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Inhibition of sirtuins 1 and 2 impairs cell survival and migration and modulates the expression of P-glycoprotein and MRP3 in hepatocellular carcinoma cell lines

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ABSTRACT

Sirtuins (SIRTs) 1 and 2 deacetylases are overexpressed in hepatocellular carcinoma (HCC) and are associated with tumoral progression and multidrug resistance (MDR). In this study we analyzed whether SIRTs 1 and 2 activities blockage was able to affect cellular survival and migration and to modulate p53 and FoxO1 acetylation in HepG2 and Huh7 cells. Moreover, we analyzed ABC transporters P-glycoprotein (P-gp) and multidrug resistance-associated protein 3 (MRP3) expression. We used cambinol and EX-527 as SIRTs inhibitors. Both drugs reduced cellular viability, number of colonies and cellular migration and augmented apoptosis. In 3D cultures, SIRTs inhibitors diminished spheroid growth and viability. 3D culture was less sensitive to drugs than 2D culture. The levels of acetylated p53 and FoxO1 increased after treatments. Drugs induced a decrease in ABC transporters mRNA and protein levels in HepG2 cells; however, only EX-527 was able to reduce MRP3 mRNA and protein levels in Huh7 cells. This is the first work demonstrating the regulation of MRP3 by SIRTs. In conclusion, both drugs decreased HCC cells survival and migration, suggesting SIRTs 1 and 2 activities blockage could be beneficial during HCC therapy. Downregulation of the expression of P-gp and MRP3 supports the potential application of SIRTs 1 and 2 inhibitions in combination with conventional chemotherapy.

1. Introduction

Sirtuins (SIRTs) are class III histone deacetylase enzymes (HDACs) that use NAD+ as a cosubstrate in their reactions. In mammals, there are seven SIRTs members (SIRT1-SIRT7), with conserved core NAD+-binding domain but diverse substrate specificities and cellular functions. SIRTs 1 and 2 are reported to play crucial roles in many biological processes, including the support of proliferation and blockage of apoptosis and the promotion of tumor initiation, progression and metastasis (Chen et al., 2013; Liu et al., 2009; Tang, 2010). In this regard, SIRTs 1 and 2 are upregulated in HCC cell lines and in a subset of human HCC tissues when compared to normal hepatocytes and nontumoral tissues, respectively (Chen et al., 2013; Hao et al., 2014; Portmann et al., 2013). SIRTs 1 and 2 have many histone and nonhistone substrates, and their role in cell survival is achieved by deacetylation of key cell cycle molecules and apoptosis regulatory proteins, including p53 (Jin et al., 2008; Vaziri et al., 2001) and forkhead box O (FoxO) 1 (Daitoku et al., 2011; Yang et al., 2005). Deacetylation by SIRTs 1 and 2 completely abolishes p53-dependent cell growth arrest and apoptosis, and acetylation of this protein seems to be

Abbreviations: ABC, ATP binding cassette; Camb, cambinol; EX, EX-527; FoxO, forkhead box O; HDAC, histone deacetylase; MDR, multidrug resistance; MRP3, multidrug resistance-associated protein 3; P-gp/MDR1, P-glycoprotein/multidrug resistance protein 1; SIRT, sirtuin

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crucial for its function as a tumor suppressor (Peck et al., 2010; Vaziri et al., 2001). In addition, SIRTs deacetylate and repress the ability of FoxO1 transcription factor to induce apoptosis (Daitoku et al., 2011; Yang et al., 2005). In this regard, silencing of SIRTs 1 and 2 by RNA interference impair cell growth and enhance cell death in HCC (Chen et al., 2013, 2011; Mao et al., 2014; Portmann et al., 2013; Wang et al., 2012).

On the other hand, although chemotherapy has become one of the main treatments for cancers, HCC is a chemorefractory malignancy (Avila et al., 2006). Multidrug resistance (MDR) limits the application of liver cancer chemotherapy, and it is also a major cause of liver cancer recurrence and metastasis. One of the major causes of MDR is the overexpression of ATP binding cassette (ABC) transporters in cancer cells, involved in the efflux of chemotherapeutic drugs (Chen et al., 2016). Two ABC transporters of relevance for HCC chemotherapy are P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1, ABCB1), and multidrug resistance-associated protein 3 (MRP3, ABCC3) (Sun et al., 2013; Tomonari et al., 2016; Yang et al., 2013). In this connection, it was described that SIRT1 overexpression is associated with prediction of poor long-term survival for patients with resected HCC and promotes tumorigenesis and resistance to chemotherapeutic drugs in HCC cell lines (Chen et al., 2012, 2011; Liang et al., 2008). In addition, it was recently demonstrated that SIRT1 overexpression induces P-gp upregulation in HepG2 cells (Jin et al., 2015) and that this phenomenon is mediated by FoxO1 overexpression in breast cancer cells (Han et al., 2008; Oh et al., 2010). Conversely, SIRT1 or FoxO1 silencing increases the cellular uptake of chemotherapeutic agents and restores the responsiveness of HCC and other cancer cell lines to these drugs (Chen et al., 2011; Han et al., 2008; Oh et al., 2010).

Based on the functional role of SIRTs 1 and 2 in tumorigenesis, metastasis, prognosis, and chemical resistance, inhibition of SIRTs might be of value in the development of new therapeutic targets for HCC.

Therefore, the aim of the present study was to analyze the effects of SIRTs 1 and 2 inhibitions on survival and migration of HCC cell lines. Additionally, we explored the actions of SIRTs 1 and 2 inhibitions on p53 and FoxO1 acetylation and on P-gp expression. Finally, since MRP3 is also involved in MDR of HCC cells, we evaluated if SIRTs 1 and 2 inhibitions were capable of modulating this ABC transporter expression.

2. Materials and methods

2.1. Chemical and antibodies

Cambinol (C0494), EX-527 (E7034) and anti-MRP3 antibody (M0318) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Anti-SIRT1 (sc-15404), anti-SIRT2 (sc-20966), anti-p53 (sc-126), anti-acetyl-FoxO1 (Lys 259/262/271; sc-49437), anti-FoxO1 (sc-11350) and anti-GAPDH (sc-25778) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-acetyl-p53 antibody (Lys382; PA5-17648) was obtained from Thermo Fisher Scientific (Rockford, IL, USA). Anti-P-glycoprotein antibody (ALX-801-002) was from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, NY, USA). All other chemicals were of the highest grade commercially available.

2.2. Cell lines and treatments

The human HCC cell lines HepG2 and Huh7 were obtained from ATCC (Manassas, VA, USA) and JCRB Cell Bank (Tokyo, Japan), respectively. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. For treatments, two inhibitors of SIRTs 1 and 2 activities were used, cambinol and EX-527, since they have shown promising results as antitumor agents, alone or in combination with other drugs (Cea et al., 2011; Gollavilli et al., 2015; Heltweg et al., 2006; Portmann et al., 2013; Zhu et al., 2012). In most experiments, cells were treated after 24 h of attachment with 50 µM cambinol (Camb 50) or with 1 or 40 µM EX-527 (EX 1 or EX 40, respectively); both drugs dissolved in dimethylsulfoxide (DMSO). Control (untreated) cells were incubated only with DMSO, with a final concentration in the culture medium always below 0.5%.

2.3. MTT assay

Cells were seeded in 96-well plates at a density of 10,000 cells/well for HepG2 and 4000 cells/well for Huh7. After 24 h of attachment, cells were treated for 72 h with different concentrations of the drugs. After treatment, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich Corp.) was added into the culture medium to assess its metabolization, as previously described (Ferretti et al., 2016). Absorbance of the metabolite produced from viable cells was detected at 540 nm (reference filter 650 nm) in a DTX 880 multimode detector (Beckman Coulter Inc., Fullerton, CA, USA). Results were expressed as percentage of absorbance in control cells. The half-maximal inhibitory concentration resulting in 50% cell-growth inhibition (IC50) was determined using CompuSyn software (ComboSyn, Paramus, NJ).

2.4. Clonogenic survival assay

Cells were seeded in 6-well plates at a density of 8000 cells/well for HepG2 and 500 cells/well for Huh7. After 72 h of treatment, cells were cultured in fresh medium without drugs for 7 days. Media were replaced every 2-3 days. Finally, cell colonies were washed twice with PBS, fixed with methanol for 10 min and stained with toluidine blue (1% (W/V) in 1% (W/V) sodium borate) for 5 min. The plates were rinsed with water, air-dried, photographed and evaluated for colony estimation. Colonies were counted and relative colony formation was determined by the ratio of the average number of colonies in treated cells to the average number of colonies in control cells.

2.5. Annexin V/propidium iodide assay

Cells were seeded in 6-well plates at a density of 500,000 cells/well for HepG2 and 250,000 cells/well for Huh7 and treated the next day for 72 h. After detachment of cells, apoptotic cell death was assessed by Annexin V-FITC and propidium iodide staining (FITC Annexin V Apoptosis Detection Kit II; BD Biosciences, San Jose, CA) coupled to flow cytometric analysis (BD FACSAria™ II cell sorter flow cytometer, BD Biosciences), as previously described (Ferretti et al., 2016). Detection of green and red fluorescence was performed, and the proportion of Annexin V (apoptotic) and propidium iodide (necrotic) positive cells were determined in the indicated experimental groups.

2.6. Wound healing assay

Cells seeded at 3×10^6/well for HepG2 and 1.5×10^6/well for Huh7 were cultured overnight in 6-well plates. After 24 h, cells were wounded by dragging a 200 µl pipette tip through the confluent monolayer, washed with PBS and treated for 24 h to allow migration. Images of wounds in the same field were captured when the scrape wound was introduced (0 h) and after 24 h of wounding using an inverted microscope (Zeiss, Axiovert 25) connected to a digital camera (Nikon Coolpix 990). The area and height of the wound were determined using ImageJ software (NIH) and the healing width was calculated as the area to height ratio. The migrated distance (µm) was then calculated with the formula = wound width at 0 h − wound width at 24 h, and expressed in percentage relative to control cells.

2.7. Spheroid growth and viability: spheroid growth delay and APH assays

Spheroids were obtained by liquid overlay technique in 96-well plates based on the protocol by Friedrich et al. (Friedrich et al., 2009), with slight modifications. Wells were coated with a mixture of 3% agarose in water (W/V): DMEM (1:1) and 2 h later HepG2 and Huh7 cells were seeded at a density of 1500 cells/well. Culture conditions were the same as for 2D cell culture. Spheroids were formed after an initiation interval of 4 days. Spheroids images were captured using an inverted microscope (Zeiss, Axiovert 25) connected to a digital camera (Nikon Coolpix 990) and diameters were determined using ImageJ software. Then, 50% of the supernatant was replaced by drug-supplemented fresh medium, containing different concentrations of cambinol or EX-527 (prepared at twice of the desired doses). Spheroid diameter at the onset of treatment was close to 400 µm, as recommended (Friedrich et al., 2009; Vinci et al., 2012). Spheroid volumes were calculated before and after treatments (0 and 72 h) using the equation: $V = (4/3)\pi R^3$; R = (D1 + D2)/4, where D1 and D2 are the maximal diameters of spheroids measured in the rectangular direction. Results were expressed as the percentage of volume at 72 h vs. 0 h (Vinci et al., 2012). Additionally, the acid phosphatase assay (APH) was performed at 72 h for viability assessment, according to the protocol of Friedrich et al. (Friedrich et al., 2007). The substrate *p*-nitrophenyl phosphate was kindly provided by Wiener Lab. (Buenos Aires, Argentina). Absorption of *p*-nitrophenol at 405 nm was measured in a DTX 880 multimode detector. Results were expressed as percentage of absorbance in control cells. The IC50 was determined using CompuSyn software.

2.8. Preparation of total cell homogenates

Cells were seeded in 6-well plates at a density of 500,000 cells/well for HepG2 and 250,000 cells/well for Huh7. After 72 h of treatment, cells were washed, scrapped, collected and resuspended in homogenization buffer (250 mM sucrose, 20 mM Tris-HCl, 5 mM EDTA; pH 7.4) containing protease inhibitors.

2.9. Protein concentration determination

The protein concentration was determined by Sedmak and Grossberg method (Sedmak and Grossberg, 1977), using bovine serum albumin as a standard.

2.10. Western blot analysis

Equal amounts of proteins (20 µg per lane) were resolved by 8% or 12% SDS-PAGE and electroblotted onto polyvinyl difluoride (PVDF) membranes (PerkinElmer Life Sciences Inc., Boston, MA, USA). Immunoblots were blocked with PBS-10% nonfat milk, washed and incubated overnight at 4 °C with primary antibodies. Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by enhanced chemiluminescence (ECL™) detection system (Thermo Fisher Scientific). The immunoreactive bands were quantified by densitometry using the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). Equal loading and transference of protein was checked by detection of GAPDH and by Ponceau S staining (latter data not shown) of the membranes.

2.11. SIRTs activity assay

For endogenous SIRTs activity measurement, total cell homogenates were obtained after 72 h of treatment and protein concentration was determined. SIRTs activity was measured with SIRT-Glo™ Assay and Screening System (Promega, Madison, WI, USA) using 20 µg of protein, according to the manufacturer's suggestions. This assay is a single reagent-addition, homogeneous, luminescent assay that measures the relative activity of SIRTs. Luminescence was measured in a DTX 880 multimode detector. Experimental values are represented as percentage of control.

2.12. RNA isolation, cDNA synthesis and real-time q-PCR

Cells were seeded in 6-well plates at a density of 500,000 cells/well for HepG2 and 250,000 cells/well for Huh7. After 72 h of treatment, total RNA was isolated from HCC cells by the TriZOL method (Life Technologies Inc, Gaithersburg, MD, USA) according to manufacturer's instructions. One microgram of total RNA was treated with DNase I (Thermo Fisher Scientific) and cDNA was made using an oligo-dT primer and M-MLV reverse transcriptase (Promega). PCR assay was performed using an Mx3000P Real-Time Thermocycler (Stratagene, La Jolla, CA, USA) with EvaGreen® dye (HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX); Solis BioDyne, Tartu, Estonia). Primer sequences for *MDR1* (encoding P-gp) and *MRP3* were: MDR1 (F): 5′ TACTTGGTGGC ACATAAAC 3′ (first nucleotide position: 3429), MDR1 (R): 5′ CCAAA GACAACAGCTGAAA 3′ (first nucleotide position: 3496); MRP3 (F): 5′ GTCCGCAGAATGGACTTGAT 3′ (first nucleotide position: 4732), MRP3 (R): 5′ TCACCACTTGGGGATCATTT 3′ (first nucleotide position: 4851). For each sample we analyzed *Cyclophillin A* (*CYPA*) expression to normalize target gene expression, using the following primers: CYPA (F): 5′ TCTGCCCACCTTAACAGACC 3′ (first nucleotide position: 2010), CYPA (R): 5′ AATTGCCCAACACACCAAAT 3′ (first nucleotide position: 2107). PCR reactions were initiated by incubation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Gene-specific amplification was confirmed by a single peak in the melting curve analysis. Relative changes in gene expression were determined by using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.13. Transfection with SIRT1 and SIRT2 short hairpin RNAs (shRNAs)

Two specific shRNAs targeting both, SIRT1 and SIRT2, were designed as 60-mers containing a 19-nt sequence derived from SIRT1 and SIRT2 mRNA, respectively, spaced by a hairpin-loop region and with *Bgl*II and HindIII enzyme sites. The control shRNA (scrambled) was designed by scrambling the nucleotides of one of these specific 19-nt sequences. Self-complementary inverted repeat sequences were synthesized as single strand oligonucleotides by Invitrogen (Carlsbad, CA, USA) and then annealed and cloned into pSUPER vectors (pSUPER RNAi System™; OligoEngine Inc., Seattle, WA, USA), following manufacturer's suggestions. Recombinant shRNA-pSUPER plasmids were used to transform competent *Escherichia coli* DH5α cells, which were selected and verified by sequencing. The 19-nucleotide sequences for shRNAs construction were as follows: shSIRT1-1: 5′ GTTGACCTCCTC ATTGTTA 3′ (first nucleotide position: 1350) and shSIRT1-2: 5′ GAAG TGCCTCAGATATTAA 3′ (first nucleotide position: 1425) targeting SIRT1 and shSIRT2-1: 5′ CCAACCATCTGTCACTACT 3′ (first nucleotide position: 735) and shSIRT2-2: 5′ TGTGGCTAAGTAAACCAT 3′ (first nucleotide position: 1843) targeting SIRT2.

For transient transfections, cells were seeded in 6-well plates at a density of 750,000 cells/well for HepG2 and 500,000 cells/well for Huh7. On the following day, cells were transfected with the plasmids using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. After 48 h, total cell homogenates were prepared for western blot analysis as described above.

2.14. Statistical analysis

Results were expressed as mean ± SE. Significance in differences was tested by one-way ANOVA, followed by Dunnett's test. Differences were considered significant when the p value was < 0.05.

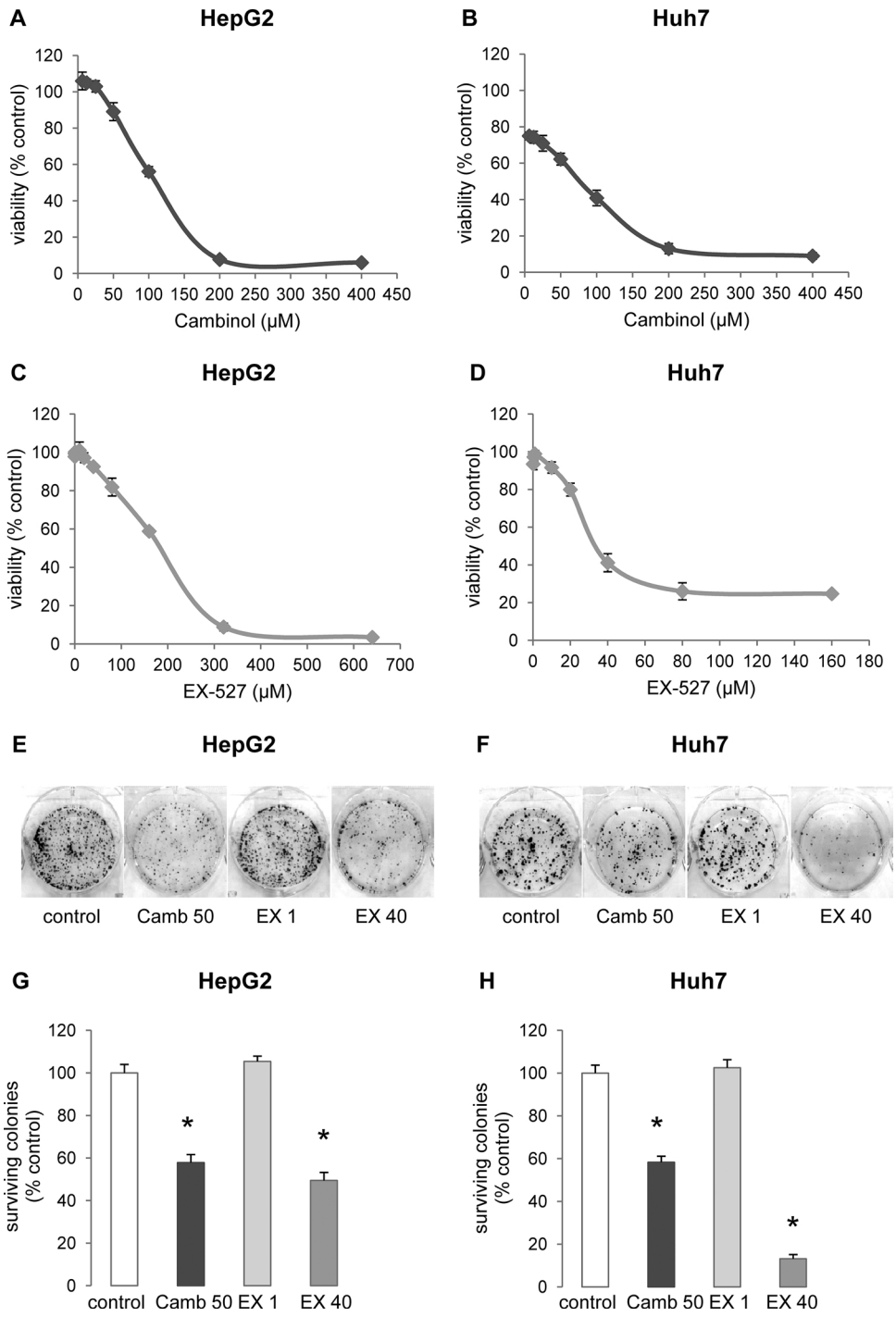

Fig. 1. Effect of cambinol and EX-527 on cellular viability and colony formation. (A-D) HepG2 and Huh7 cells were incubated for 72 h with different doses of cambinol and EX-527. Dose-response curves for cambinol (A HepG2, B Huh7) and EX-527 (C HepG2, D Huh7). Cell viability is expressed in percent value with control cells arbitrarily considered 100%. At least 3 independent experiments; n = 4 in each one. (E-H) HepG2 and Huh7 cells were incubated for 72 h with 50 μM cambinol (Camb 50), 1 μM or 40 μM EX-527 (EX 1 or EX 40) followed by growth in fresh culture media for 7 days. Representative wells are shown for HepG2 (E) and Huh7 (F) cells. Data are expressed as a percentage of colonies in control cells (arbitrarily considered 100%) of three independent experiments, each of which was performed in duplicate (G HepG2, H Huh7). Mean ± SE; *$p < 0.05$ *vs.* control.

3. Results

3.1. Cambinol and EX-527 treatments exert a cytotoxic effect on HCC cell lines

In order to evaluate the sensitivity of HepG2 and Huh7 cells to cambinol and EX-527, these HCC cell lines were treated with different doses of the drugs for 72 h to obtain the dose response curves and the IC50 values. As seen in Fig. 1, SIRTs 1 and 2 inhibitors significantly reduced cellular viability in a dose-dependent manner compared with untreated cells in both cell lines. The IC50 (μM) for cambinol and EX-527 was 104.13 ± 5.46 and 195.33 ± 11.57 in HepG2 (Fig. 1A and C, respectively) and 78.01 ± 9.08 and 33.37 ± 5.41 in Huh7 cells (Fig. 1B and D, respectively).

The inhibitors used in this study target both SIRT1 and SIRT2. However, while cambinol inhibits SIRT1 and SIRT2 with similar IC50s, EX-527 is much more selective for SIRT1 than for SIRT2 (Lugrin et al., 2013). Taken this information into consideration, together with the results on Fig. 1A–D, doses of 50 μM cambinol (Camb 50), 1 μM EX-527 (EX 1; only inhibits SIRT1) or 40 μM EX-527 (EX 40; inhibits both sirtuins) were chosen to perform all further experiments.

The clonogenic assay confirms the toxicity of these drugs as the% of colonies significantly diminished for Camb 50 and EX 40-treated cells compared to untreated cells (Fig. 1E–H).

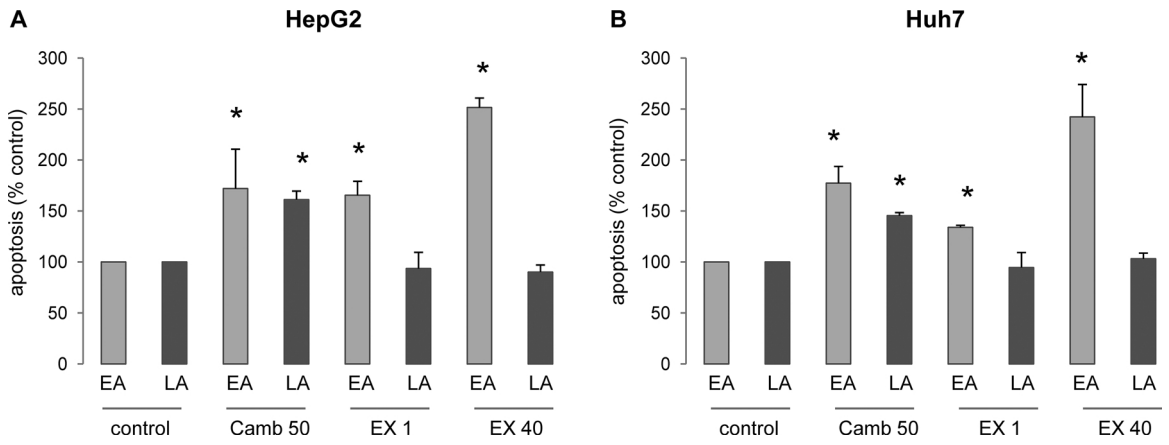

Fig. 2. Effect of cambinol and EX-527 on apoptosis. HepG2 (A) and Huh7 (B) cells were incubated for 72 h with 50 μM cambinol (Camb 50), 1 μM or 40 μM EX-527 (EX 1 or EX 40). Early (EA) and late (LA) apoptosis is expressed in percent value with control cells arbitrarily considered 100%. Three independent experiments, each of which was performed in duplicate. Mean ± SE; *p < 0.05 vs. control.

3.2. Cambinol and EX-527 treatments trigger apoptosis in HCC cell lines

Cytometric Annexin V-PI assay at 72 h demonstrated that both drugs augmented apoptosis in HepG2 and Huh7 cells (Fig. 2). Nevertheless, whereas Camb 50 incremented early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptosis, only early apoptosis was increased in the presence of EX-527 (both doses). No changes were found with Camb 50, EX 1 or EX 40 in primary necrotic death as assessed by determination of the Annexin V-/PI+ ratio (data not shown).

3.3. SIRTs 1 and 2 inhibitors lead to a decrease in cellular migration in HepG2 and huh7 cells

Since cellular migration is a key event during the metastatic process, it was our interest to study the effect of these inhibitors on the migratory capacity of HCC cell lines. To minimize potential proliferation or apoptosis effects we performed wound healing assays within a short time period (24 h), before the doubling time of the HCC cells (Sun et al., 2011). Besides, we performed MTT assays to check the effect of Camb 50, EX 1 and EX 40 on viability/cytotoxicity at 24 h. Wound healing assay shows that the average distance migrated was significantly reduced to a similar extent, irrespective of the cell line, drug or dose used (Fig. 3). Since MTT results at 24 h showed that cell viability was always > 90% (data not shown) while migration rate was significantly decreased at that time, we can infer that inhibition of migration by cambinol and EX-527 is not due to cytotoxic effect.

3.4. Cambinol and EX-527 inhibit spheroid growth and reduce its viability

In spite that 2D culture is a standard first line to evaluate anticancer agents, tumor cells grow *in vivo* in a three-dimensional space. Culture of spheroids become an attractive tool to study 3D growth, since it recreates the tumor microenvironment in the absence of an external matrix and resembles a solid tumor better than a monolayer (Mehta et al., 2012; Vinci et al., 2012). We evaluated and compared the sensitivity of HCC cells to cambinol and EX-527 in 2D and 3D cultures.

The volume of the spheroids was calculated before and after treatment in order to evaluate the effects of drugs on 3D growth (Vinci et al., 2012). Both inhibitors induced concentration-dependent growth inhibition in HepG2 and Huh7 spheroids as shown in Fig. 4.

In addition, cambinol and EX-527 significantly reduced cellular viability in a dose-dependent manner compared with untreated spheroids from both cell lines (Fig. 4). The IC50 values (μM) for cambinol and EX-527 were 135.36 ± 3.00 and 567.31 ± 40.58 in HepG2 and 130.38 ± 2.48 and 67.25 ± 15.45 in Huh7 cells. Importantly, these values were higher than those from the respective 2D cultures (Fig. 1) ($p < 0.05$), indicating a lower sensitivity of the spheroids to inhibitors treatment.

3.5. SIRTs 1 and 2 inhibitors modulate p53 and FoxO1 acetylation

It is known that SIRTs 1 and 2 inhibit proapoptotic effects of many tumor suppressor genes including p53 (Jin et al., 2008; Peck et al., 2010; Vaziri et al., 2001) and FoxO1 (Daitoku et al., 2011; Yang et al., 2005) as a consequence of their deacetylase activities, thus promoting cell survival.

To analyze whether cambinol and EX-527 modulate p53 and FoxO1 acetylation, we examined the levels of the total and acetylated forms of p53 and FoxO1 by western blot. Fig. 5A, B shows that acetyl-p53/p53 ratio significantly increased with EX 1 and EX 40 in both cell lines compared to the same ratio for control cells. In contrast, Camb 50 led to a significant decrease of this ratio in Huh7 cells. As seen in the representative western blot images in Fig. 5, there was a significant increase in p53 total protein levels in HepG2 (199.38 ± 5.23%*) and Huh7 cells (191.87 ± 15.56%*) treated with Camb 50 (*p < 0.05 vs. control cells).

On the other hand, the acetyl-FoxO1/FoxO1 ratio significantly increased in HCC cells treated with Camb 50, EX 1 and EX 40 compared to the same ratio for control cells (Fig. 5C,D). As seen from the representative western blot images in the same figure, the levels of total FoxO1 significantly diminished in HepG2 (Camb 50: 66.58 ± 7.64%*, EX 1: 51.94 ± 1.30%* and EX 40: 51.82 ± 8.59%*) and Huh7 (Camb 50: 36.85 ± 7.95%*, EX 1: 60.65 ± 6.03%* and EX 40: 11.65 ± 1.63%*) treated cells (*p < 0.05 vs. control cells).

Similarly, we explored if cambinol and EX-527 affected the expression of SIRTs 1 and 2. As seen in Fig. 5(E, F), SIRT1 protein levels decreased in HepG2 treated with EX-527 and in Huh7 treated with both drugs, whereas SIRT2 protein levels diminished after Camb 50 and EX 40 treatments only in Huh7 cells.

Finally, we measured SIRTs deacetylase activity after cambinol and EX-527 treatments. SIRTs activity decreased after exposure to Camb 50 and EX 40 in HCC cells compared to control group (Fig. 5G).

3.6. Cambinol and EX-527 are capable of modulating P-glycoprotein and MRP3 expression

MDR is the primary cause of chemotherapy failure against HCC, and the evidence strongly supports the role of energy-dependent efflux systems, like P-gp/MDR1 and MRP3, that pump chemotherapeutic drugs out of cells (Sun et al., 2013; Tomonari et al., 2016; Yang et al., 2013). In order to evaluate the effect of cambinol and EX-527 on the expression of both transporters, we measured their mRNA levels by

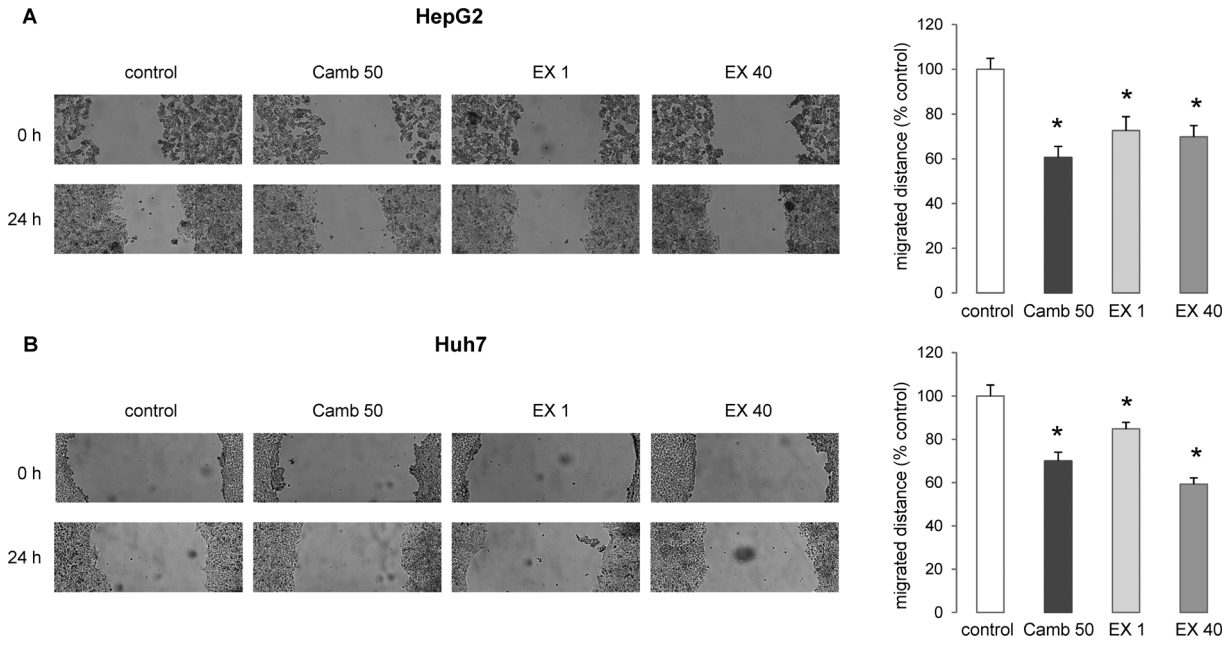

Fig. 3. Effect of cambinol and EX-527 on cellular migration. HepG2 (A) and Huh7 (B) cells were incubated for 72 h with 50 μM cambinol (Camb 50), 1 μM or 40 μM EX-527 (EX 1 or EX 40). Representative wound images are shown (left panel). The distance migrated by cells is expressed in percent value with control cells arbitrarily considered 100% (right panel). Three independent experiments, each of which was performed in duplicate. Mean ± SE; *p < 0.05 *vs.* control.

real-time q-PCR and their protein levels by western blot. As seen in Fig. 6(A, C), Camb 50 and EX 40 treatments led to a significant decrease of *MDR1* mRNA content in HepG2, whereas that of *MRP3* mRNA was significantly increased by EX 40. In Huh7 cells, the levels of *MDR1* transcript significantly raised in the presence of Camb 50 and EX 40, whereas *MRP3* transcript increased in Camb 50 group but decreased in response to EX 40 (Fig. 6B, D).

Regarding protein expression, Fig. 6(E, G) shows that P-gp and MRP3 were significantly downregulated in HepG2 cells after treatment with both inhibitors, except for the lowest dose of EX-527 that did not affect P-gp. In Huh7 cells, P-gp was significantly upregulated in Camb 50 and EX 40 groups, whereas the effects registered on MRP3 were more variable since Camb 50 increased but EX 40 reduced its content (Fig. 6F, H). Clearly, these drugs modulated the expression of ABC transporters differentially in the cell lines studied.

To confirm whether SIRTs 1 and 2 mediate the expression of ABC transporters as found with SIRTs inhibitors, we used shRNAs to knockdown SIRT1 (shSIRT1-1, shSIRT1-2) and SIRT2 (shSIRT2-1, shSIRT2-2) in HepG2 and Huh7 cells (Fig. 6I, J). The shRNAs effective inhibition of SIRT1 and SIRT2 expression was confirmed by western blotting analysis. For HepG2 cell line, the transcriptional silencing of SIRT1 or SIRT2 showed similar results to those found with SIRTs inhibitors; that is, a diminution in P-gp and MRP3 protein levels. In the case of Huh7 cells, transfection with shRNAs led to a diminution in MRP3 protein levels without an apparent change in P-gp levels.

4. Discussion

HCC is one of the most chemoresistant and lethal type of tumors, being the second most common cause of death from cancer worldwide (Ferlay et al., 2015). MDR is a key determinant of cancer chemotherapy failure and one of the major causes is the enhanced efflux of chemotherapeutics drugs due to the increase in the expression of one or more ABC transporters such as P-gp/MDR1 or MRP3 (Chen et al., 2016). Nowadays, no effective systemic therapy is available for HCC and there is an urgent need for new therapies. Since SIRTs 1 and 2 are overexpressed in HCC and are associated with tumoral progression and MDR (Chen et al., 2012, 2013; Xu et al., 2016), blocking SIRTs 1 and 2 activities may be beneficial for HCC patients. Small-molecule SIRTs 1 and 2 inhibitors have shown antiproliferative and/or proapoptotic effects in other types of cancers (Carafa et al., 2016; Olmos et al., 2011) and, more recently, it was described their ability to reduce cellular migration (Kim et al., 2016). However, further studies should be done to test the effect of these inhibitors on HCC and, more importantly, whether such effect is associated with reversion of ABC transporter-dependent MDR. Three aspects were particularly considered: i) the effects of SIRTs 1 and 2 inhibition on cell survival and migration, ii) the impact of this inhibition on p53 and FoxO1 acetylation, and iii) whether SIRTs 1 and 2 inhibition affects P-gp and MRP3 mRNA and protein expression.

In the first part of the study we found that simultaneous inhibition of SIRT1 and SIRT2 activities increased cellular toxicity in HepG2 and Huh7 cells in a dose-dependent manner, as shown by MTT assay and colonies counting. However, no significant changes were observed when only SIRT1 activity was blocked (EX 1). This suggests that both sirtuins activities need to be inhibited to produce cytoxicity or that the 1 μM dose of EX-527 is too low. We also found that SIRTs 1 and 2 inhibitors increased apoptotic cell death. Little information is available regarding the effect of cambinol or EX-527 on HCC cell lines growth. In fact, only one work explored the action of cambinol on HepG2 cells and found reduced colony formation and cell proliferation but no signs of cell death (Portmann et al., 2013). Further studies are necessary to address this contradictory aspect of SIRTs inhibition on apoptosis when using cambinol in HCC cells. On the other hand, EX-527 was used previously to treat HepG2 cells but in combination with Trichostatin A or H_2O_2, leading to increased apoptosis (Hu et al., 2015; Schuster et al., 2014); up to present there were no studies testing the cytotoxic effects of EX-527 alone. In line with our results, cambinol and other SIRTs inhibitors were effective in triggering apoptosis in diverse cancer cell lines (Carafa et al., 2016; Cea et al., 2011; Heltweg et al., 2006; Olmos et al., 2011; Stenzinger et al., 2013) and SIRT1 silencing sensitizes HepG2 and other HCC cell lines to apoptosis (Chen et al., 2011; Mao et al., 2014; Wang et al., 2012), as well as other human epithelial cancer cells (Tang, 2010).

It was reported that increased SIRT1 and SIRT2 expressions promoted migration and invasion in HCC cell lines and enhanced HCC tumor metastasis *in vivo* while SIRTs 1 and 2 knockdowns significantly reduced these phenomena (Chen et al., 2013; Hao et al., 2014; Li et al.,

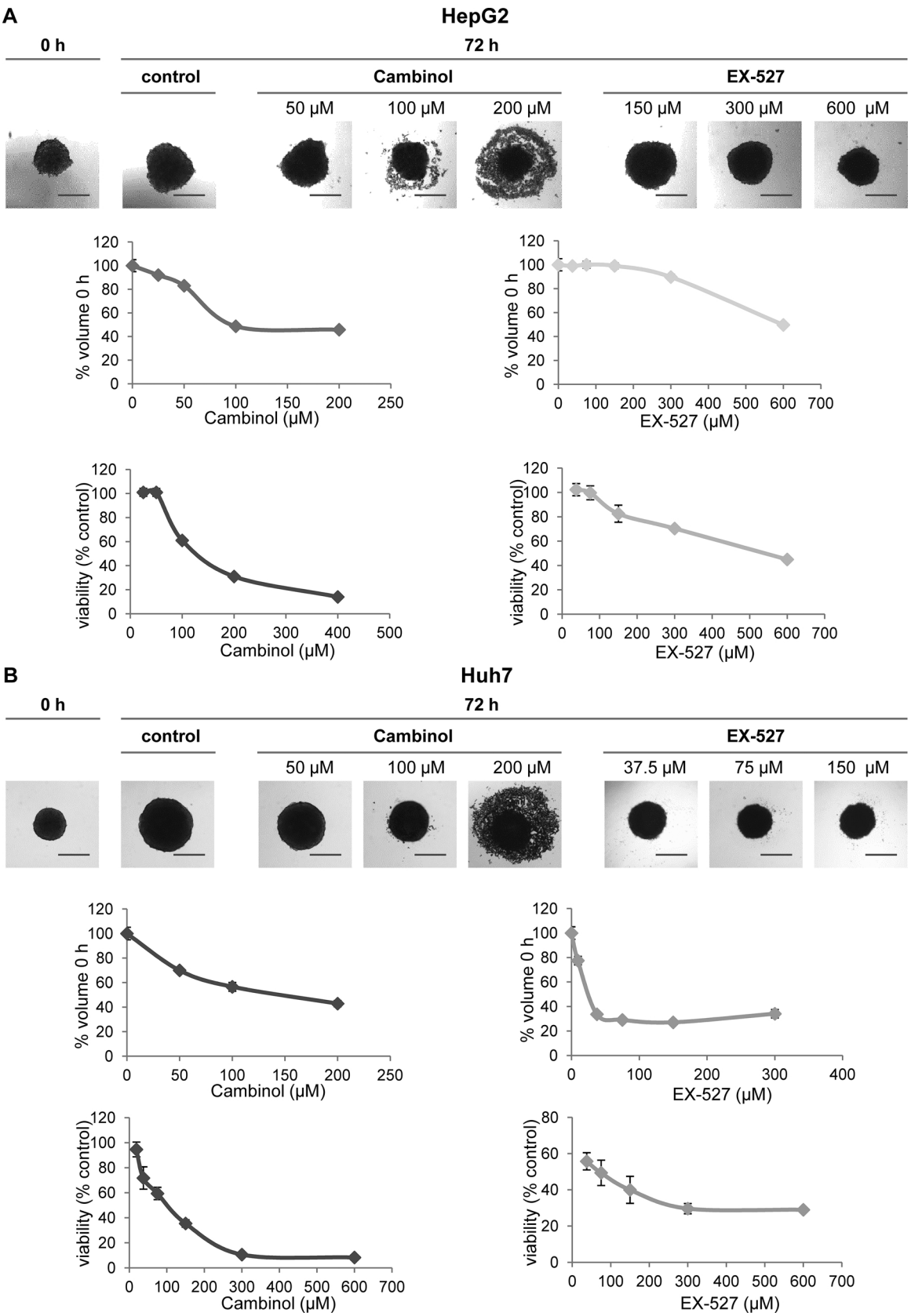

Fig. 4. Effect of cambinol and EX-527 on spheroid growth and viability. 3D cultures of HepG2 (A) and Huh7 (B) cells were incubated for 72 h with different doses of cambinol and EX-527. Representative spheroids images are shown for some doses (upper panel). Scale bar 400 μm. Results of spheroid growth are expressed as the percentage of volume at 72 h vs. 0 h with control cells arbitrarily considered 100% (middle panel). Cell viability in dose-response curves is expressed in percent value with control cells arbitrarily considered 100% (lower panel). Three independent experiments; n = 8 in each one. Mean ± SE; *p < 0.05 vs. control.

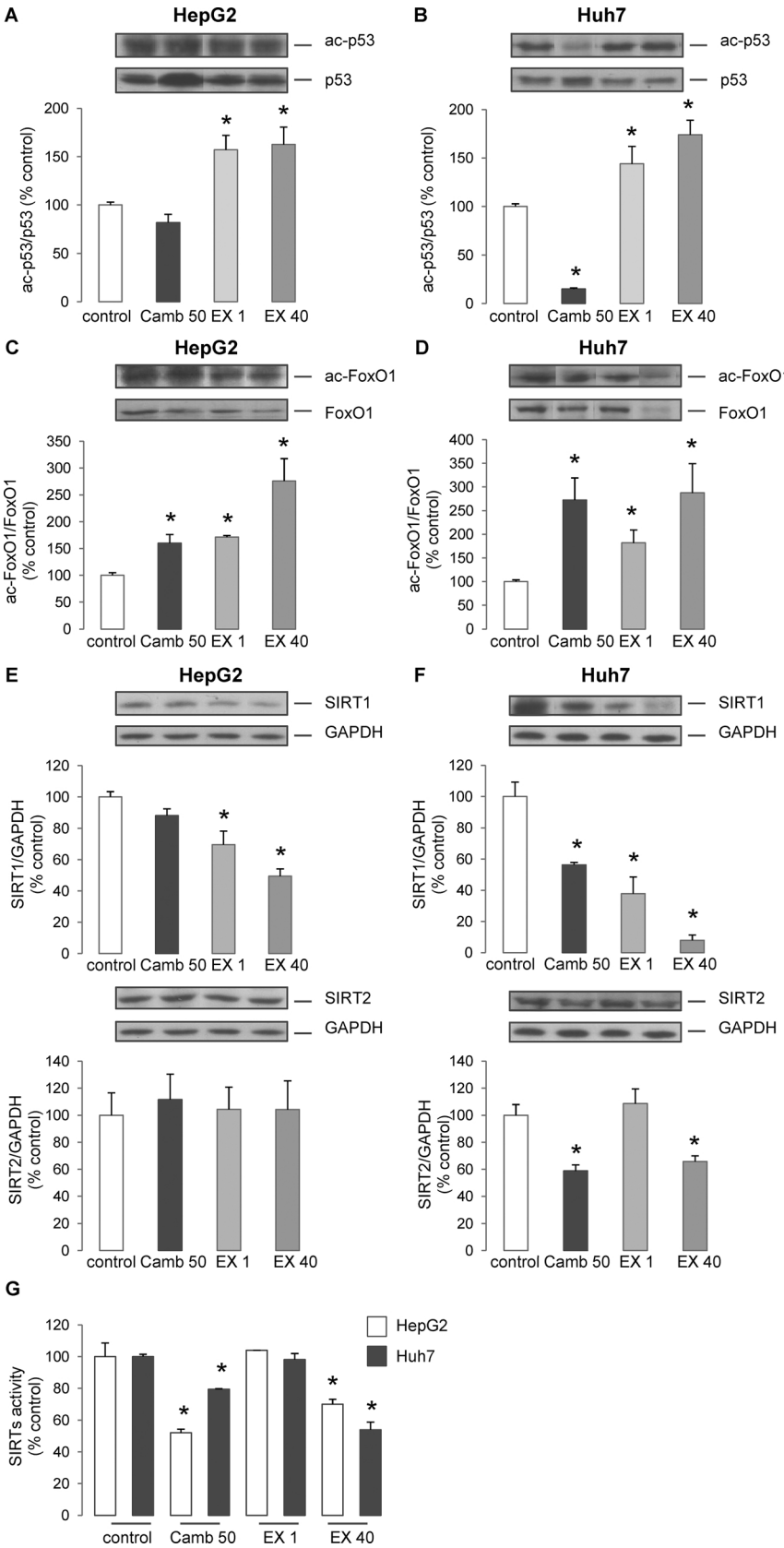

Fig. 5. Effect of cambinol and EX-527 on p53 and FoxO1 acetylation and on SIRTs 1 and 2 expression and activity. HepG2 and Huh7 cells were incubated for 72 h with 50 μM cambinol (Camb 50), 1 μM or 40 μM EX-527 (EX 1 or EX 40). Levels of the acetylated and total forms of p53 (A HepG2, B Huh7) and FoxO1 (C HepG2, D Huh7). Densitometric analysis was performed and results are expressed as the acetylated protein/total protein ratio and showed in percent values with control cells arbitrarily considered 100%. SIRT1 and SIRT2 protein levels (E HepG2, F Huh7). GAPDH was probed as loading control. Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. SIRTs deacetylase activity (G). Luminescent signal measurement was carried out and results are expressed in percent values with control cells arbitrarily considered 100%. Three independent experiments; n = 3 in each one. Mean ± SE; *p < 0.05 *vs.* control.

2016). In addition, these reports revealed a positive correlation between SIRT1 and SIRT2 expressions in HCC tumors and adverse patient prognosis. Thus, it was of interest to analyze if cambinol and EX-527 were capable of regulating cellular migration. We found a significant impairment of wound-healing ability in HepG2 and Huh7 cells treated with both inhibitors, including the low dose of EX-527 (1 μM). This suggests that inhibition of SIRT1 alone is sufficient to reduce migration. This finding does not exclude the additional involvement of SIRT2 in

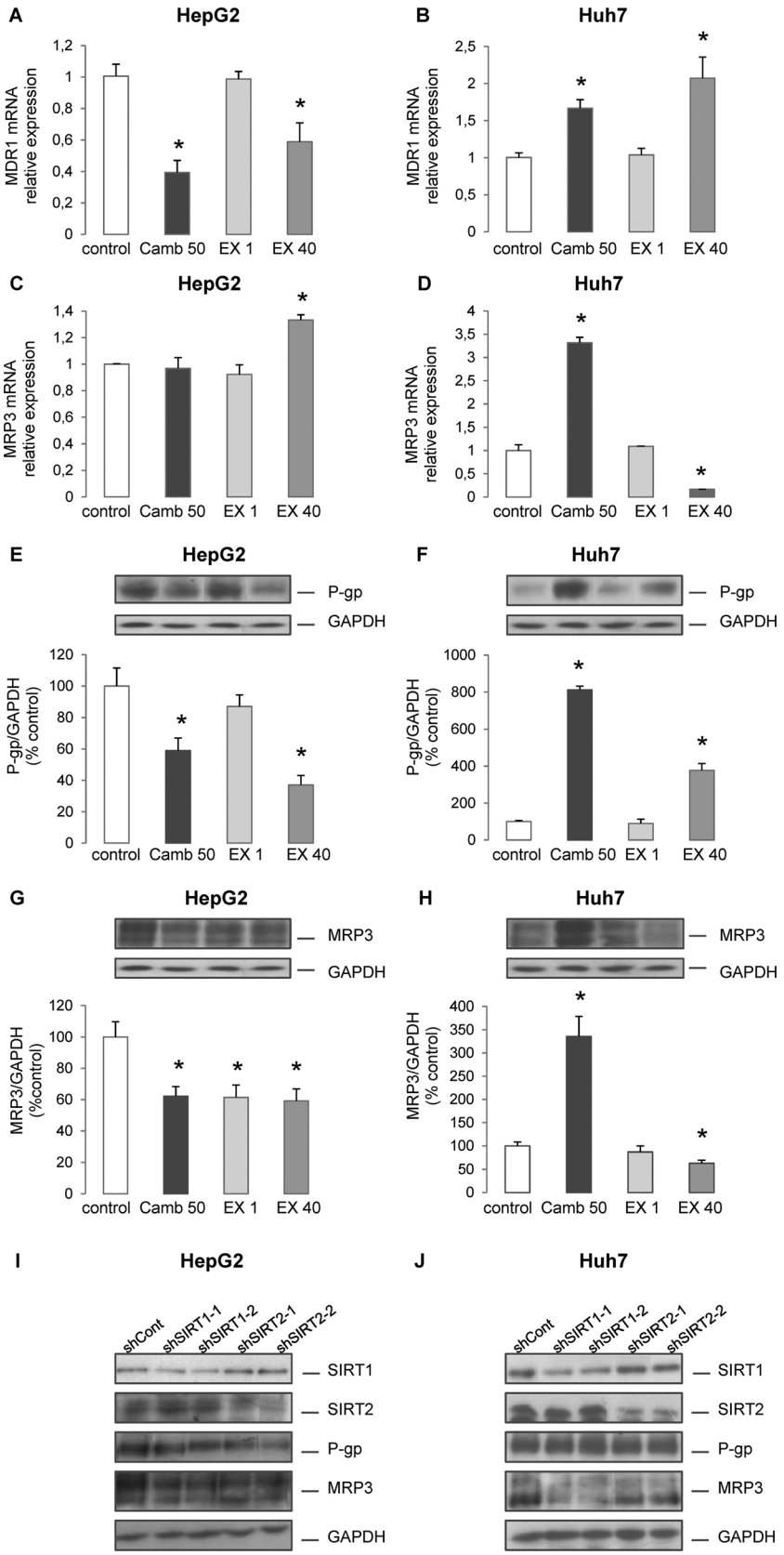

Fig. 6. Effect of cambinol and EX-527 on P-gp and MRP3 expression. HepG2 and Huh7 cells were incubated for 72 h with 50 μM cambinol (Camb 50), 1 μM or 40 μM EX-527 (EX 1 or EX 40). Expression levels of the genes encoding P-gp (MDR1) (A HepG2, B Huh7) and MRP3 (C HepG2, D Huh7). mRNA values in treated cells were calculated relative to the amount found in control cells, which was arbitrarily defined as 1. P-gp (E HepG2, F Huh7) and MRP3 (G HepG2, H Huh7) protein levels. GAPDH was probed as loading control. Densitometric analysis was performed and results are expressed in percent values with control cells arbitrarily considered 100%. Three 3 independent experiments; n = 3 in each one. Mean ± SE; *p < 0.05 *vs.* control. HepG2 and Huh7 cells were transfected for 48 h with control (shCont), SIRT1-targeting (shSIRT1-1 and shSIRT1-2) or SIRT2-targeting (shSIRT2-1 and shSIRT2-2) shRNAs. Protein levels of SIRT1, SIRT2, P-gp and MRP3 (I HepG2, J Huh7). GAPDH was probed as loading control.

this process. Our results extends those from Portmann et al. (Portmann et al., 2013) reporting dose-dependent reduction of cell migration by cambinol in the HCC cell line Hep3 B and, more importantly, identified SIRT1 as a potential target to modulate cell migration in HCC.

It is well accepted that spheroids resemble the native tissue from which the component cells originated, offering *in vitro* models that closely mimic *in vivo* conditions (Pampaloni et al., 2007). Importantly, most therapeutic approaches were found to be less effective in 3D than

in 2D cultures (Friedrich et al., 2009; Mehta et al., 2012; Vinci et al., 2012). To establish whether cambinol and EX-527 are capable of affecting the growth and viability of 3D cultures as demonstrated for the 2D cultures (Fig. 1), we compare the IC50 values between these models. Our results revealed that both drugs were able to reduce the growth and viability of spheroids in a dose-dependent manner. Furthermore, spheroids presented higher IC50 values for both compounds, which is in agreement with the overall higher drug resistance reported in the literature for 3D cultures (Friedrich et al., 2009; Mehta et al., 2012; Vinci et al., 2012). To our knowledge, this is the first work aimed to explore the effect of SIRTs inhibitors on 3D cultures. The data suggest that they could be effective in reducing growth and viability of HCC cells *in vivo*.

It is widely documented that SIRTs 1 and 2 are linked to cellular survival pathways by keeping the tumor suppressors p53 and FoxO1 deacetylated (Daitoku et al., 2011; Jin et al., 2008; Peck et al., 2010; Yang et al., 2005). Consistent with these results, we found that EX-527 raised the acetyl-p53/p53 ratio in both cell lines, probably favoring the proapoptotic behavior of this tumor suppressor protein. This was achieved with EX 1 and EX 40, in agreement with the fact that SIRT1 and SIRT2 can acetylate p53 indistinctly. Surprisingly, Camb 50 led to a significant decrease of this ratio in Huh7 cells and showed a trend to lower it in HepG2 cells. No information is available concerning the impact of cambinol on p53 acetylation in HCC cell lines. It was recently demonstrated that, unlike the specific inhibitor EX-527, cambinol lacks specificity and acts by targeting more than just SIRT1 and SIRT2 (Lugrin et al., 2013). Thus, it is possible that cambinol affects p53 acetylation through other pathways. While EX-527 did not affect total p53 protein levels, cambinol did so. This could be tentatively associated with the increment produced in early and late apoptosis shown above. In additional studies we observed that the acetyl-FoxO1/FoxO1 ratio significantly increased in HCC cells treated with cambinol and EX-527. Again, this was observed with both doses of EX-527, consistent with the notion that SIRT1 and SIRT2 indistinctly acetyate FoxO1. As for p53, the increment of FoxO1 acetylation levels probably exacerbates its ability to induce apoptosis. In addition, the levels of total FoxO1 significantly diminished in the presence of both inhibitors. This is the first study reporting an effect on both FoxO1 acetylation and expression in HCC cell lines. We also analyzed if these drugs modified SIRTs 1 and 2 protein levels in addition to their inhibitory action. The data showed a decrease in SIRT1 protein levels for EX 1 and EX 40 treatments in HepG2 cells and for all treatments in Huh7 cells. In the case of SIRT2 protein levels, there was a diminution after Camb 50 and EX 40 exposure only in Huh7 cells. The lack of change in SIRT2 levels with EX 1 was in accordance with the fact that EX-527 inhibits only SIRT1 enzymatic activity at this particular concentration. When measured the activity of intracellular sirtuins, we found a decrease upon Camb 50 and EX 40 treatments. Despite the commercial kit detects total SIRTs activity without discrimination between SIRT members, it can be stated that both Camb 50 and EX 40 treatments affected the deacetylase activity of SIRTs. In spite that EX 1 did not change SIRTs activity, the increment found in p53 and FoxO1 acetylation in this group suggests a reduction of SIRT1 deacetylase activity for this dose of EX-527.

Since it was described that SIRT1 overexpression promotes MDR in HCC (Chen et al., 2012; Liang et al., 2008), we analyzed the effect of cambinol and EX-527 on the expression of P-gp and MRP3. Despite it was recently reported the inhibitory effect of EX-527 on P-gp expression and activity in diverse cancer cell lines (Kim et al., 2015; Zhu et al., 2012), there is no information linking EX-527 and P-gp in HCC cell lines. In our study, we found a decrease in P-gp expression after inhibition of SIRTs 1 and 2 in HepG2, probably consistent with the raise of FoxO1 acetylation and/or the decrease of FoxO1 level. These regulations could ultimately result in decreased chemoresistance. In support to this possibility, SIRT1 inhibition reduced total protein levels of FoxO1 and P-gp expression, favoring the uptake of a chemotherapeutic agent in breast cancer cells (Oh et al., 2010). In our study, transfection of HepG2 cells with shRNAs against SIRT1 or SIRT2 also led to a diminution of P-gp and MRP3 protein levels. Importantly, this is the first study describing a link between MRP3 and SIRTs. However, and similarly, Ling et al. (Ling et al., 2017) demonstrated recently that SIRT1 downregulation induced the suppression of MRP1, which is the closest homologue of MRP3 among the MRP family, and increased the intracellular concentration of adriamycin in a HCC cell line resistant to 5-fluorouracil (BEL/FU). In view of the fact that HCC cells resistant to sorafenib, the only approved drug for advanced HCC (Wilhelm et al., 2008), exhibited increased expression of P-gp (Wu et al., 2016) and MRP3 (Chow et al., 2013; Tomonari et al., 2016), our finding that these ABC transporters are downregulated in the presence of SIRT inhibitors in HepG2 cells could be of extreme value in finding therapeutic strategies directed to overcome MDR. In contrast to the findings on the effect of SIRT inhibitors in HepG2 cells, treatment with these drugs resulted in induction of P-gp and MRP3 levels in Huh7 cells, except for the higher dose of EX-527 that downregulated MRP3. Transfection of Huh7 cells with shRNAs targeting SIRT1 or SIRT2 generated the reduction of MRP3 protein levels without apparent changes of P-gp protein levels. Although we do not know the reason for the dissimilar behavior regarding ABC expression between cell lines, a candidate could be the p53 suppressor protein. While HepG2 cells carry wild-type p53, Huh7 cells have a mutant p53 gene (Hsu et al., 1993) which maintain its transcriptional activity (Hsieh et al., 2003) and can be acetylated by SIRT1 (Zhang and Zhou, 2013). However, it was demonstrated that whereas wild-type p53 generally represses MDR1 promoter, some mutants of p53 stimulate its activity (Zastawny et al., 1993). In this way, certain p53 mutants upregulated P-gp expression and activity in cancer cells, including in a HCC cell line (Chan and Lung, 2004; Tsou et al., 2015). This mechanism could be also the responsible for the dissimilar behavior between P-gp and MRP3 in Huh7 cells, when considering only the specific SIRTs 1 and 2 inhibitor EX-527. This phenomenon should be considered in the eventuality of combining SIRTs inhibitors with conventional chemotherapy. Nonetheless, even when a scenario for ABC transporter regulation similar to Huh7 cells is presented, the benefit of the combination treatment should not be ruled out when the chemotherapeutic drug is not a P-gp substrate or when transported by MRP3 in addition to P-gp.

Taking altogether, effects of cambinol, EX-527 and shRNAs targeting SIRTs 1 and 2 on P-gp and MRP3 indicate that SIRT2, in addition to SIRT1, plays a role in MDR. In this relationship, Karwaciak et al. (Karwaciak et al., 2015) found that SIRT2 inhibition reduced MRP1 gene expression sensitizing melanoma cells to an anticancer agent. In line with this, Xu et al. (Xu et al., 2016) reported that SIRT2 expression was higher in MRP1-mediated multidrug resistance acute myeloid leukemia cells than in responding cells and that SIRT2 silencing decreased MRP1 level and enhanced drug accumulation. Thus, SIRTs 1 and 2 may cooperate not only to deacetylate specific targets and affect cellular survival or migration but also to modulate MDR.

5. Conclusions

Our findings clearly show a negative modulation of both SIRTs 1 and 2 inhibitors on cell survival and migration in HCC cell lines, even in the absence of a chemotherapeutic drug. These results suggest that SIRTs 1 and 2 activities blockage could be beneficial during HCC therapy. Data on p53 and FoxO1 acetylation are consistent with proapoptotic behavior of these tumor suppressor proteins. Downregulation of P-gp and MRP3 in HepG2 cells supports an additional potential application of SIRTs 1 and 2 inhibitors if used in combination with conventional chemotherapeutic drugs to overcome MDR during HCC therapy. However, it should be always taken into account the genetic background of each HCC since in Huh7 cells P-gp was upregulated. Finally, the cytotoxic effects exerted by SIRTs 1 and 2 inhibitors were confirmed in 3D model cultures of both cell lines. Taken together, our findings provide a rationale for clinically exploring the use of SIRTs 1 and 2 inhibitors in HCC therapy.

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