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The natural flavonoid silybin improves the response to Photodynamic Therapy of bladder cancer cells

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ABSTRACT

Photodynamic Therapy (PDT) is an anticancer treatment based on photosensitisation of malignant cells. The precursor of the photosensitiser Protoporphyrin IX, 5-aminolevulinic acid (ALA), has been used for PDT of bladder cancer. Silybin is a flavonoid extracted from *Silybum marianum*, and it has been reported to increase the efficacy of several anticancer treatments.

In the present work, we evaluated the cytotoxicity of the combination of ALA–PDT and silybin in the T24 and MB49 bladder cancer cell lines. MB49 cells were more sensitive to PDT damage, which was correlated with a higher Protoporphyrin IX production from ALA.

Employing lethal light doses 50% (LD_{50}) and 75% (LD_{75}) and additional silybin treatment, there was a further increase of toxicity driven by PDT in both cell lines. Using the Chou–Talalay model for drug combination derived from the mass-action law principle, it was possible to identify the effect of the combination as synergic when using LD_{75}, whilst the use of LD_{50} led to an additive effect on MB49 cells. On the other hand, the drug combination turned out to be nearly additive on T24 cells.

Apoptotic cell death is involved both in silybin and PDT cytotoxicity in the MB49 line but there is no apparent correlation with the additive or synergic effect observed on cell viability. On the other hand, we found an enhancement of the PDT-driven impairment of cell migration on both cell lines as a consequence of silybin treatment.

Overall, our results suggest that the combination of silybin and ALA–PDT would increase PDT outcome, leading to additive or synergistic effects and possibly impairing the occurrence of metastases.

1. Introduction

Photodynamic Therapy (PDT) is an antitumour treatment based on the administration of a tumour located photosensitiser. Through the illumination of the tumour area using a wavelength that activates the molecule, a series of free radicals mediated reactions are triggered, inducing death of the cells [1,2] through direct damage to tumour cells and vasculature, as well as modulating the immune response [3]. Our group has been studying for many years the use of Protoporphyrin IX (PpIX) as an endogenous photosensitiser, which is biosynthesised from 5-aminolevulinic acid (ALA). This treatment is known as ALA–PDT. The easy access to the tumour area in bladder cancer allows ALA instillation and endoscopic access to laser carrying optical fibers in order to irradiate the area, becoming this type of cancer to be particularly interesting to PDT [4,5].

Several approaches have been designed to improve or potentiate the action of PDT. Combination with antioxidant inhibitors [6], bioreductive drugs [7], antineoplastics [8,9], hyperthermia, adjuvants such as oxygen carriers, antiangiogenenic drugs [10,11], and inhibitors of arachidonic acid metabolism [12] among others, have been successfully developed.

Silybin, also known as silibinin, a natural polyphenolic flavonoid, is a major bioactive component of silymarin which is isolated from the plant milk thistle *Silybum marianum* (L.) Gaertn., and has been extensively used for its hepatoprotective effects in Asia and Europe. Nevertheless, it also owns antitumour activity due to its involvement in regulation of several intracellular signal transduction pathways exhibiting strong anticancer efficacy towards various human cancer cell lines and also in several animal cancer

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models of several tissues: prostate, bladder, skin, lung, colon, etc. [13–16]. Silybin regulation is mediated by cellular proliferative pathways such as receptor tyrosine kinases, androgen receptor, STATs, NF-kappaB, cell cycle regulatory and apoptotic signalling pathways [15]. Several studies have shown that silybin activates apoptotic pathways in cells of different origin [17–20].

Silybin has also shown to inhibit the invasion and migration of cancer cells [21–25] by down regulation of metalloproteinases, urokinase plasminogen activator, MAPK pathway signalling proteins and upregulation of E-cadherin, among other molecules.

In previous works we found that silymarin and silybin inhibit bladder cancer cell growth and enhance the effects of both Bacillus Calmette–Guérin immunotherapy and radiotherapy [26,27].

On the other hand, ALA–PDT has been shown to induce apoptotic response, including both the mitochondrial and death receptor pathways [28,29], although the difference between apoptotic and necrotic death is highly dependent on the photosensitiser and light dose employed [30].

Since the plasma membrane is the target for various photosensitisers [31], it is not surprising to find that PDT induces changes in cell adhesion, invasion and metastasis. In addition, tumour cells treated with PDT release prostanoids [32] that have been shown to influence the *in vivo* dissemination, and *in vitro* migration of carcinoma cells. There are several reports on the influence of PDT on migration and invasion of tumour cells [33–36].

Silybin has been used in combination with chemotherapeutic drugs showing enhanced effects on growth inhibition, cell cycle regulation, and apoptosis in prostate, breast, and lung cancer systems. Together, the results indicate a synergistic effect of silybin on growth inhibition, reversal of chemoresistance, apoptosis induction, and a strong increase in G2-M checkpoint arrest when given in combination with these drugs. The criteria for combination with antineoplastics is that the response has to be synergistic or additive and that the drugs should not share common mechanisms of resistance and not overlap in their major side-effects [37].

The aim of this work was to study the efficacy of ALA–PDT in two bladder cell lines, and the effect of the combined action with silybin treatment, with emphasis on its effects on apoptosis and invasion pathways.

2. Materials and methods

2.1. Chemicals

ALA, silybin, acridine orange and MTT were obtained from Sigma (Palo Alto, USA). 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Life Technologies (Oregon, USA).

2.2. Cell lines

T24, a human bladder cancer cell line, was obtained from ATCC (Bethesda, MD) and cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 80 μg/ml gentamycin and 5% fetal calf serum and incubated at 37 °C in an atmosphere containing 5% CO_2. Murine bladder cancer line MB49 generously provided by Dr E.C. Lattime, Thomas Jefferson University, Philadelphia, PA, was cultured in the same medium. Cultures were free of mycoplasma as determined by periodic cytoplasmic DNA staining.

2.3. Silybin cytotoxicity

T24 and MB49 cells seeded in 24-well plates were exposed to increasing silybin concentrations for 24 and 48 h. The cell viability was determined by the MTT assay after withdrawal of silybin.

2.4. ALA–PDT treatment

Cells were incubated in serum-free medium containing 0.6 or 1 mM ALA and 3 h later, irradiations were performed. After irradiation, medium was replaced by ALA-free medium + serum, the cells were incubated for another 21 h and then tested for viability. Lethal light doses 50 and 75 (LD_{50} and LD_{75}) were defined as the light dose expressed in Joules/cm^2, necessary to kill 50% and 75% of cells.

2.5. Silybin-PDT combined treatment

2.5.1. 24 h treatment

Cells were incubated in serum-containing medium, in the presence of several concentrations of silybin for 24 h. Afterwards, medium was replaced by serum-free medium without silybin containing 0.6 mM ALA, and cells were incubated for 3 h. Afterwards, PDT was carried out using LD_{50} and LD_{75}. Cells were left in serum-containing medium for other 21 h until the MTT assay was performed.

2.5.2. 48 h treatment

In addition to 24 h previous treatment with different silybin concentrations, silybin was added during ALA–PDT in serum-free medium containing 0.6 mM ALA, and cells were incubated for 3 h. After withdrawal of ALA-containing medium, the cells were incubated for further 21 h in complete medium with silybin, and the flavonoid was withdrawn to perform the MTT assay.

2.6. MTT assay

A 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was added to each well, and plates were incubated at 37 °C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was read at 560 nm in a Spectracount plate reader (Packard, USA).

2.7. Toluidine blue staining

Twenty one hours after treatment, the cells grown on cover slides were fixed with paraformaldehyde. Afterwards, they were incubated with 0.025 mg/ml Toluidine blue during 1 min, and subsequently washed with distilled water. The cover slides were mounted on Canadian Balsam and cellular structures were observed under light microscope.

2.8. Porphyrins extraction from cells

Cells were seeded in 24-well plates. After ALA incubation for 3 h in serum-free medium at 37 °C, porphyrins accumulated within the cells were extracted with 5% HCl. The excitation and emission wavelengths producing the highest fluorescence were 406 nm and 604 nm respectively. These wavelengths were employed to measure the samples in a Perkin Elmer LS 55 Luminescence Spectrometer. PpIX from Frontier Scientific (Logan, UT, USA), was used as a reference standard.

2.9. In vitro wound-healing assay

cells in medium containing 10% FBS were seeded into wells of 6-multiwell plates and allowed to grow for 24 h. Forty eight hours after plating, that is, 24 h after 80 μM silybin treatment and immediately after ALA–PDT treatment, cells were gently wounded through the central axis. Cells were washed with PBS and refreshed with medium with serum. After overnight incubation (21 h) at 37 °C, the cells were fixed, stained with violet crystal and