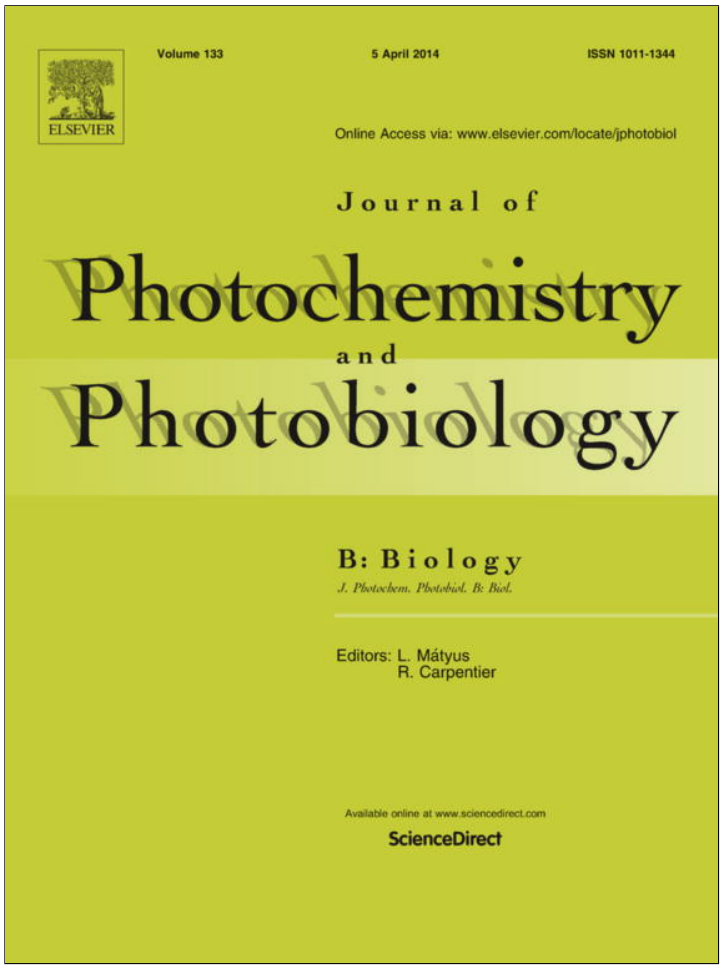

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The natural flavonoid silybin improves the response to Photodynamic Therapy of bladder cancer cells

L. Gándara [a], E. Sandes [b], G. Di Venosa [a], B. Prack Mc Cormick [b], L. Rodriguez [a], L. Mamone [a], A. Batlle [a], A.M. Eiján [b], A. Casas [a,*]

[a] Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET and Hospital de Clínicas José de San Martín, University of Buenos Aires, Córdoba 2351 1er subsuelo, Ciudad de Buenos Aires CP1120AAF, Argentina
[b] Area Investigaciones, Instituto de Oncología Ángel H. Roffo, Argentina

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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

* Corresponding author. Address: Viamonte 1881 10A, 1056 Buenos Aires, Argentina. Tel.: +54 1159508346; fax: +54 11 4811 7447.
E-mail address: adriana@qb.fcen.uba.ar (A. Casas).

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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

photographed. Controls of time 0 were carried out at 24 h after plating, that is, immediately before PDT treatment. Quantification of cell motility was performed by measuring the distance between the invading front of cells in six random selected microscopic fields for each condition [38]. The degree of motility is expressed as percent of wound closure as compared with the zero time point. Images were acquired with an Olympus microscope with 4× objectives and processed using image processing software. Differences in cell migration distances were tested using two-sided *t* test for comparing means.

2.10. Determination of apoptosis after silybin and PDT treatment

Tumour cells were seeded on cover slides in 6-well plates, and allowed to grow for 24 h. Afterwards, the 48 h silybin protocol alone or combined with PDT, or PDT alone, was carried out. After overnight incubation, cells adhered to cover slides were stained with DAPI and apoptotic bodies were centrifuged at 8000*g*, suspended in 50 μl of acridine orange (10 μg/ml), counted in a haemocytometer and expressed as apoptotic bodies per ml of medium. Adherent cells with condensed chromatin were recorded as apoptotic [39].

2.11. Statistical treatment and synergy determination

The values in the figures and table are expressed as means ± standard deviations of the means. A two-tailed Student's *t*-test was used to determine statistical significance between means in apoptosis and migration assays. *P* values <0.05 were considered significant. At least 3 independent experiments were performed in duplicate.

The synergistic effect of combined therapy was assessed using CompuSyn (Chou, T.C. and Martin, N. ComboSyn, Inc. Paramus, NJ, USA. www.combosyn.com) a software programme based on the Chou–Talalay model for drug combination derived from the mass-action law principle, already employed to test combinations of PDT with another treatments [40]. The Combination Index (CI) value is the quantitative measure of the dose-effect analysis of a drug combination. Roughly, CI values <1 represent synergism, CI > 1 corresponds to antagonism and CI ≈ 1 represents an additive effect for the drug combination, whilst the precise biological significance of the quantitative value was interpreted as suggested by Reynolds and Maurer [41].

3. Results

3.1. Response to ALA–PDT of T24 and MB49 cells

We first attempted to characterise the response of T24 and MB49 bladder cells to ALA–PDT treatment employing two ALA concentrations: non-saturating (0.6 mM ALA) and saturating (1 mM ALA).

Fig. 1 shows that the human T24 cells were more sensitive to ALA–PDT than the murine MB49 cells. LD_{50}s for MB49 cells were 0.05 J/cm^2 and 0.01 J/cm^2 using 0.6 mM and 1 mM ALA respectively.

For T24 cells the LD_{50}s were 0.0025 and 0.0035 J/cm^2 employing 0.6 mM and 1 mM ALA respectively.

To explain these differences in sensitivity to ALA–PDT, we analysed porphyrin synthesis from ALA in both cell lines (Table 1). T24 cells synthesise 2-fold higher porphyrins from 0.6 and 1 mM ALA as compared to MB49. In addition, cell exposure (24 h or 48 h) to silybin did not modify porphyrin synthesis normalised per cell number.

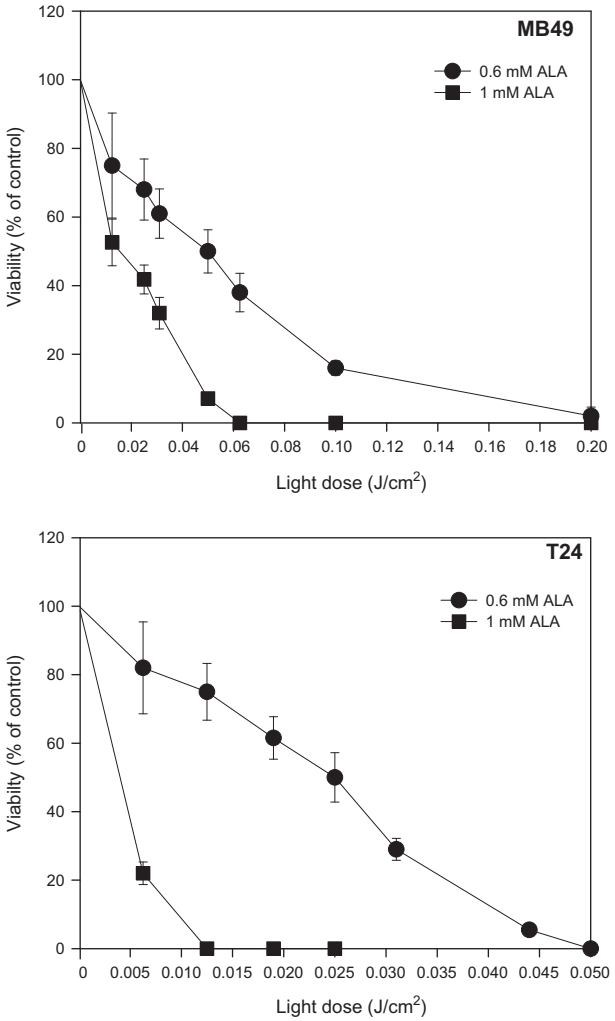

Fig. 1. ALA–PDT effectiveness in MB49 and T24 cells. Cells were exposed 3 h to 0.6 or 1 mM ALA and afterwards, they were illuminated with different light doses. After 21 h the MTT assay was carried out. Results are expressed as viability percentage relative to the control without treatment.

Table 1
Comparison of porphyrin biosynthesis from ALA in MB49 and T24 cells.

	MB49	T24
0.6 mM ALA	7.5 ± 0.9	15.3 ± 1.8
0.6 mM ALA + 48 h silybin	6.8 ± 0.7	17.1 ± 1.2
1 mM ALA	10.3 ± 1.3	18.7 ± 2.3
1 mM ALA + 48 h silybin	12.1 ± 1.5	19.2 ± 2.0

The cells were exposed for 3 h to 0.6 or 1 mM ALA with or without previous exposure to silybin in medium containing serum and afterwards, intracellular porphyrins were extracted. The results are expressed in μg porphyrins/10^5 cells.

3.2. ALA–PDT and silybin combined treatment) on MB49 cells

We have chosen 0.6 mM ALA to use in combined treatments with silybin, since we assumed that milder PDT toxicity would become combined effects more marked.

In Fig. 2A it can be observed that after 24 h exposure to silybin induces *per se* a concentration-dependent toxicity on MB49 cells. Whereas cytotoxicity is negligible at a concentration of 10 μM, from 20 μM onwards, a reduction in cellular viability becomes evident. A concentration of 80 μM silybin induces *per se* a 50% impairment of cell viability. Upon silybin-PDT combined treatment (24 h exposition), from 30 μM onwards, an increased damage is noticed

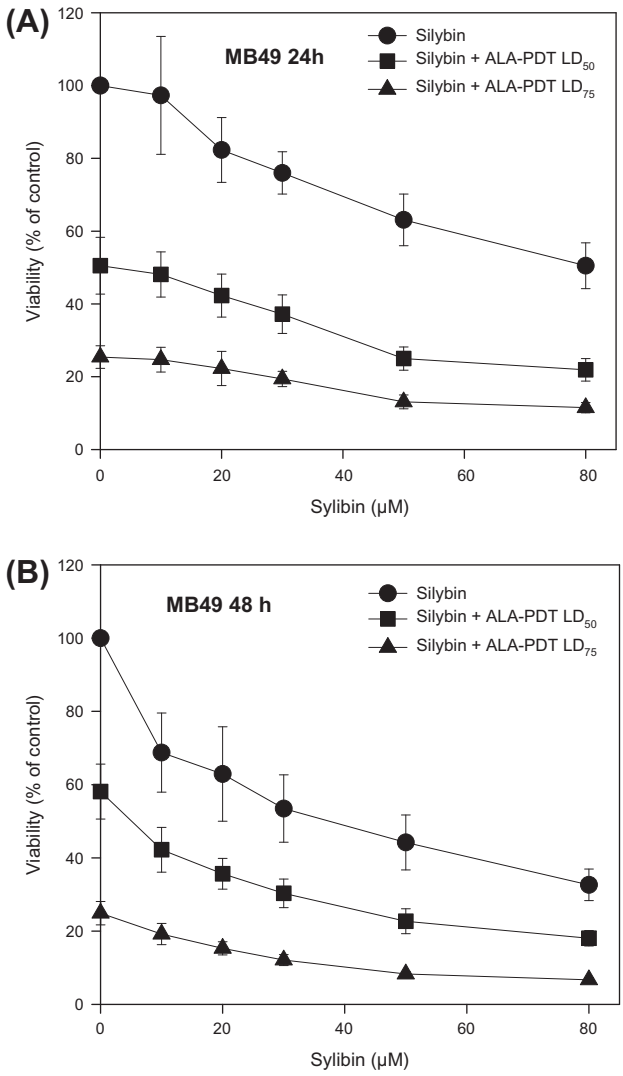

Fig. 2. Combined effects of silybin and ALA–PDT in MB49 cells. MB49 cells were incubated with several silybin concentrations for 24 h prior to 0.6 mM ALA–PDT (A) or 24 h previous to ALA–PDT, during, and 21 h after ALA–PDT (B) using LD_{50} and LD_{75}. MTT assay was performed. Results are expressed as viability percentage relative to the control without silybin.

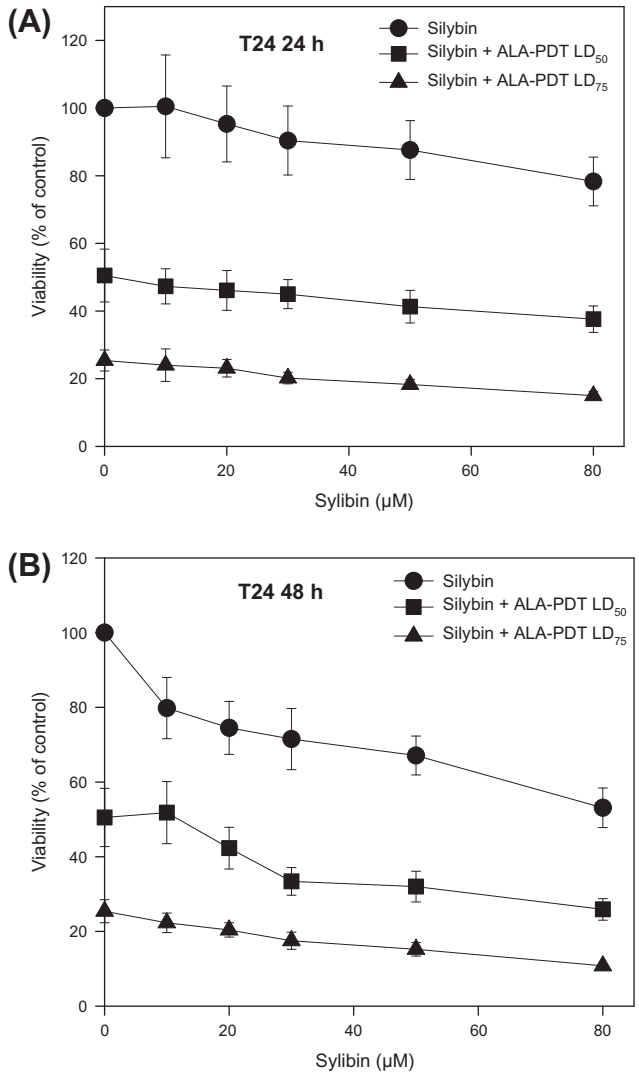

Fig. 3. Combined effects of silybin and ALA–PDT in T24 cells. T24 cells were incubated with several silybin concentrations for 24 h prior to 0.6 mM ALA–PDT (A) or 24 h previous to ALA–PDT, during, and 21 h after ALA–PDT (B) using LD_{50} and LD_{75}. MTT assay was performed. Results are expressed as viability percentage relative to the control without silybin.

as compared to PDT alone (0 μM silybin). This effect is evidenced upon irradiation with both LD_{50} and LD_{75} light doses.

Fig. 2B shows that in MB49 cells silybin *per se* induces a concentration-dependent toxicity after 48 h exposure, that is, cells exposed to silybin 24 h previously, during, and 24 h after PDT. This cytoxicity is 20-30% higher than the one caused after 24 h incubation. In silybin + PDT combination, an increased toxicity as compared to PDT alone can be appreciated from silybin 10 μM onwards both for LD_{50} and LD_{75}.

3.3. ALA–PDT and silybin combined treatment on T24 cells

In Fig. 3A we can see that upon 24 h exposition, silybin toxicity was less marked in T24 cells as compared to MB49. A mild effect of silybin slightly higher than 20% is observed even at high concentrations (80 μM). This slight cytotoxicity *per se* is reflected in an apparent increased photodamage at 80 μm silybin upon LD_{50} irradiation, and from 30 μM silybin onwards, upon application of LD_{75}.

Fig. 3B shows in T24 cells, a similar pattern to MB49. Silybin *per se* induces a concentration-dependent toxicity at 48 h incubation, and this toxicity is also 20–30% higher than the one obtained with 24 h incubation.

The 48 h silybin + PDT combination shows a higher cytotoxic effect than the one obtained with 24 h exposition to the drug, and a enhancement of the photodamage can be appreciated from a concentration of 30 μM silybn onwards for both light doses.

We also determined whether the interactions between ALA–PDT employing LD_{50} and LD_{75} and silybin are synergistic or additive using the combination index (CI) method of Chou and Talalay [42].

3.4. Determination of additivity and synergism for the combination of ALA–PDT and silybin

The CI values were calculated employing the data from Figs. 2 and 3 at different degrees of effect (affected fraction or Fa) for the combined treatments, using the CompuSyn software developed by Chou and Martin. The CI–Fa plots are presented in Figs. 4 and 5.

Fig. 4 shows that the CI values are mostly between 1 and 1.1 employing MB49 cells and 24 h silybin exposure, over a range of

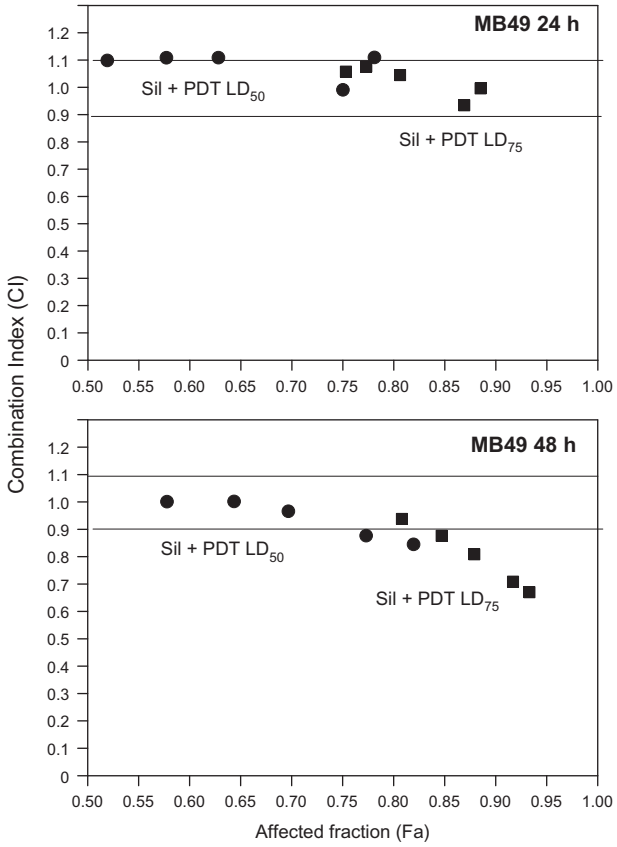

Fig. 4. Fa–CI plots generated according to Chou and Talalay for MB49 cells treated with silybin and ALA–PDT employing LD_{50} and LD_{75}. The fractions of cells affected (Fa) by each dose of the silybin and PDT combination were calculated from Fig. 2 data. (•) Silybin + ALA–PDT LD_{50}, (■) silybin + ALA–PDT LD_{75}. The CI values <1 indicate synergistic interactions between ALA–PDT and silybin, whereas the values = 1 indicate additivity and <1, antagonism. Values between 1.1 and 0.9 are considered as indicators of a nearly additive effect according to Reynolds and Maurer [41].

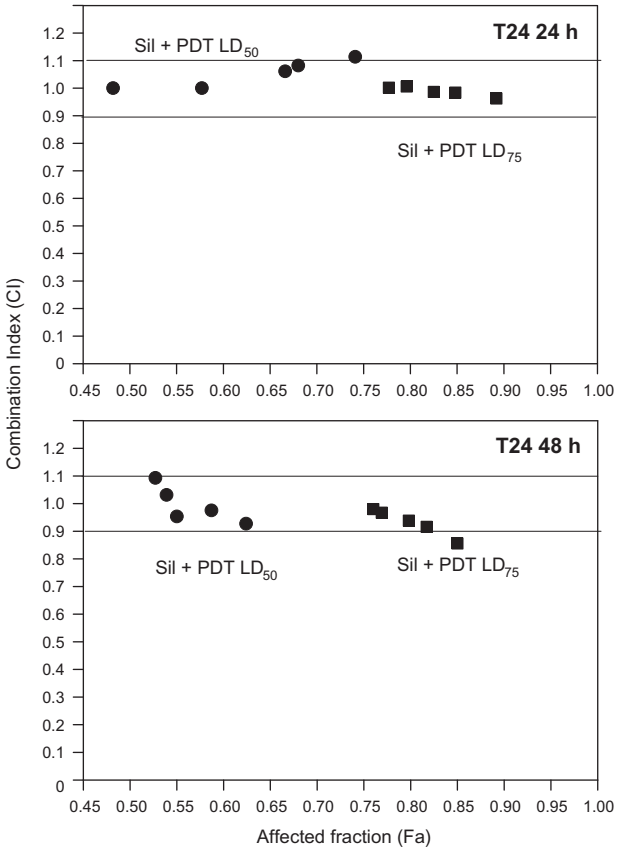

Fig. 5. Fa–CI plots generated according to Chou and Talalay for T24 cells treated with silybin and ALA–PDT employing LD_{50} and LD_{75}. The fractions of cells affected (Fa) by each dose of the silybin and PDT combination Fa (affected Fractions) were calculated from Fig. 3 data. (•) Silybin + ALA–PDT LD_{50}, (■) silybin + ALA–PDT LD_{75}. The CI values <1 indicate synergistic interactions between ALA–PDT and silybin, whereas the values = 1 indicate additivity and <1, antagonism. Values between 1.1 and 0.9 are considered as indicators of a nearly additive effect according to Reynolds and Maurer [41].

Fa values including ALA–PDT with LD_{50} and LD_{75} and silybin from 10 to 80 μM. These values suggest the existence of nearly additive effects for the combined treatments.

On the other hand, when MB49 cells were exposed to 48 h silybin, the CI values were = 1 for the two lowest silybin doses and PDT LD_{50}, and <1 for the higher doses and most of PDT LD_{75} affected fractions, indicating synergistic interactions between both treatments.

Fig. 5 shows CI–Fa plots calculated from combination treatments on T24 cells. Most CI values are ≈1 employing both PDT doses, suggesting an additive effect, when silybin exposure was 24 h.

Similarly, when T24 cells were exposed to silybin during larger periods of 48 h, the effects are largely additive for silybin and PDT employing both LD_{50} and LD_{75}, except for the highest dose of both silybin and PDT for which a moderate but clear synergism was established. Overall, the analysis suggests that synergism is observed in both bladder cells employing the highest PDT and silybin doses or drug exposure.

3.5. Morphology of bladder cancer cells after treatment with silybin and ALA–PDT

We have also examined cell morphology of MB49 and T24 cells after silybin and ALA–PDT treatment (Fig. 6). MB49 cells are smaller and show less spreading than T24 ones. Upon both silybin and PDT treatments, a percentage of condensed cells become evident mainly in T24-treated population. Cytoplasmatic vacuolisation is also observed in some PDT-treated T24 cells (see figure inset). The combination of both treatments induces cytoplasm but not chromatin condensation in most T24 cells, and to a much lesser extent in MB49-treated cells. On the other hand, cell detachment of MB49 cells is characteristic of ALA–PDT and silybin treatments but not of T24. The absence of chromatin condensation as a consequence of both treatments independently on the cell line was confirmed in adherent cells by DAPI staining (data not depicted).

3.6. Apoptosis induced by silybin and ALA–PDT

Counting of Acridin orange-labelled apoptotic bodies (Fig. 7) revealed that silybin and ALA–PDT can induce *per se* in MB49 cells an increased number of apoptotic cells within the detached cell population as compared with the non-treated control. However, the combination of both treatments did not increase the number of apoptosis observed for the separated conditions. On the other hand, in accordance with the lack of cell detachment depicted in Fig. 6, neither in individual nor in combined treatments, the number of apoptotic bodies did increase over the controls in T24 cells.

3.7. Impact of PDT + silybin treatment on cell migration

We investigated the effect of ALA–PDT and silybin treatment on the migration of the tumour bladder cells *in vitro*. As shown in

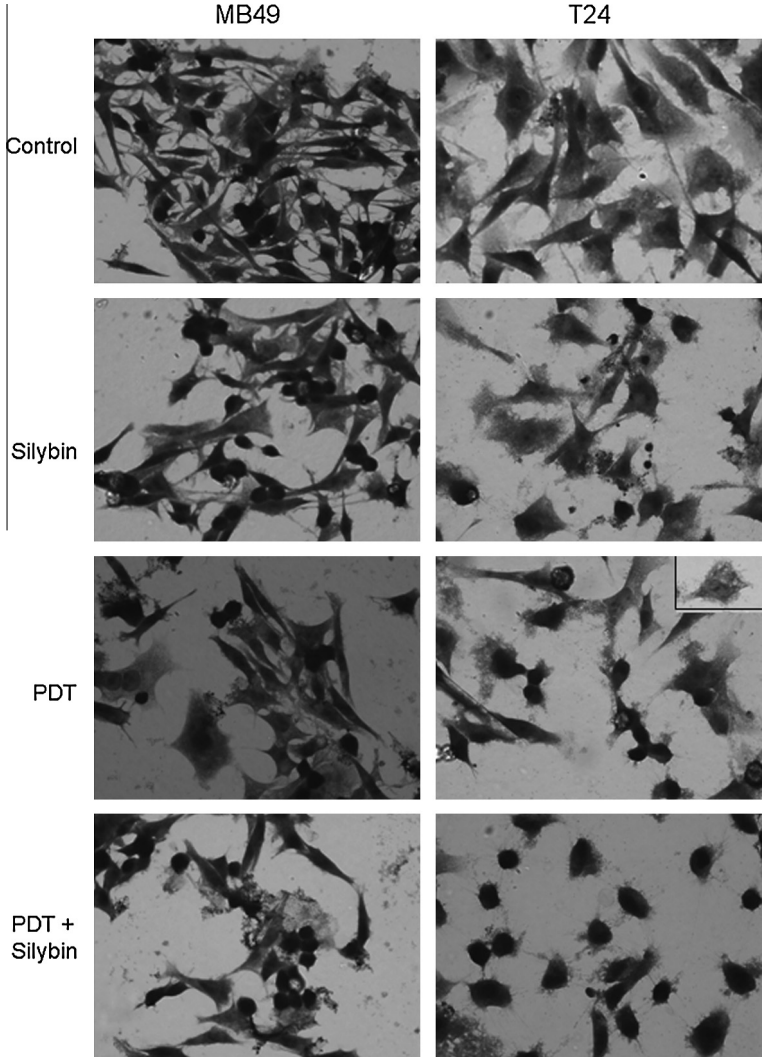

Fig. 6. Cellular morphology of murine MB49 and human T24 lines treated with ALA–PDT, silybin or combined treatment. Cell staining with Toluidine blue showing details of morphology 21 h after treatment of PDT: 0.6 mM ALA–PDT employing LD_{50} light doses; Silybin: 48 h 80 μM silybin treatment; PDT + Silybin: combination of ALA–PDT and silybin treatments (48 h protocol); Control: without treatment. Magnifications 100×.

Fig. 8, MB49 is a low migratory cell line, whereas T24 cells covers 94% of the wound surface after 21 h of migration. Whereas silybin induces significant decreases on migration of both MB49 and T24 cells as compared to the controls, PDT alone impairs even more the percentage of migration. In both cell lines, the combination of silybin + PDT leads to a stronger decrease of cell migration, which is 2% and 20% of wound closure in MB49 and T24 cell lines respectively as compared to 40% and 93.8% of controls.

4. Discussion

In the present work we found a differential response to ALA–PDT treatment of the bladder cancer lines employed. These differences correlate well with the rate of porphyrin bioynthesis from ALA. Silybin proved to be an inhibitor of cytochromes P450 [43]. However, although cytochromes are haemproteins, porphyrin biosynthesis from ALA was not affected by exposure to silybin. In addition, antioxidant or protective effects of flavonoids were not observed under our experimental conditions.

The effect of silybin on the cellular redox status shows a very complex dynamic, which seems to be highly context-dependent, and it has been usually reported to have pro-oxidant or anti-oxidant properties depending on the exposure conditions. Silybin recovers both expression and activity of several antioxidant enzymes towards its control values in rats treated with alloxan [44], enhances superoxide dismutase activity in erythrocytes and lymphocytes of patients with chronic alcoholic liver disease [45], and exerts antioxidant activity in numerous *in vitro* and *in vivo* models of oxidative stress-induced hepatocellular injury. Other cells types have also proven to be sensitive to its protective action against the induction of oxidative stress including endothelial cells, keratinocytes and fibroblasts [46].

However, a reactive oxygen species (ROS) production enhancement mediated by silybin has also been reported in several experimental models. Silybin has been shown to induce oxidative stress against colorectal cancer cells leading to mild apoptosis or autophagy depending on the exposure to the compound [47]. Moreover, it has been recently described a silybin- induced ROS generation, associated with significant depletion on intracellular glutathione in human hepatocarcinoma HepG2 cells, that leads to the activation of a pro-death response [48]. In addition, silybin also exerted a dual action on UVB-induced carcinogenesis. The natural compound strongly prevented apoptosis of HaCaT keratinocytes at lower UV doses, whereas it enhanced UVB-caused apoptosis at higher light doses, suggesting that silybin possibly works as a

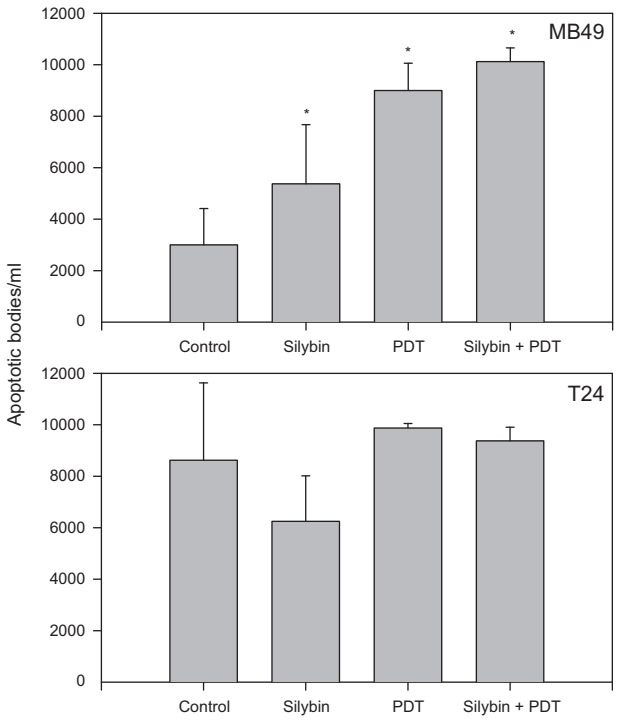

Fig. 7. Apoptotic bodies induced by ALA–PDT, silybin or combined treatments in MB49 and human T24 lines. Acridine orange-labelled apoptotic bodies 21 h after treatment of PDT: 0.6 mM ALA–PDT employing LD_{50} light dose; Silybin: 48 h 80 μM silybin treatment; PDT + Silybin: combination of ALA–PDT and silybin treatments (48 h protocol); Control: without treatment. $^{*}p < 0.05$ respect to the control.

UVB damage sensor to exert its biological action [49]. In the light of this reports the study of PDT-silybin combined treatment becomes particularly interesting. Photodynamic Therapy acts through free radicals forming species, and targets intracellular sites such as mitochondria, cell membrane and lysosomes [31,50]. However, to the best of our knowledge, there are no previous works showing the effectiveness of silybin combination with PDT.

T24 cells are more sensitive to photodynamic damage mediated by ALA-induced porphyrins than MB49 ones. On the other hand, MB49 are more sensitive to silybin cytotoxicity. Notably, these different susceptibilities to the treatments would suggest the existence of different mechanisms of action driven by these therapies, and this fact may have an impact on the effect of the combination treatments for each cell line. The combination of PDT and silybin turned out to be additive on T24 cells under all the conditions assessed and only employing the mildest conditions of both therapies in MB49 cells. On the other hand, synergistic interactions appeared in MB49 cells upon exposure to the more severe toxicity conditions. This pattern may be probably related to the different cell line susceptibility to the treatments, being the more sensitive to silybin the cell line likely to exhibit synergistic interactions upon combination with the higher light doses, and under conditions of maximal time exposure to silybin as well. It is noticeable that the silybin dose employed for the combination of treatments, induced similar percentages of cell death as compared to other reports in other cell lines [51].

Interestingly, silybin addition to MB49 cells before PDT (24 h silybin) only produced an additive effect, whereas the exposure to silybin before, during, and after PDT (48 h silybin) drove to a synergic effect for the treatments combination. The 24 h after irradiation period is generally considered the lapse on which the cell death mechanisms triggered by ALA–PDT fulfill their biological role leading to the impairment of cell viability [52]. Thus, it is highly noteworthy that this time period is required for silybin in order to develop a synergic effect when combined with PDT. One possible explanation for this phenomenon is that the effectors of silybin-induced synergism have a short half-life, allowing surviving cells to partially recover after silybin withdrawal. A possible enhancement of ROS generation mediated by silybin would also explain the synergic effect of silybin-PDT combination at 48 h silybin. Many tumour cells are significantly more sensitive to oxidative stress since they have heightened basal levels of ROS-mediated signals, which contribute to their increased rates of growth, metabolism and proliferation [53,54]. Therefore, the mechanisms underlying numerous antitumour treatments, including PDT, involve an enhancement of ROS generation that leads to cell death [54]. Similarly, silybin treatment could induce a higher basal level of ROS on surviving cells, turning them more sensitive to PDT. An enhancement on ROS production in tumour cells induced by silybin has been reported by Raina et al. [47] and Zhang [48]. However, further studies are needed to test these hypotheses.

Thereafter, silybin mechanism of action supports the combined chemotherapy approach, wherein the criteria for combination 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]. It has been largely shown that silybin enhances antitumour activity of a range variety of antineoplastics as well as other natural compounds [21,37,51,55–57].

It is interesting to note that T24 cells, which are more sensitive to PDT as compared to MB49 cells but more resistant to silybin treatment, did not detach from the surface and did not undergo apoptosis after either of the treatments. On the other hand, MB49 cell line undergoes apoptosis upon both ALA–PDT and silybin, but the combined treatments did not induce further effects. Since MB49 is the cell line that shows higher response to silybin, it is likely that apoptosis is playing a main role in its cytotoxic action, but since it is neither blocked nor enhanced in the combination of treatments, we suggest that apoptosis is not the cell death mode involved in PDT enhancement by silybin.

Silybin has clearly demonstrated inhibition of multiple cancer cell signalling pathways, including growth inhibition, impairment of angiogenesis and chemosensitisation. The molecular mechanisms of silybin-mediated antiproliferative effects are mainly via receptor tyrosine kinases, androgen receptor, STATs, NF-kappaB, cell cycle regulatory and apoptotic signalling pathways in various cancer cells [15,16]. Recent preclinical studies have also shown strong efficacy of silybin to target cancer cell's migratory and invasive characteristics as well as their ability to metastasis to distant organs. Detailed mechanistic analyses revealed that silybin targets signalling molecules involved in the regulation of epithelial-to-mesenchymal transition, proteases activation, adhesion, motility, invasiveness as well as the supportive tumour-microenvironment components, thereby inhibiting invasion and metastasis [14–16].

A reduction of metastasis has been reported *in vivo* after PDT compared to surgery [58–60]. Rousset et al. [61] also reported a decrease of metastasis induced by colon adenocarcinoma cells treated with Photofrin-PDT respect to the control. Etminan et al [34] found that ALA–PDT caused long-lasting, nearly complete suppression of glioma cell migration in a spheroid model, and matrix invasion, and that was not simply due to phototoxicity because 50% of tumour cells remained vital throughout the observation period. In previous work we have also observed that cells resistant to ALA–PDT are much less migratory and metastatic than the parental cells [33], and these features have mainly been related to a dramatic disorganisation of actin structure, among other cytoskeleton proteins [62].

In the present work we have found that silybin enhanced ALA–PDT effect on cell migration in two bladder cancer cell lines, but mainly in T24 cells, which have a marked migratory and invasive phenotype, in spite of its lower responsiveness to silybin effects.

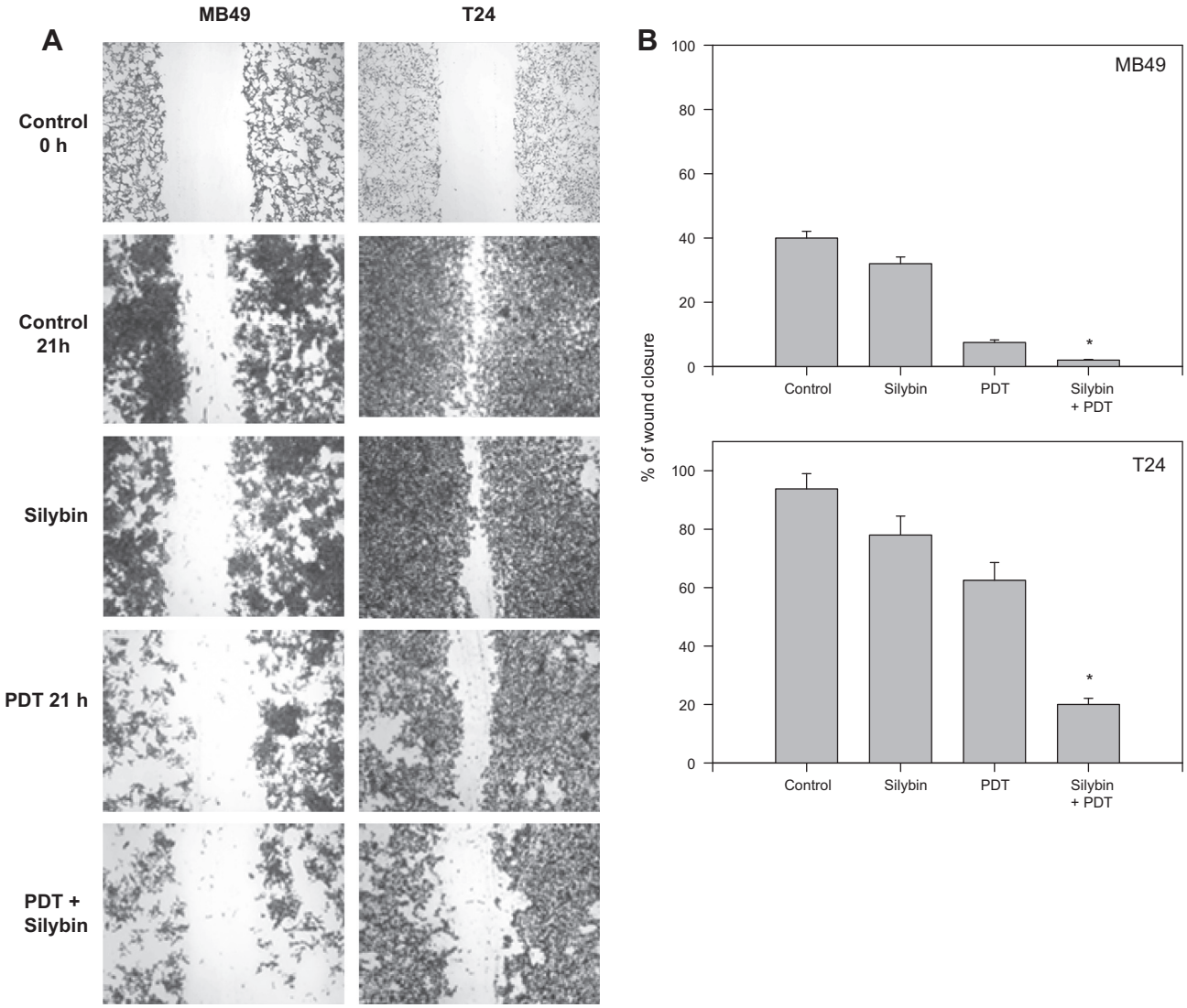

Fig. 8. Wound healing assay of MB49 and T24 cells treated with ALA–PDT, silybin or combined treatment. (A) Images were taken at 21 h after the wound assay for each treatment. Controls of time 0 were carried out immediately after making the wound. PDT: 0.6 mM ALA–PDT employing LD_{50} light dose; Silybin: 48 h 80 μM silybin treatment; PDT + Silybin: combination of ALA–PDT and silybin treatments (24 h protocol); Control: without treatment. (B) Quantification of cell motility by measuring the distance between the invading fronts of cells in six random selected microscopic fields. The degree of motility is expressed as percent of wound closure as compared with the control. $^{*}p < 0.05$ respect to silybin and ALA–PDT alone.

This feature suggests that inhibition of cell motility driven by PDT is probably induced by different signalling pathways as compared to those activated by silybin.

Overall, these results suggest that the addition of a natural compound such as silybin to ALA–PDT, would increase the effectiveness of the photodynamic treatment, leading to additive or synergistic effects and possibly impairing the occurrence of metastases.

5. Abbreviations

ALA	5-aminolevulinic acid
CI	combination index
FA	affected fraction
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
PDT	Photodynamic Therapy
PpIX	Protoporphyrin IX

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