Article

Flavonoid Silybin improves the response to radiotherapy in invasive bladder cancer[†]

Running Head: Silybin improves radiotherapy response

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ABSTRACT

Conservative treatment for invasive bladder cancer (BC) involves a complete transurethral **tumor resection** combined with chemotherapy (CT) and radiotherapy (RT). The major obstacles of chemo-radiotherapy are the addition of the toxicities of RT and CT, and the recurrence due to RT and CT resistances. The flavonoid Silybin (Sb) inhibits pathways involved in cell survival and resistance mechanisms, therefore **the purpose of this paper was to study** *in vitro* and *in vivo*, the ability of Sb to improve the response to RT, in two murine BC cell lines, with different levels of invasiveness, placing emphasis on radio-sensitivity and pathways involved in radio-resistance and survival. *In vitro*, Sb radio-sensitized murine invasive cells through the inhibition of RT-induced NF-κB and PI3K **pathways**, and the increase of oxidative stress, while non-invasive cells did not show **to be sensitized**. *In vivo*, Sb improved RT-response and overall survival in invasive murine tumors. Since **Sb is already being tested in clinical trials** for other urological cancers and it improves RT-response in invasive BC, these results could have translational relevance, supporting further research.

Keywords: bladder cancer; bladder preservation; Silybin; radiotherapy; radiosensitizer.

LIST OF ABBREVIATIONS

BC = Bladder Cancer

CT = Chemotherapy

RT = Radiotherapy

Sb = Silybin

NF-κB = Nuclear transcription Factor kappa B

RSF = Radiosensitizing Factor

DEF = Dose Enhancement Factor

ROS = Reactive Oxygen Species

INTRODUCTION

Bladder cancer (BC) ranks second as a cause of death from genitourinary cancer in men. Transitional bladder tumors are the most frequent type of BC. Although most patients present non-invasive disease, 10-20% eventually progress to muscle-invasive disease. In addition about one-third of the new cases are muscle-invasive when diagnosed [Jemal et al., 2010].

Radical cystectomy is the gold standard treatment for patients with invasive BC [Hautmann et al., 2006; Stein et al., 2001]. However, it is associated with high complication rates and compromises the quality of the patient's life[Konety et al., 2006; Manoharan et al., 2009]. The main treatment for bladder preservation includes complete transurethral tumor resections combined with chemotherapy (CT) and radiotherapy (RT)[Caffo et al., 2013]. While chemo-radiotherapy represents an effective strategy in many patients, its major obstacles are the

addition of the toxicities of RT and CT, and the recurrence due to RT or CT

resistances. It is then important to study new molecular targets to optimize the

conservative treatment [Caffo et al., 2013].

Nuclear transcription factor kappa B (NF-κB) regulates several genes involved in

tumor development, progression and treatment resistance in a wide range of

tumors. Among others, NF-κB overexpression has been proposed as a predictor of

radio-resistance and a potential target to overcome resistance in muscle-invasive

BC[Ahmed and Li, 2008; Koga et al., 2011].

Silybin (Sb) [(2R,3R)-3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-

2- (hydroxy-methyl)-2,3-dihydrobenzo[b][1,4]doxin-6-yl] chroman-4-one] is a

flavonoid and the main compound of silymarin, the extract obtained from the milk

thistle (*Silybum marianum*)[Kvasnicka et al., 2003]. Sb has clearly demonstrated

inhibition of multiple cancer cell features including growth, angiogenesis, multi-drug

resistance, invasion and metastasis[Gazak et al., 2007; Ramasamy and Agarwal,

2008; Singh and Agarwal, 2005]. Sb mechanisms of action include regulation of

transcription factors including NF-κB, cell cycle regulatory and apoptotic signaling

pathways[Li et al., 2010]. Sb also possesses anti-inflammatory and antioxidative

properties[Kim et al., 2013; Kren and Walterova, 2005]. Furthermore, in previous

works, we found that Sb inhibited BC cell growth and enhanced the effects of

photodynamic therapy [Gandara et al., 2014].

The aim of this work was to study *in vitro* and *in vivo*, the ability of Sb to improve

the response to RT, in two murine BC cell lines, with different levels of

invasiveness [Lodillinsky et al., 2009], making emphasis on radio-sensitivity and

pathways involved in radio-resistance and survival.

MATERIALS AND METHODS

Cell culture: Murine non-invasive MB49 **was gently donated by the Tomas Jefferson University (Philadelphia)** and invasive MB49-I, **was derived from MB49 as described in** [Lodillinsky et al., 2009]. **Both cell lines** were cultured in RPMI-1640 from Gibco (31800-014;**USA)** supplemented with 10% heat-inactivated fetal bovine serum (FBS) from Internegocios S.A. **(Argentina)** and 80 µg/ml gentamycin, at 37ºC in a humidified atmosphere of 5% CO_2.

Silybin and irradiation: Sb (C25H22O10 ≥98% purity) from Sigma-Aldrich **(USA).** Stock solution was prepared in Dimethylsufoxide (DMSO; Protide-Pharmaceutical) for *in vitro* and *in vivo*assays. Cells and tumors were irradiated using a Theratron 780 with ^{60}Co source gamma-radiation emitter (dose rate: 1 Gy/minute). Sb treatments or DMSO as a control, were performed in RPMI with 2% of FBS.

Cell viability: Cytotoxic effects of Sb, +/-RT treatment,were analyzed using MTS-assay (Promega; **USA**) **in a Multiskan Ascent (Labsystem) at 492nm/620nm**. To determine Sb inhibitory dose 50% (ID_{50}), $8x10^3$ cells were cultured in 96-well plates and treated with Sb (**0.01, 0.1**, 1, 10, 20, 40, 60, 80, 100 and 150 µM) for 24 hours. To assess the cytotoxic effect of the combined treatment (Sb+RT), $1x10^3$ cells were cultured in 96-well plates and viability was measured 5 days after RT (0, 2, 4 and 6 Gy). Radio-sensitivity factors (RSF) were calculated by *RSF= optical density reading with RT/optical density reading without RT* for each Sb concentration.

Colony formationassay: To analyze radio-sensitivity to Sb, 500-1500 MB49 or MB49-I cells were cultured **in triplicate** in 6-well plates, treated +/-Sb (60 μM) for 24 hours and then irradiated (0, 2, 4 and 6 Gy). After RT, medium was changed maintaining Sb treatment but supplementing with 10% FBS, and plates were incubated for 10-14 days until cell colonies were visible to the naked eye, **adapted from [Nambiar et al., 2015]**. Colonies were fixed with methanol/**acetic acid**(3:1), stained with Giemsa and those with more than 50 cells were registered to calculate plating efficiency (PE) and surviving fractions (SF), as previously[Franken et al., 2006]. *PE= mean colony number/cells plated*; and *SF= sample PE/PE of its non-irradiated control*. Dose enhancement factors (DEF) were calculated as*DEF= dose with RT-only/ dose with RT and Sb, for a same biological effect*.

Cell cycle assay: cell cycle was analyzed using DNA content measurement with Propidium Iodide (PI). Sub-confluent monolayers were treated +/-Sb (60 μM) for 24 hours. **Attached** and **detached** cells were harvested, suspended in 0.5 ml of PBS with minimal cell aggregation and fixed with 4.5 ml of 70% ethanol on ice. **The cell suspension was centrifuged and cells were suspended and** incubated with PI (0.1% (v/v) Triton X-100, 50 μg/mL PI and 100 μg/mL DNase-free RNase A) in PBS for 30 minutes at room temperature. 20,000 cells were identified on a Partec-Cyflow flow cytometer and analyzed using Cyflogic V.1.2.1.

Gene reporter assay: cells were transiently transfected with a plasmid carrying the luciferase gene under control of NF-κB-response element, as previously [Langle et al., 2012]. Luciferase activity was determined using the Dual-

Luciferase® Reporter–Assay-System (**Promega; USA**) in a Triathler luminometer liquid scintillation counter (Hidex, Turku, Finland) and normalized to constitutive renilla activity.

Immunoblot: proteins (80 μg) from whole-cell or nuclear extracts were electrophoresed on SDS–PAGE and transferred to PVDF membranes. Antibodies: NF-κB-p65 protein (**Santa Cruz Biotechnology**, sc-109, **USA**), survivin (Cell-Signaling 2802, **USA**), pAkt (**Santa Cruz Biotechnology**,sc-7985-R, **USA**), Akt (**Santa Cruz Biotechnology**,sc-8312, **USA**),**caspase-3** (**Cell-Signaling 9662S**, **USA**) **and PARP (Cell-Signaling 9532S, USA)**. Densitometry units were referred to β-actin (Sigma A5441, **USA**).

Sub-confluent monolayers were treated +/-Sb (60 μM) for 24 hours, +/-RT (8 Gy). To assess NF-κB nuclear translocation, nuclear extracts were obtained 30-60 minutes after RT using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents **(Thermo Scientific™ 78833, USA) according to manufacturer's instructions**. Survivin, Akt, p-Akt and procaspase-3 were evaluated in **whole-cell** extracts **obtained using a lysis and extraction buffer composed of 50 mM tris-HCl (pH 8), 100 mM NaCl, 1% Tritón and 10mM EDTA, with the addition of protease inhibitor (1:10000).Protein content was determined by the Bradford method.**

Reactive oxygen species (ROS) generation: intracellular ROS study was based on ROS mediated conversion of non-fluorescent 2´,7´-DCFH-DA into fluorescent DCFH. After pre-treatment +/-Sb (60 μM) **for 24 hours**, cells were incubated for 20

minutes with 2´,7´-DCFH-DA (20 µM) in PBS and then irradiated (6 Gy). DCFH fluorescence was measured in the cell suspension **by flow cytometry** (Partec Cyflow) and analyzed (Cyflogic V.1.2.1). A group without 2´,7´-DCFH-DA was used as background and defined as 1.

Tumor growth *in vivo*: C57BL/6J mice between 8-10 weeks were subcutaneously injected with $2.5x10^5$ MB49 or $2x10^5$ MB49-I into the flank. When the average tumor volume reached 50 mm^3, animals were randomly divided into four groups of five-seven mice each: control, Sb, RT or Sb+RT. Sb (400 mg/kg) or equivalent DMSOdissolved in 0.1 mL of cell medium were administered by gavage five days/week, **adapted from [Yan et al., 2015].** RT (18 Gy) was administered as six fractions of 3 Gy, being three fractions/week (Figure 5 A). RT was performed as previously [Prack Mc Cormick et al., 2017], mice were anesthetized with ketamine (70 mg/kg) and xylazine (5 mg/kg) and placed on paraffin stretchers with acrylic lids so that only tumors remained in the irradiation field (Figure 5 B). Tumor volumes were calculated as *0.4xwidth^{2}xlength***[Girit et al., 2008]**. **For tumor weights comparison**, final tumor weights and **tumor weights from mice euthanized not more than three days before the end of the assay,** were registered.

Mice were obtained from our Institute's animal facility and handled in accordance with the international **procedures** for Care and Use of Laboratory Animals. Protocols were approved by the Institutional Review Board CICUAL, protocol number 2012/02, "Instituto de Oncología Angel H. Roffo".

Tumor bearing mice survival and criteria for euthanasia: mice survival was carried out by the Kaplan Meier analysis based on the days of death recorded **during the above described *in vivo* assays**. Euthanasia was performed according to the ethical standards in force for national and international animal facilities. The ethical endpoint was determined by the presentation of at least two of the signs of animal discomfort or suffering listed below: decrease of more than 10% of body weight, pilo-erection, isolation, or anorexia.

Statistical analysis: *In vitro* results are expressed as **means**±SD of at least six replicates/group. Statistical significances were calculated by ANOVA, **Student´s t-test** or Kruskal-Wallis with Dunns comparisons using 5.0 Graph-Pad-Prism. The Log-rank (Mantel-Cox) was used for global comparison and Gehan-Breslow-Wilcoxon was used for comparisons between individual treatments in survival analysis. **p<0.05 was considered significant**. All experiments were repeated at least three times with similar results. For the graphics representing *in vivo* results, data from all experiments **were** combined for each cell line.

RESULTS

Silybin inhibits *in vitro* bladder cancer cell lines growth through different mechanisms

Our results showed that Sb inhibited *in vitro* growth of both non-invasive and invasive murine BC lines, in a dose dependent manner. Sb ID_{50s} were 120 μM and

105 µM for MB49 and MB49-I respectively (Figure 1 A). Clonogenic assays performed to evaluate single cell growth revealed that Sb 60 µM was able to inhibit MB49 surviving fraction while 80 µM was required for MB49-I (Figure 1 B). Despite that, in the invasive line we detected inhibition of the **colony diameters** with Sb 40 µM (Figure 1 C-D). A cell cycle assay (Figure 1 E) showed that Sb increased the number of cells in S-phase in detriment of G2/M-phase **in MB49-I but not in MB49.** These results suggest that Sb-mediated MB49-I cell growth inhibition is related to a cell cycle detention in S-phase. On the other hand, **in MB49, 48 hours of treatment with Sb induced apoptosis, throw caspase-3 activation followed by PARP cleavage. This effect was almost absent in MB49-I** (Figure 1 F)**.**

Silybin enhances radiotherapy effect

To determine whether Sb improves RT response we combined concentrations of Sb lower than the ID_{50} with RT. The cells were treated with Sb 24 hours before RT, andthe response to this experiment was evaluated 5 days after RT. Concentrations equal to or lower than 40 µM were harmless while 60 µM inhibited cell growth of both lines (Figure 2 A). When comparing these results with the toxicity showed in Figure 1 A we can see that Sb has time-dependent toxicity. Based on Sb+RT combinations, we calculated the radio-sensitivity factors (RSF) at 2 Gy for different Sb concentrations. The maximum RSF for each cell line was 1.33 and 3.08 for MB49 and MB49-I respectively, suggesting that invasive cells are more sensitive to Sb radio-sensitization.

Then, in **colony formation** assays, cells were treated with RT with or without Sb 60 µM. Inhibition of plating efficiency was evident for MB49 cells treated either with

Sb-only or RT-only (Figure 2 B), but this response did not improve when treatments were combined. Improvement did not appear either when cells were treated with Sb 40 µM (data not shown). In MB49-I, Sb alone did not show inhibition (Figure 2 B), however its combination with RT did (Figure 2 C). The calculated DEF at 0.5 survival was 1 for MB49 and 2.1 for MB49-I, indicating that murine invasive cells, but not the non-invasive ones, could be radio-sensitized by Sb treatment.

Combined treatment inhibits NF-κB activation and survivin expression

NF-κB transcription factor is a major molecule associated with radio-resistance and/or tumor recurrence. Our results showed that Sb alone or combined with RT, inhibited NF-κB transcriptional activity in both lines. RT was not able to activate this factor in MB49, while a slight but significant activation of NF-κB was shown in MB49-I (Figure 3 A). Immunoblotting for nuclear NF-κB-p65 supported these results (Figure 3 B).

Survivin is an apoptosis inhibitor that is known to be deregulated in BC and may be involved in RT resistance. Survivin expression was decreased **after RT in MB49-I** and after Sb+RT in both lines (Figure 3 C).

Silybin increases ROS and the combined treatment inhibits pro-survival Akt phosphorylation

It is known that RT increases ROS levels that have cytotoxic effect but can also activate pathways involved in radio-resistance as NF-κB and survival as PI3K/Akt. Although Sb has antioxidant activity, under certain conditions it can behave as pro-

oxidant. MB49-I cells had twice the level of ROS present in MB49 cells and Sb increased these levels in both cell lines. RT alone slightly modified ROS in both cell lines when compared with non-treated controls. **ROS levels, in cells pretreated with Sb, were increased in response to RT in MB49 but not in MB49-I. However, in both cell lines, the combined treatment increased ROS as compared to RT alone** (Figure 4 A).

Akt expression and its activation by phosphorylation in response to RT and Sb were evaluated by immunoblot. RT induced an increase in Akt expression (2.5 and 120 folds) and its phosphorylation (3.5 and 30 folds) for MB49 and MB49-I respectively. **In MB49 cells, pAkt expression reached a peak at 30 minutes after RT**, while in MB49-I pAkt continued increasing for 180 min (Figure 4 B-C). Sb reduced Akt and pAkt levels after RT in both lines. Given that MB49-I showed a greater increase in PI3K pathway after RT, its inhibition by Sb could be associated with the better response that this invasive line has compared to the non-invasive one.

Silybin improves *in vivo* radiotherapy response in invasive bladder cancer

To evaluate the radio-sensitizing effect of Sb on BC *in vivo,* we employed a murine model of tumor growth. As a measure of response we assessed tumor size, weight and tumor-bearing mice survival. These results were consistent with those observed *in vitro.* In MB49 tumors RT partially inhibited tumor growth (Figure 5 C-D) and improved overall survival (Figure 5 E) without additional effects with Sb. On the other hand, when analyzing MB49-I tumors, RT partially inhibited tumor growth and this response was improved by Sb (Figure 5 F-G). There was also a significant

improvement in MB49-I mice survival treated with Sb+RT, as compared with either Sb or RT alone (Figure 5 H).

DISCUSSION

Invasive BC is one of the most aggressive epithelial tumors and is characterized by a high rate of early systemic dissemination. Therefore, when it is detected, cystectomy is the election therapy. Taking into account the morbidity and mortality associated with this therapy, bladder preservation is indicated in some patients. One of the problems associated with the conservative therapy is the development of CT and RT resistances. NF-κB constitutive activation has been associated with tumor development and RT resistance. Since it was reported that Sb could inhibit NF-κB activation, in the present work we assessed the role of Sb as a radio-sensitizer.

   Our results showed that Sb caused time-and dose-dependent inhibition of murine and human BC cells viability. Sb inhibited clonogenic survival of murine BC cells, by cell dependent mechanisms. In MB49, Sb induced **apoptosis throw** caspase-3 activation **followed by PARP cleavage** while in MB49-I it caused cell cycle arrest in S-phase. **Tyagi *et al* reported apoptosis in transitional cell papilloma of the bladder (RT4) treated with Sb, throw induction of p53 followed by caspase-2 and -3 activation and PARP cleavage [Tyagi et al., 2006]. Apoptosis as a result of induction of ROS followed by ATM/p53/caspase-3 signal transduction pathway activation was recently reported for MB49 cells after treatment with the traditional Chinese herbal formula, Guizhi Fuling Wan [Lu et al., 2016]. In BC, p53 mutations have been**

associated with progression to muscle invasion [Smith et al., 2003], and therefore the invasive cell line MB49-I could have a less functional p53 than the parental MB49, being a putative hypothesis for the selective efficacy of Sb treatment in transitional BC cells.

The colony formation assays showed MB49-I cells to be more resistant to radiation than MB49 cells, however pretreatment with Sb made MB49-I cells similarly sensitive to RT than MB49 cells without Sb. In agreement with our hypothesis Sb strongly decreased constitutive and RT-induced NF-κB activity in our murine BC model. Consistently, Sb was capable of partially inhibiting NF-κB-p65 nuclear translocation in the BC cells used in this study. NF-κB-p65 RT-induced translocation was higher in invasive MB49-I than in non-invasive MB49 cell line, **possibly justifying the radiosensitizing potential of Sb in the invasive one**. Also, Sb-induced decrease of survivin expression, a target of NF-κB, was evident 48 hours after RT. **However, for future studies, further evaluations including other prosurvival molecules should be conducted in order to better understand the mechanism of radiosensitization triggered by Sb.**

Induction of ROS is one of the major mechanisms involved in electromagnetic radiation-induced cell death. An increase in ROS levels is capable to induce cell death by different mechanisms involved in DNA, membrane and protein damage among others[Panganiban et al., 2013]. Sb alone or combined with RT enhanced ROS in MB49 and MB49-I and in consequence could be responsible for the increased growth inhibition. Similar results were described for hepatic-cellular carcinoma cells treated with Sb alone[Zhang et al., 2013]. On the other hand, it is known that high levels of ROS could activate PI3K/Akt pathway and NF-κB. Here

we showed that Sb was able to attenuate the enhancement of PI3K/Akt pathway induced by RT, since either total Akt as pAkt expressions were decreased in irradiated cells. One of the possible mechanisms of Sb could be to diminish the stock of Akt to be phosphorylated, through proteasome degradation. However, we cannot exclude that Sb could also act directly in phosphorylation mechanisms. Thus, our results suggest that Sb could be considered as a radio-sensitizercompound, enhancing oxidative stress but at the same time controlling survival pathways such as PI3K/Akt and NF-κB.

Sometimes, radio-sensitizers seem effective in*in vitro*but do not work under *in vivo* conditions and also have toxicity associated problems. RT significantly reduced tumor growth, in accordance to our *in vitro* results, while its combination with Sb only improved the response of invasive BC tumors. It is possible that a higher growth rate and higher activation of NF-κB and PI3K/Akt pathways in response to RT could make invasive tumors more sensitive to Sb treatment than non-invasive ones. On the other hand, it is noteworthy that even though the differences in tumor response between RT and Sb+RT were not statistically significant,the combined treatment improved invasive tumor-bearing mice overall survival with respect to RT. This was probably one of the most relevant results of our study.

There are only a few studies involving the combination of Sb and RT for the treatment of cancer, and to our knowledge the present report is the first one evaluating the combination in bladder cancer. Possible combinations of Sb with chemoradiotherapy treatment for invasive bladder cancer [James et al., 2012] could be considered for future studies. Recently, Sb-based nutraceutical Legasil® resulted in significant clinical and radiological improvement of brain metastasis

from lung cancer patients with poor performance status that progressed after whole brain radiotherapy and chemotherapy[Bosch-Barrera et al., 2016].

Furthermore, it has been reported that Sb could radio-sensitize prostate cancer cells, while protecting normal tissue from RT associated toxicity [Nambiar et al., 2015].The authors suggest that Sb radio-sensitizing activity depends on cell malignancy. In our study, histologic analysis of MB49 tumors treated with Sb, revealed a less aggressive phenotype with a growth pattern characterized by pseudo-papillae with neovascularization, absent in, control tumors (**supplementary Figure-1**) **as well as untreated and Sb-treated MB49-I tumors (supplementary Figure-2)**. **Given that Sb protects normal tissue from RT,** we can hypothesize that in non-invasive tumors **this Sb-induced differentiation effect** could be preventing its radio-sensitizing activity.

Moreover, tumor progression from diploid MB49 line to MB49-I line was associated with an enrichment in polyploid cells, with new structural abnormalities and additional chromosomal imbalances [Fabris et al., 2012], supporting higher instability. Given that Sb targets DNA repair systems [Nambiar et al., 2015], and that higher instability has also been related with better response to sensitizers which target those repair systems [Tofilon and Camphausen, 2009], the differences associated with tumor progression could be justifying in part the fact that Sb only improves the response to RT in the invasive bladder cancer line both *in vitro* and *in vivo*, but not the non-invasive line.

CONCLUSION

Our results demonstrate that Sb improves the overall survival of MB49-I tumor bearing mice treated with RT, and the response to RT in invasive BC MB49-I. This supports further studies of this therapeutic approach.

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Conflict of Interest Statement

None of the authors have any conflict of interest with this study nor personal relationships with other people or organization that could influence their work.

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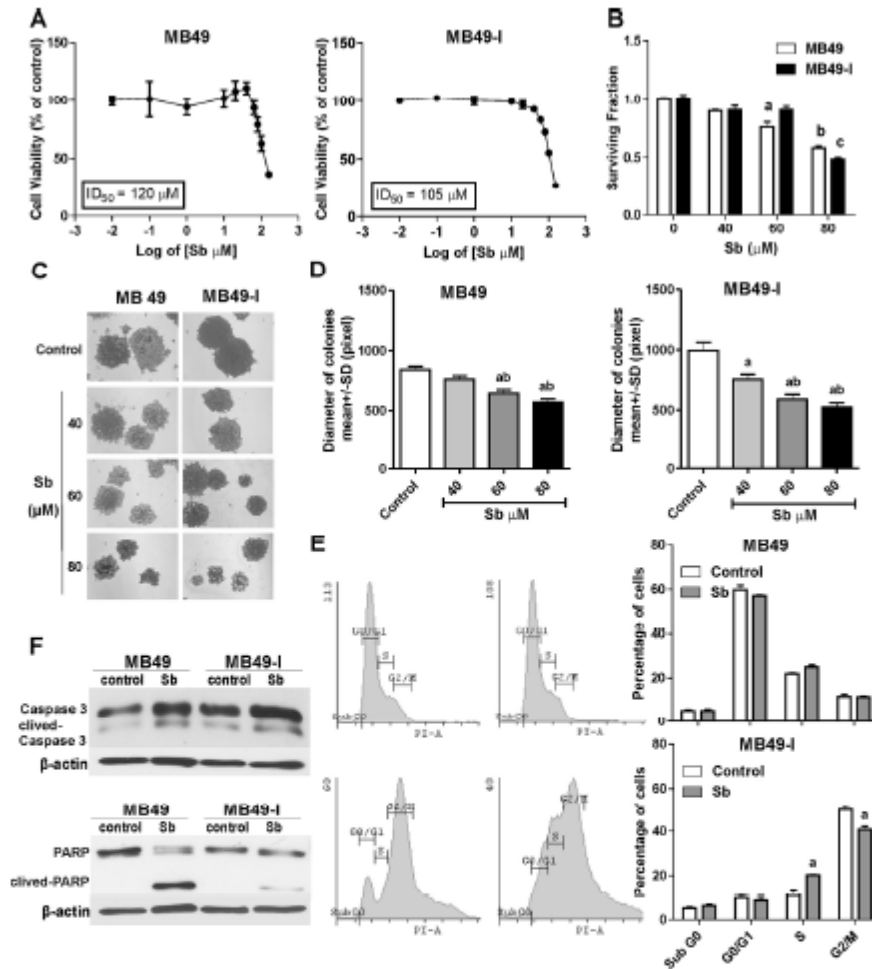
Figure 1. Silybin inhibits in vitro BC growth throw different mechanisms. A, MTS assays were used to assess cell viability and calculate the inhibitory dose effective 50 (ID50) for Sb at 24 hours of treatment, in BC cell lines. B, Clonogenic assays of cells treated with Sb (0-80 μM) and cultured for 12 days, were performed to evaluate the influence of Sb over the surviving fraction. a: p<0.05 and b: p< 0.01 for Sb (60 μM and Sb 80 μM respectively) compared with untreated MB49 cells. c: p<0.01 compared with untreated MB49-I cells. C, Microphotographs of the colonies (magnification 40X) and D quantification of the colonies' diameter. MB49: a: p<0.001 compared with control, b: p<0.05 compared with 40 and 60 μM respectively. MB49-I: a: p<0.01 compared with control, b: p<0.05 compared with Sb 40 μM. E, Cell cycle analysis for MB49 and MB49-I. In MB49-I, Sb significantly increased the cell number in S-phase (a: p<0.01) and decreased the cell number in G2/M (a: p<0.01). F, Immunoblot to assess caspase-3 and PARP cleavage in MB49 and MB49-I cells treated with Sb 60 μM for 48 hours.
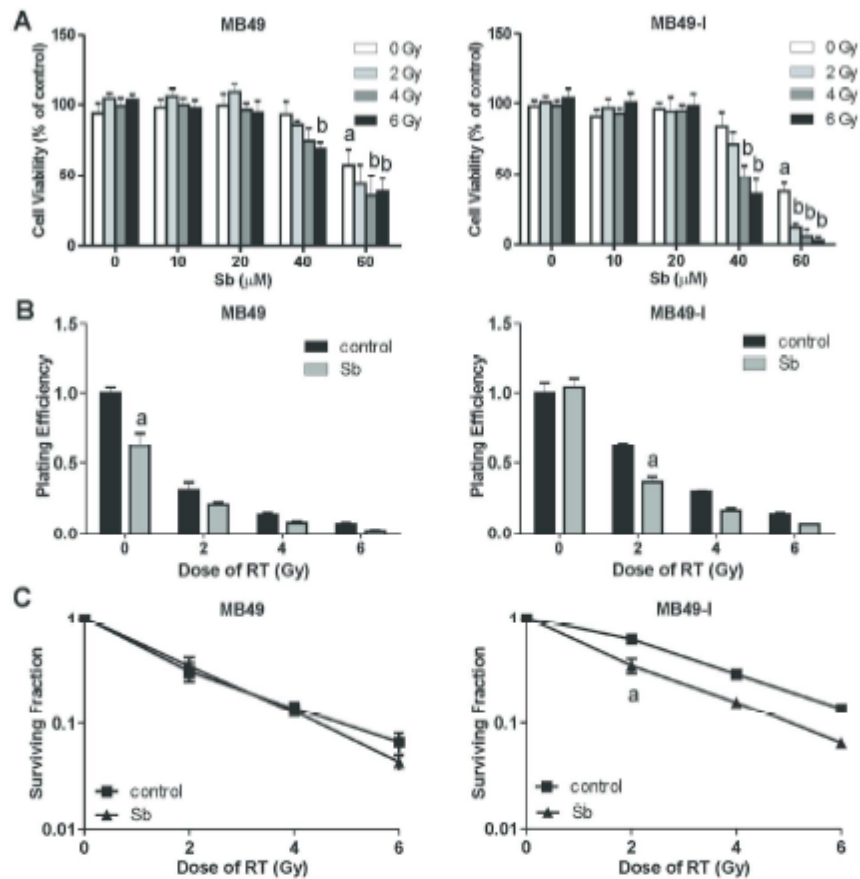
Figure 2. Silybin exhibits cell dependent radiosensitizing capability. A, MTS assays were performed to test the effect of a range of Sb dosis in combination with RT treatment, 6 days after RT, and calculate the resultant RSFs. The maximum RSF at 2 Gy for each cell line was 1.33 and 3.08 for MB49 and MB49-I, respectively. An ANOVA of two ways showed interaction between both treatments in MB49-I, but not in MB49 (a: $p<0.05$ compared with untreated cells, b: $p<0.01$ compared with Sb-only). B, Platting efficiency and C surviving fraction obtained from clonogenic assays using Sb 60 µM in combination with increasing doses of RT, performed to confirm the information above. Calculated DEF at 0.5 survival are 1 for MB49 and 2.1 for MB49-I. An ANOVA of two ways showed the same results than those obtained from the MTS. (B) a: $p<0.001$ compared with control without RT, b: $p<0.01$ compared with control without Sb. (C) a: $p<0.01$ compared with control without Sb.
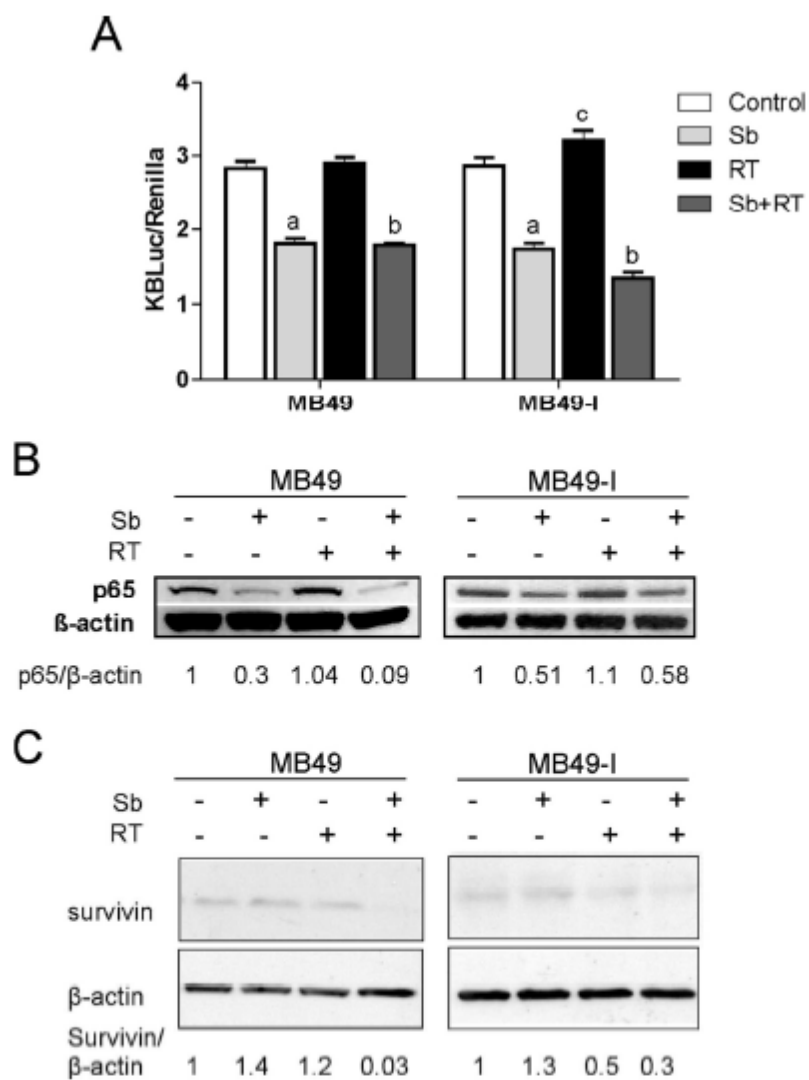
Figure 3. Combined treatment inhibits NF-κB transcriptional activity and survivin expression. MB49 and MB49-I cells were treated with Sb 60 μM in with or without RT (8 Gy). A, a gene reporter assay based on a plasmid carrying the luciferase gene under control of the NF-κB response element, was performed to assess NF-κB activity 18 hours after RT treatment; ANOVA with "Newman-Keuls" multiple comparisons, a: $p < 0.01$ compared to control, b: $p < 0.01$ compared to RT and c: $p < 0.05$ compared to control. B, an immunobloting was performed to assess p65 NF-κB subunit appearance in the subcellular nuclear fraction, 1 hour after RT. C, inmunoblottings were perform using cell lysates obtained 48 hours after RT, to assess the expression of survivin protein. Survivin/β-actin shows the result of the quantification of three independent experiments.
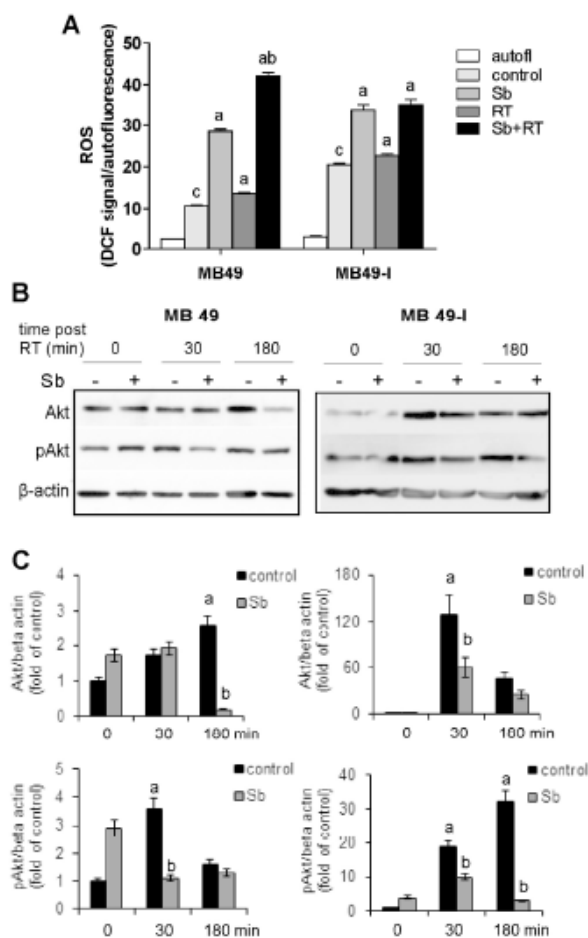
Figure 4. Silybin increases ROS, and inhibits Akt expression and phosphorylation in response to radiotherapy. MB49 and MB49-I cells were treated with Sb (60 μM) with or without RT to assess ROS status (6 Gy) and Akt expression and phosphorilation (8 Gy) in response to treatment. A, intracelular ROS levels were measured using the cell-permeable fluorogenic probe 2',7'-dichlorofluorescein diacetate, 1 h after treatment. ANOVA (p<0,0001 for both cell lines) and Bonferroni post test; a: p<0.001 compared to control, b: p<0.01 compared to RT, c: p<0.001 compared to autofluorescence. B, Immunoblottings of total cell lysates were done with an anti-Akt antibody or an anti-pAkt antibody. Immunoblotting for β-actin was used as a loading control. C, graphics show the result of the quantification of three independent immunoblotings. MB49: both Akt and pAkt levels augmented after RT treatment, and this effect was reverted in the combined treatment with Sb (a: p<0.01 compared to control at time zero, b: p< 0.01 compared with respective RT without Sb). MB49-I: both Akt and pAkt levels augmented after RT treatment, and this effect was diminished by the combined treatment with Sb (a: p<0.01 compared with time zero without Sb, b: p< 0.01 compared with respective RT without Sb).
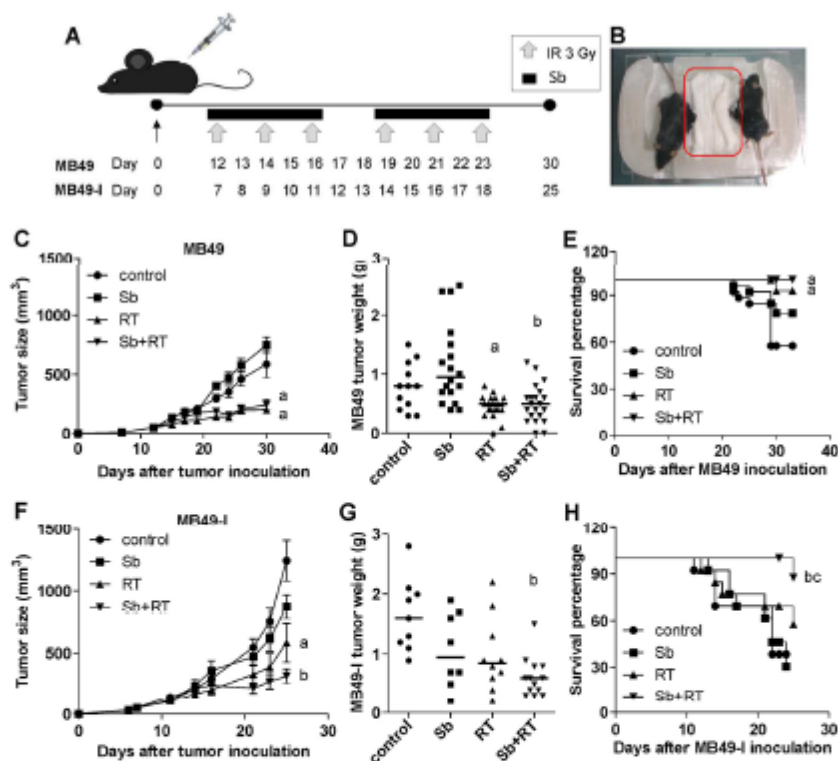
Figure 5. Silybin improves RT-response in invasive BC. C57BL/6J mice were injected with MB49 or MB49-I cells subcutaneously into the left flank and tumors were allowed to grow. Animals were then randomly divided into four groups of five-seven mice each: control (DMSO), Sb, RT (DMSO) or Sb+RT. Sb treatment (400 mg/kg) or an equivalent solution of DMSO was administered orally by gavage 5 days/week until the end of the experiment. RT was administered in fractionated doses of 3 Gy, 3 days/week reaching a total dose of 18 Gy. A, experimental design. B, photograph of the animals prepared to undergo radiotherapy. C and F, effect of Sb on tumor growth in vivo. The effect of the combined treatment of Sb and RT was dependent on the cell line, for MB49 tumors (C) there were no differences between RT-only and the combined treatment. On the contrary Sb combined with RT induced the greatest growth delay of MB49-I tumors (F). D and G, effect of Sb on tumor weight at the end of the assay. C, D, F, and G Kruskal Wallis Test with Dunn's multiple comparisons. E and H, effect of Sb on tumor bearing mice survival. Kaplan Meier survival curves for MB49 and MB49-I tumor bearing mice. Sb+RT increased survival of MB49 tumor bearing mice respect control group (a:p< 0.001; Hazard ratio 10.30, 95% CI 2.98-35.54). Sb+RT increased survival of MB49-I tumor bearing mice respect either control (p<0.01, Hazard ratio 12.26, 95% CI 2.80-53.58) or RT group (Hazard ratio 4.80, 95% CI 0.94-24.53), Log-rank (Mantel Cox) Test. a: p<0.05 and b: p<0.001 compared with control, c: p<0.05 compared with RT.