from Mrs. Rodica-Mariana Ion from the National Institute of R&D for Chemistry and Petrochemistry – ICECHIM, Bucharest, Romania (Fig. 1a). Firstly, the meso-tetra(4)-phenyl porphyrin (TPP) was synthesized from pyrrole and benzaldehyde in a reaction medium of propionic acid which was then purified with 2,3-dichloro-5,6-dicyanoquinone. To synthesize the tetra-sulpho-phenyl-porphyrin (TSPP), TPP were dissolved in fuming H_2SO_4 (30% free SO_3, heated at 85 °C and stirred). The resulting dark green precipitate was filtered and washed with HCl, then redissolved in NaOH and re-filtered from the water insoluble impurities. The filtrate was then neutralized with HCl, analysed by HPLC and stored at 0 °C till purification with chromatography [9].

2-Thiobarbituric acid, 2,4-dinitrophenyl-hydrazine and guanidine hydrochloride were obtained from Merck KgA Darmstadt (Germany). Metformin (Fig. 1b), sodium dodecyl sulphate (SDS), Tris, Triton X-100, Coomassie brilliant blue R-250, NADPH-dependent nitrate reductase, β-NADPH, N-(1-naphthyl) ethylenediamine hydrochloride, sulfanil-amide, trichloroacetic acid, Bradford reagent and o-phthalaldehyde were purchased from Sigma–Aldrich Chemicals GmbH (Germany), while absolute ethanol and *n*-butanol were provided by Chimopar (Bucharest).

The TUNEL kit used to quantify apoptosis was supplied by Roche Applied Science, Mannheim (Germany), whereas the ELISA test for evaluation of nitrotyrosine levels was obtained from Blue Gene (China). Goat polyclonal IgG antibody for COX-2 and secondary antibody mouse anti-goat and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rabbit polyclonal iNOS antibody from Thermo-Scientific (Germany).

2.2. Experimental design

The experimental design included 6 groups ($n = 48$) of Wistar male rats (180 ± 20 g, 3 months old) bearing Walker 256 carcinosarcoma: group 1 received TSPP 10 mg/kg b.w. (TSPP), groups 2 and 4 received TSPP in the same dose and Metf, respectively, and were irradiated 24 h thereafter (TSPP + IR, Metf + IR), while group 3 received only Metf (20 mg/kg b.w.). The last two groups received the combined treatment with Metf prior to irradiation (TSPP + Metf + IR) and following irradiation (TSPP + IR + Metf). TSPP was administered intraperitoneally (i.p.) in 0.75 ml of phosphate bovine serum (PBS) and Metf was administered orally dissolved in distilled water. This pattern of drug (i.e. Metf) administration was chosen because of its similarity to human administration.

Our experiment was designed at the Physiology Department and the Wistar albino rats were obtained from the Animal Department of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca. In order to acclimatize the animals, they were kept

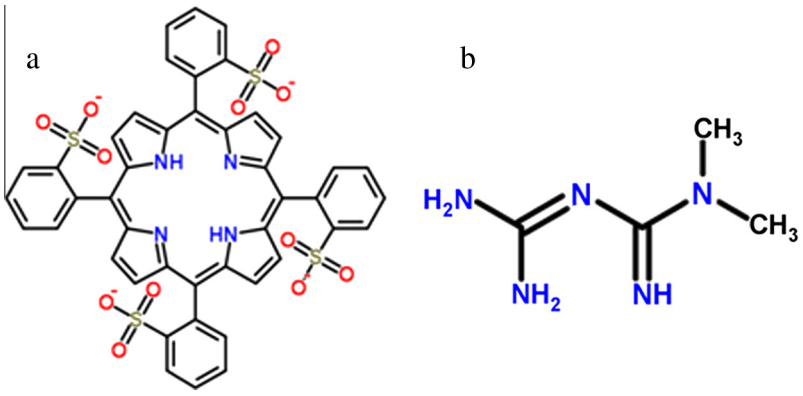

Fig. 1. The chemical structures of photosensitiser (a) and metformin (b).