Cancer Letters xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Cancer Letters

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

Targeted therapy against chemoresistant colorectal cancers: Inhibition of p38α modulates the effect of cisplatin *in vitro* and *in vivo* through the tumor suppressor FoxO3A

Aldo Germani [a,1], Antonio Matrone [a,1], Valentina Grossi [b,1], Alessia Peserico [a], Paola Sanese [a], Micaela Liuzzi [c], Rocco Palermo [d], Stefania Murzilli [e], Antonio Francesco Campese [d], Giuseppe Ingravallo [c], Gianluca Canettieri [d], Tugsan Tezil [a,*], Cristiano Simone [a,f,*]

[a] *Laboratory of Signal-Dependent Transcription, Department of Translational Pharmacology (DTP), Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Ch), Italy*
[b] *Cancer Genetics Laboratory, IRCCS S. de Bellis, 70013 Castellana Grotte, Italy*
[c] *Division of Pathological Anatomy, DETO, University of Bari, 70124 Bari, Italy*
[d] *Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy*
[e] *Animal Care Facility, Department of Translational Pharmacology (DTP), Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Ch), Italy*
[f] *Division of Medical Genetics, DIMO, University of Bari, 70124 Bari, Italy*

ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form 22 October 2013
Accepted 22 October 2013
Available online xxxx

Keywords:
Dual therapy
p38 MAPK
Chemoresistance
Colorectal cancer
Cell death

ABSTRACT

Chemoresistance is a major obstacle to effective therapy against colorectal cancer (CRC) and may lead to deadly consequences. The metabolism of CRC cells depends highly on the p38 MAPK pathway, whose involvement in maintaining a chemoresistant behavior is currently being investigated. Our previous studies revealed that p38α is the main p38 isoform in CRC cells. Here we show that p38α pharmacological inhibition combined with cisplatin administration decreases colony formation and viability of cancer cells and strongly increases Bax-dependent apoptotic cell death by activating the tumor suppressor protein FoxO3A. Our results indicate that FoxO3A activation up-regulates transcription of its target genes (p21, PTEN, Bim and GADD45), which forces both chemosensitive and chemoresistant CRC cells to undergo apoptosis. Additionally, we found that FoxO3A is required for apoptotic cell death induction, as confirmed by RNA interference experiments. In animal models xenografted with chemoresistant HT29 cells, we further confirmed that the p38-targeted dual therapy strategy produced an increase in apoptosis in cancer tissue leading to tumor regression. Our study uncovers a major role for the p38-FoxO3A axis in chemoresistance, thereby suggesting a new therapeutic approach for CRC treatment; moreover, our results indicate that Bax status may be used as a predictive biomarker.

1. Introduction

Colorectal cancer is one of the most frequent causes of cancer death for both genders with one million new cases recorded annually worldwide [1]. Thanks to the recent advances in clinical oncology, colorectal tumors usually respond to chemotherapy; however, most patients succumb to recurrent tumors originated from chemoresistant clones. Cisplatin is one of the most frequently used drugs in chemotherapy, but some tumor types, including colon, ovarian and lung cancer, have been shown to be more likely to develop chemoresistance. These neoplasms may respond well to cisplatin at the beginning (up to 70% of cell death); however, the death rate of cancer cells gradually decreases to 15–20% over time [2–4]. Nevertheless, in some clinical settings cisplatin remains the most suitable therapeutic option. Therefore, the need for developing new chemosensitization strategies is essential to improve patient survival.

Cisplatin most prominent mode of action is the induction of the intrinsic apoptotic pathway through activation of the DNA damage response. Most cisplatin-resistant tumor cells show similar characteristics at the molecular level. First, they commonly display a decreased uptake and/or increased efflux of cisplatin, which is mediated by specific transporters, such as MDRs, ATP7B and CTR1, interfering with the formation of cisplatin-DNA adducts [5–9]. Second, they show high activity of DNA repair pathways, which

* Corresponding authors. Address: Laboratory of Signal-Dependent Transcription, DTP, Consorzio Mario Negri Sud, Via Nazionale 8/A, 66030 Santa Maria Imbaro (Ch), Italy. Tel.: +39 0872570 210; fax: +39 0872570 299 (T. Tezil). Address: Division of Medical Genetics, DIMO, Faculty of Medicine, University of Bari 'Aldo Moro', Policlinico, Piazza Giulio Cesare 11, 70124 Bari, Italy. Tel.: +39 0805478270; fax: +39 0805593618 (C. Simone).

E-mail addresses: teziltugsan@sabanciuniv.edu, tezil@negrisud.it (T. Tezil), cristiano.simone@uniba.it, simone@negrisud.it (C. Simone).

[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.canlet.2013.10.035

helps chemoresistant cells to efficiently reverse cisplatin-DNA adducts [10]. MAPKs (Mitogen-Activated Protein Kinases) have an important role in cisplatin-mediated changes in gene expression due to their ability to sense small molecular alterations within the cell. These signaling networks respond to a wide range of external stimuli including growth factors and chemical stresses [11]. In particular, the p38 MAPK pathway is involved in metabolism, cell cycle and cell death by regulating the activity of several transcription factors in a signal- and tissue-specific manner. This cascade is actually thought to represent a central node in the response to cisplatin through the interplay with various signaling pathways such as JNK, ERK, AMPK and PI3K [12,13]. Interestingly, high levels of p38 expression and activity have been reported in CRC cells compared to their normal counterparts, suggesting its involvement in cell survival [14–17]. We previously showed that p38α, one of the four p38 MAPKs identified so far, is required for CRC cell survival and proliferation. Indeed, pharmacological blockade of its kinase activity or silencing of its expression by RNA interference induces autophagy, growth arrest and cell death [18,19]. Although the p38 MAPK pathway has been shown to enhance apoptosis induction in response to several chemotherapeutics, in most chemoresistant colon/colorectal cancers p38α is believed to support cell survival. Irinotecan, for instance, further activates p38α by promoting its phosphorylation, and inhibition of p38α sensitizes chemoresistant colon cancer cells to drug treatment [20,21]. When the p38 MAPK pathway is pharmacologically inhibited by SB203580 or SB202190, CRC cells appear to be more sensitive to chemotherapy with 5-fluorouracil due to increased Bax expression [22]. There are several molecular players that are modulated in response to p38α inhibition and more and more are being identified. However, the exact mechanism of chemoresistance in CRC cells has not been elucidated yet.

Here we show that the p38 pathway is activated in chemoresistant (HT29) and chemosensitive (HCT116) CRC cells in response to cisplatin. The dual therapy based on specific p38α inhibition and cisplatin treatment not only sensitizes chemoresistant cells, but also modulates the effect of cisplatin in sensitive CRC cells. In particular, it inhibits cell division and promotes Bax-dependent apoptosis. Our data also reveal that these effects are mediated by the tumor suppressor protein FoxO3A through the regulation of a subset of its target genes, including p21, PTEN, Bim and GADD45. Finally, tumor xenograft experiments support the efficacy of p38α inhibition combined with cisplatin treatment *in vivo*. Overall, our study suggests that targeting p38 might be a goal-directed and effective pharmacological intervention in the therapy of advanced colorectal cancer.

2. Materials and methods

2.1. Cell culture and reagents

HT29, Caco2, SW480, LoVo, LS174T (all from ATCC), HCT116 $Bax^{+/-}$ and $Bax^{-/-}$ (kindly provided by Dr. Bert Vogelstein, John Hopkins University, Baltimore) [23] cells were grown in DMEM including 10% FBS (HT29, SW480, LS174T and HCT116) or 20% FBS (Caco2 and LoVo), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2 avoiding confluence at any time. SB202190 was purchased from Calbiochem or Sigma-Aldrich. Cisplatin was purchased from Sigma–Aldrich. For *in vivo* experiments, cisplatin was dissolved in 0.9% sodium chloride solution and stored in the dark.

2.2. In vivo studies

Female CD-1 athymic nude mice (6-8-week old) were obtained from Charles River Laboratories. For developing xenograft tumors, 10×10^6 HT29 cells were injected subcutaneously into the flanks (0.2 ml per flank) of CD-1 mice. The volume of the tumors was measured every 2–3 days and calculated using the following formula: volume $(mm^3) = (width)^2 \times length \times 0.5$. When the tumor volume reached 60 mm^3, mice were randomized into four different treatment groups: vehicle (DMSO, $n_{mice} = 7$, $n_{tumors} = 10$), cisplatin (2 mg/kg, $n_{mice} = 7$, $n_{tumors} = 11$), SB202190 (25 μg/kg, $n_{mice} = 7$, $n_{tumors} = 11$), and SB202190 plus cisplatin (SB202190: 25 μg/kg, cisplatin: 2 mg/kg, $n_{mice} = 7$, $n_{tumors} = 13$). SB202190 (daily) and cisplatin (once every three days) were both given by intraperitoneal injection. At the end of the *in vivo* studies, mice were sacrificed. All procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

2.3. Quantitative real-time PCR and RNA interference

Total RNAs were extracted using TRI Reagent (Sigma). Samples were treated with DNase-1 (Ambion) and retro-transcribed using the High Capacity DNA Archive Kit (Applied Biosystems). PCRs were carried out using the SYBR Green PCR Master Mix on an ABI 7500HT machine (Applied Biosystems). Relative quantification was done using the ddCT (Pfaffl) method. For RNA interference, cells were transfected with either 50 nM Stealth siRNAs directed against FoxO3A or non-silencing siRNAs, by using RNAiMAX (Invitrogen). siRNA and primer sequences are available upon request.

2.4. Microscopic quantification of viability and cell death

Cell viability and cell death of the reported cell lines were scored by counting. The supernatants (containing dead/floating cells) were collected, and the remaining adherent cells were detached by Trypsin/EDTA (Sigma). Cell pellets were resuspended in 1X PBS and 10 μl were mixed with an equal volume of 0.01% trypan blue solution. Viable cells (unstained, trypan blue negative cells) and dead cells (stained, trypan blue positive cells) were counted with a phase contrast microscope. The percentages of viable and dead cells were calculated. The data shown in the Results section are representative of 3 or more independent sets of experiments.

2.5. Immunoblot analysis

Immunoblotting analyses were performed according to Cell Signaling instructions. Briefly, cells were homogenized in 1X lysis buffer (50 mM Tris-HCl pH 7.4; 5 mM EDTA; 250 mM NaCl; 0.1% Triton X-100) supplemented with protease and phosphatase inhibitors (1 mM PMSF; 1.5 μM pepstatin A; 2 μM leupeptin; 10 μg/ml aprotinin, 5 mMNaF; 1 mM Na3VO4). 15–20 μg of protein extracts from each sample were denatured in 5× Laemmli sample buffer and loaded into an SDS–polyacrylamide gel for western blot analysis. Western blots were performed using anti-β-Actin (Sigma), anti-β-tubulin, (Santa Cruz Biotechnology), anti-p38α, anti-phospho-MAPKAPK-2(Thr222) (MK2), anti-caspase 3 (all from Cell Signaling), anti-PARP-p85 (Promega), anti-MAP1LC3 (Novus Biologicals), anti-FoxO3A (Cell Signaling). Western blots were developed with the ECL-plus chemiluminescence reagent (GE Healthcare) as per manufacturer's instructions.

2.6. Colony formation assay

CRC cells were cultured in 60 mm dishes in the presence or absence of SB202190, cisplatin or their combination. After 48 h, media were discarded and cells were washed twice with 1X PBS. 2 ml of Coomassie brilliant blue (Bradford) were added into each dish for 5 min and then cells were washed with ethanol 70% to remove the excess of Coomassie. Plates were dried at room temperature.

2.7. FACS analysis

Cells were harvested and live-stained with FITC-conjugated Annexin-V (Sigma). Then, they were subjected to cell death analysis with a FACS Vantage flow cytometer and the Cell Quest-PRO software (BD Bioscience).

2.8. Histology, immunohistochemistry and apoptosis assays

HT29-derived mice colorectal tumor specimens were fixed overnight in 10% neutral-buffered formalin, embedded in paraffin, sectioned at a thickness of 4 μm and stained with hematoxylin-eosin. Additional sections, collected on poly-L-lysine coated slides, were used for immunohistochemical stains that were performed with avidin-biotin-based detection systems. Sections were incubated overnight at 4 °C with antibodies against phospho-p38 (Cell Signaling). Appropriate negative controls were obtained by replacing primary antibodies with pre-immune serum, and positive controls were included in the procedure. The TUNEL assay (Roche) was performed on tissue sections according to the manufacturer's instructions.

2.9. Immunofluorescence

Immunofluorescence assays were performed using an anti-FoxO3A antibody (Cell Signaling). Nuclei were counterstained using PI (propidium iodide) (Invitrogen) and the images were acquired using a Zeiss LSM5 Pascal confocal microscope.

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2.10. Cell proliferation assay (WST-1)

Cell proliferation was determined using the Cell Proliferation Reagent WST-1 (Roche) as per manufacturer's instructions. Briefly, cells were seeded into 96-well plates one day before treatment. After 24 h, 48 h or 72 h drug (or DMSO) exposure, 10 μl of the Cell Proliferation Reagent WST-1 were added to each well and incubated at 37 °C in a humidified incubator for 1 h. The absorbance was measured on a microplate reader (BioTek) at 450/655 nm. Each assay was performed in 6 replicates and the experiment was repeated twice. The proliferation index was calculated as the ratio of WST-1 absorbance of treated cells at the indicated time point (24 h or 48 h) to the WST-1 absorbance of the same experimental group at 0 h.

2.11. Statistical analysis

The statistical significance of the results was analyzed using Student's *t*-tail test, and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

3. Results

3.1. p38α activation is involved in cisplatin response in CRC cells

The p38 MAPK pathway has been proposed as a key intermediate determining the cellular effect of various chemotherapy drugs. Activation of the p38 MAPK pathway has been previously shown to be a protective mechanism in gastric cancer cells, where it sustains cell survival and thereby supports chemoresistance against doxorubicin, cisplatin and vincristine [24,25]. Increased levels of p38 phosphorylation have been reported as one of the major mediators of tumor progression and chemoresistance in lung cancer [26]. Activated p38 was also proposed as a marker of chemoresistance against irinotecan, and has been found to be an important target for sensitizing chemoresistant CRC cells to etoposide [20]. According to our previous findings, p38α represents the main p38 isoform in CRC cells [18,27]. To evaluate the effect of cisplatin on p38α activation in two different CRC cell lines, we treated HCT116 and HT29 cells with cisplatin for 48 h; then, total protein extracts were subjected to immunoblotting (Fig. 1A). The results showed phosphoactivation of the p38α pathway in both HCT116 and HT29 cells. Indeed, MK2 (MAPK-activated protein kinase 2), a direct p38α substrate and one of its main downstream effectors, was also phosphoactivated after cisplatin exposure, thus confirming cisplatin-dependent activation of the p38 MAPK pathway through p38α in CRC cells. To monitor the effect of cisplatin on colony formation, HCT116 and HT29 cells were treated with a physiologically relevant concentration of cisplatin (30 μM) and colony densities were measured. Following cisplatin exposure, HCT116 cells

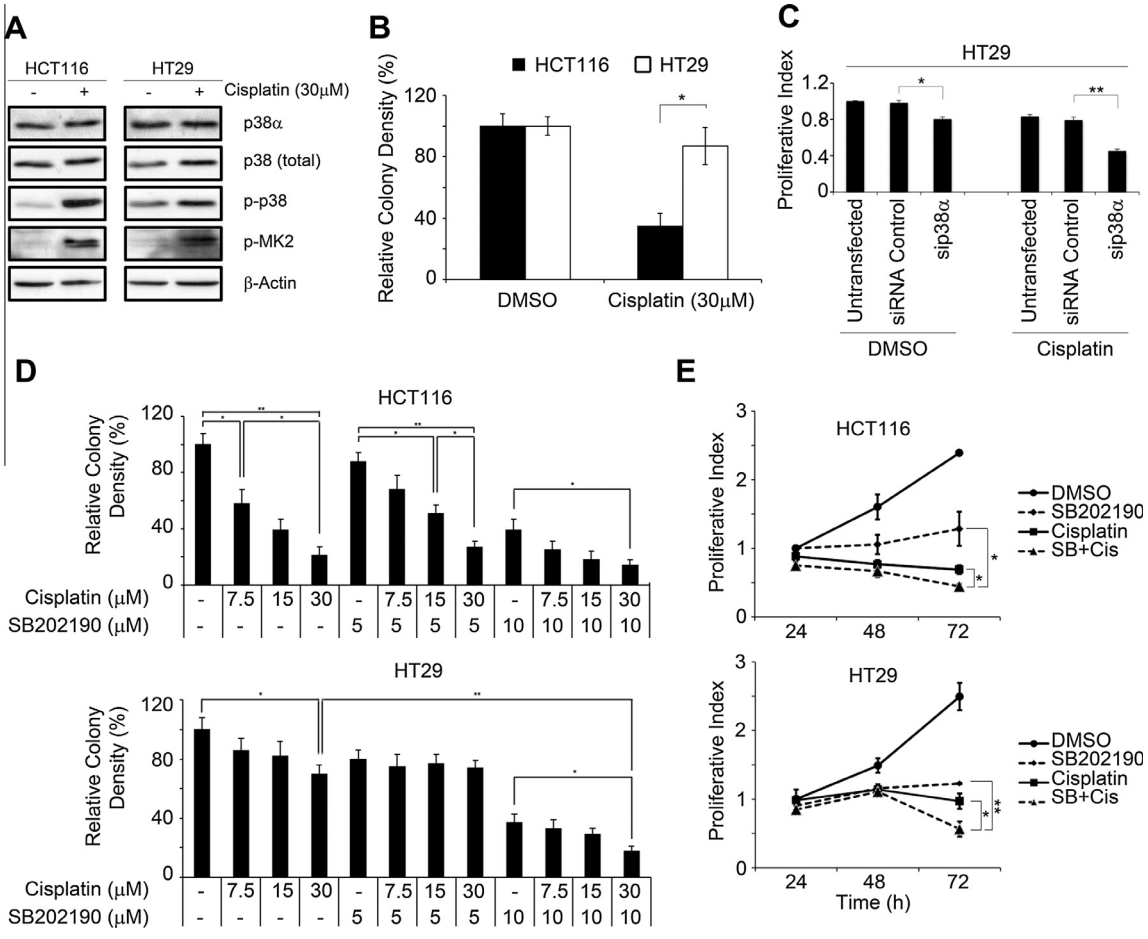

Fig. 1. Inhibition of p38 sensitizes chemoresistant HT29 cells to cisplatin. (A) Cisplatin induces the activation of the p38 MAPK pathway. HCT116 and HT29 CRC cells were treated with 30 μM cisplatin and total proteins were extracted for immunoblotting analysis. β-Actin was used as a loading control. (B) HT29 cells are more resistant to cisplatin than HCT116 cells. CRC cell lines were treated with cisplatin for 72 h and relative colony densities were determined. (C) HT29 cells were transfected or not with control siRNA or siRNA-p38α for 48 h and then treated with DMSO or cisplatin for additional 36 h. At the end of the treatment, WST-1 assay was performed and proliferation was calculated. (D) Administration of cisplatin together with a p38 inhibitor (SB202190) abolishes growth of chemoresistant HT29 cells in a dose-dependent manner. Colony densities of CRC cells were analyzed 72 h after treatment with the indicated concentrations of cisplatin and/or SB202190. (E) Cisplatin and SB202190 co-treatment decreases cell viability in a time-dependent manner. Cells were treated with cisplatin (30 μM) and/or SB202190 (10 μM), analyzed by WST-1 assay and scored for proliferation index. SB + Cis: SB202190 (10 μM) and cisplatin (30 μM) co-treatment. Statistical analysis was performed using Student's *t*-tail test; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

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showed a 65% decrease in colony formation, while HT29 cells showed only a modest inhibition (13%), which suggests that HT29 cells are more resistant to cisplatin than HCT116 cells when colony formation is taken into consideration (Fig. 1B).

Inhibition of p38 was previously reported as a sensitizing therapy for chemoresistant cells upon co-treatment with certain chemotherapeutics. Indeed, a p38 inhibitor co-administered with etoposide was found to reduce cell migration and invasion in neuroblastomas, while combination of p38 inhibition and 5-fluorouracil treatment has been shown to sensitize human CRC cells to chemotherapy [28,22]. Since HT29 cells displayed a more resistant behavior in terms of response to cisplatin compared to HCT116 cells, we investigated the involvement of p38α in chemoresistance in those cells. For this purpose, we ablated p38α expression by a specific siRNA (Supplementary Fig. 1) and measured the proliferation index in HT29 cells treated or not with cisplatin. Our results showed that removing p38α significantly relieved chemoresistance (Fig. 1C).

These interesting results prompted us to evaluate pharmacological inhibition of p38α in cisplatin-treated CRC cells. Thus, we treated HCT116 and HT29 cells for 72 h with different concentrations of cisplatin and of the p38 inhibitor SB202190, and found that increasing concentrations of cisplatin led to a dose-dependent growth inhibition in chemosensitive HCT116 cells both in the absence and in the presence of SB202190. Conversely, in chemoresistant HT29 cells, administration of 5 μM SB202190 along with cisplatin (all concentrations) caused only a 20–25% decrease in colony formation, while growth was dramatically inhibited (more than 80%) when 10 μM SB202190 was co-administered with 30 μM cisplatin (Fig. 1D). This is in agreement with the observation that 10 μM, but not 5 μM SB202190, was sufficient to effectively inhibit p38α activity in CRC cell lines (Supplementary Fig. 2).

To confirm the additive effect of p38 blockade at this specific inhibitor concentration, HCT116 and HT29 cells were treated with 30 μM cisplatin or 10 μM SB202190 or both and subjected to WST-1 assay in order to assess cytotoxicity and proliferation (Fig. 1E). The results confirmed that SB202190 modulates the cytotoxic effect of cisplatin *in vitro*, especially in chemoresistant HT29 cells. Since HCT116 and HT29 cells bear different genotypic backgrounds and the WST-1 assay results are dependent on the metabolic activity of the cells, we also performed a trypan blue staining to further visualize viable cells. HCT116 and HT29 cells were exposed to different concentrations of cisplatin and/or SB202190, then trypan blue positive and negative cells were counted at various time points to analyze relative cell viability. The results showed that the number of viable HCT116 cells was reduced to 10% and 5% when the combination therapy was applied for 72 h and 96 h, respectively (Fig. 2A). Additionally, the co-treatment was able to take the viability of chemoresistant HT29 cells down to 10% after 96 h, while the viability at this time point was more than 40% with cisplatin alone (Fig. 2B).

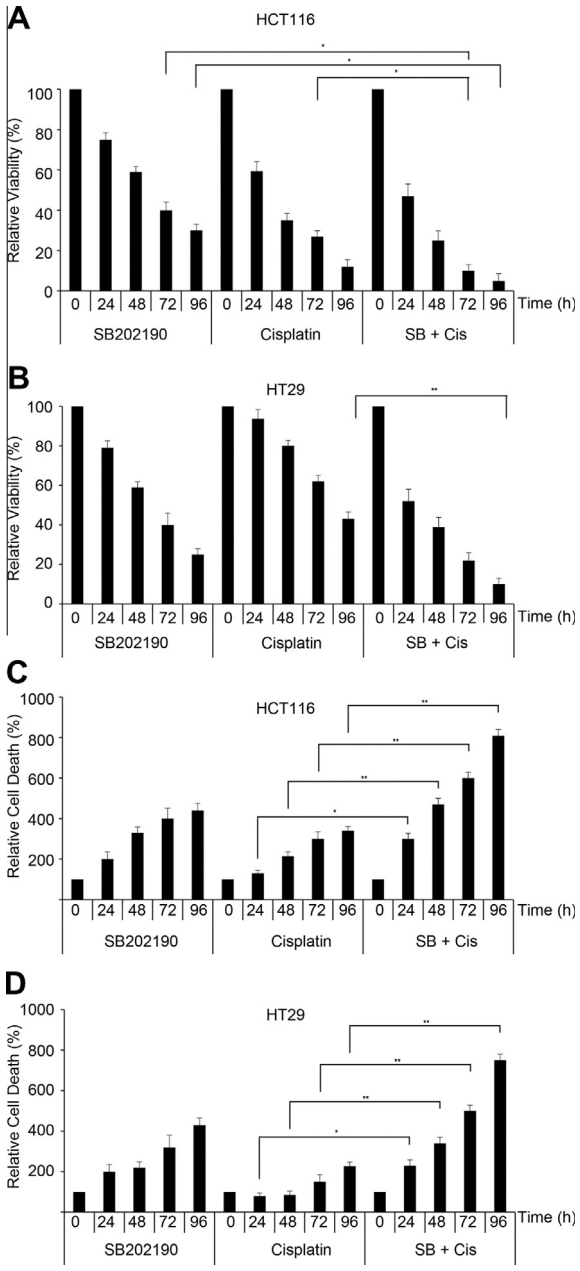

Fig. 2. Dual treatment of CRC cells with cisplatin and SB202190 decreases relative viability while increasing relative cell death. (A) HCT116 and (B) HT29 cells were treated with cisplatin (30 μM) and/or SB202190 (10 μM) and relative cell viability was calculated at the indicated time points. Administration of SB202190 with cisplatin strongly increases the number of dead cells in both (C) chemosensitive HCT116 and (D) chemoresistant HT29 cells. SB + Cis: SB202190 (10 μM) and cisplatin (30 μM) co-treatment. Statistical analysis was performed using Student's *t*-tail test; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

3.2. p38 inhibition increases cell death response of both HCT116 and HT29 cells

To determine the cell death response in single and dual treatment conditions, trypan blue staining scores were analyzed to obtain the relative cell death rates in HCT116 and HT29 cells at various time points. In HCT116 cells (Fig. 2C), the cell death rate showed an about 2-fold increase when SB202190 was administered together with cisplatin compared to cells treated with cisplatin alone. Similarly, the combination therapy caused an over 3-fold increase in the cell death response of chemoresistant HT29 cells (Fig. 2D).

3.3. SB202190 promotes apoptotic cell death in chemoresistant HT29 cells

We also performed an Annexin-V staining 48 h and 72 h after cisplatin and/or SB202190 treatment to monitor drug-induced cell death in HCT116 and HT29 cell lines. Cells were harvested after various time points and subjected to FACS analysis. Following cisplatin treatment alone, 17.4% of HT29 cells were found to be Annexin-V positive, indicating they had undergone cell death, whereas co-administration of SB202190 and cisplatin increased the cell death percentage up to 28.5% (Fig. 3A). Similarly, co-treatment

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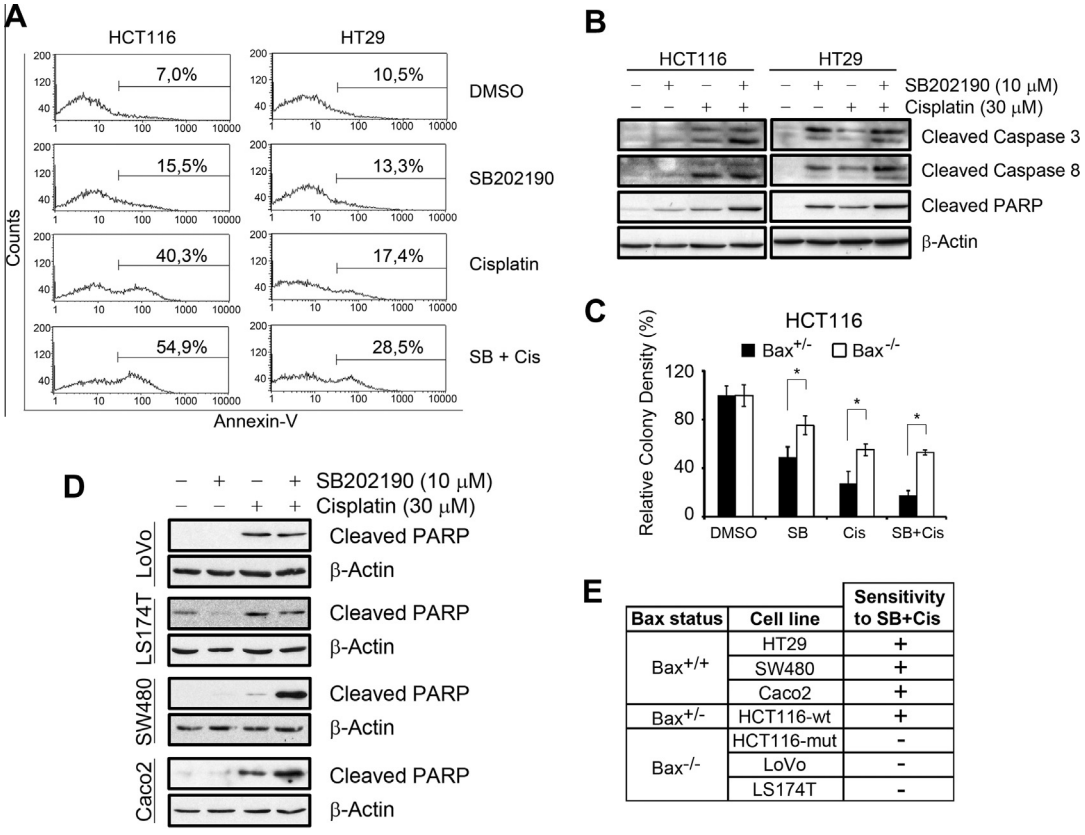

Bax status	Cell line	Sensitivity to SB+Cis
$Bax^{+/+}$	HT29	+
	SW480	+
	Caco2	+
$Bax^{+/-}$	HCT116-wt	+
$Bax^{-/-}$	HCT116-mut	-
	LoVo	-
	LS174T	-

Fig. 3. SB202190 increases the apoptotic effect of cisplatin and overcomes chemoresistance in HT29 cells. (A) SB202190 treatment enhances the apoptotic effect of cisplatin in chemoresistant cells. HT29 and HCT116 cells were treated with the indicated compounds for 72 h and FACS analysis was performed following Annexin-V-FITC staining. SB + Cis: SB202190 (10 μM) and cisplatin (30 μM) co-treatment. (B) The dual treatment further triggers the activation of apoptosis markers in HCT116 and HT29 cells. CRC cells were subjected to single or dual treatment for 72 h with the indicated drugs and then apoptosis markers were detected by immunoblotting. β-Actin was used as a loading control. (C) Cisplatin-induced apoptosis in HCT116 cells is Bax-dependent. Wild type ($Bax^{+/-}$, black bars) and Bax knock-out ($Bax^{-/-}$, white bars) HCT116 cells were subjected to 48-h drug treatments and colony formation was evaluated. Cis: 30 μM cisplatin, SB: 10 μM SB202190, SB + Cis: 10 μM SB202190 and 30 μM cisplatin co-treatment. (D) SW480, Caco2, LS174T and Lovo CRC cells were subjected to single or dual treatment for 48 h with the indicated drugs and then apoptosis markers were detected by immunoblotting. β-Actin was used as a loading control. (E) Bax status predicts dual therapy response. Table summarizing the data concerning the seven cell lines used in this study. Statistical analysis was performed using Student's *t*-tail test; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

with SB202190 and cisplatin showed an additive effect in HCT116 cells, with increased cell death (54.9%) compared to the single agents (15.5% for SB202190 and 40.3% for cisplatin) (Fig. 3A).

It is known that cisplatin induces a wide range of signaling pathways that lead to apoptotic cell death in cancer cells [29]. To analyze the putative apoptotic cell death observed upon co-treatment, we assessed the cellular levels of apoptosis markers such as cleaved caspase 8, cleaved caspase 3 and cleaved PARP1 in HCT116 and HT29 cells. According to our immunoblotting results, cisplatin alone increased the levels of active caspase 8, active caspase 3 and cleaved PARP1, indicating induction of apoptosis in both cell lines. On the other hand, HCT116 cells treated with SB202190 alone showed only a slight increase in the expression of these apoptosis markers, whereas a significant upregulation was observed after co-treatment with cisplatin. These results revealed a strong induction of apoptosis in HCT116 and HT29 cells exposed to SB202190 plus cisplatin (Fig. 3B).

One of the most important protein groups involved in intrinsic apoptosis is the Bcl-2 family. Within this apoptotic pathway, Bax and Bak are subjected to hetero- or homo-oligomerization upon appropriate stimuli, which induces the formation of lipidic pores in the mitochondrial membrane and triggers cytochrome c release. However, the hetero- or homo-oligomerization process is highly cell type-specific and determines differential apoptotic responses in cancer cells. To test Bax dependency in the HCT116 cell line isogenic model, we treated wild-type $Bax^{+/-}$ and mutated $Bax^{-/-}$ HCT116 cells with cisplatin, SB202190 or cisplatin plus SB202190 for 48 h and analyzed the resulting colony densities. HCT116 $Bax^{+/-}$ cells exposed to SB202190, cisplatin or the dual treatment showed 49%, 28% and 18% relative colony densities, respectively. On the other hand, the relative colony densities observed in HCT116 $Bax^{-/-}$ cells were always above 50% (76%, 56% and 54%, respectively). These results indicate that cisplatin-induced, SB202190-enhanced apoptosis is highly Bax-dependent in HCT116 cells (Fig. 3C). These results are of great interest for cancer therapy, since they suggest that Bax may represent a predictive factor for response to this combined therapy. To support this hypothesis, we examined various cell lines with different Bax statuses in order to obtain data from diverse genetic backgrounds. The analysis of apoptosis induction in four more CRC cell lines with different Bax statuses – Caco2 and SW480 ($Bax^{+/+}$), LoVo and LS174T ($Bax^{-/-}$) – clearly showed that, albeit these cell lines display different sensitivity to cisplatin, they respond to p38α inhibitor and cisplatin co-treatment in a Bax-dependent manner (Fig. 3D). Taken together, the data presented in Fig. 3 indicate that the co-treatment is effective only in the presence of at least one BAX wild type allele, and that Bax may represent a promising biomarker for response to p38α-targeted therapy (Fig. 3E). In agreement with these data, SB202190 treatment induces Bax protein expression in a time-dependent manner in HT29 cells (Supplementary Fig. 3).

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3.4. Co-treatment with a p38 inhibitor and cisplatin does not induce autophagic cell death

It has been previously shown that SB202190 may lead to autophagosome formation in some cell lines [18]. To investigate induction of autophagy, we immunoblotted total cell lysates obtained from HCT116 and HT29 cells with the autophagosome marker protein LC3 following treatment with SB202190, cisplatin or both. The results showed that SB202190 alone induced LC3 conversion, indicating induction of an autophagic response; however, autophagy was not affected by the co-treatment since no further increase in LC3-II levels was observed (Supplementary Fig. 4). This result indicates that the decrease in cell number and cell viability and the increase in cell death after co-treatment are independent of autophagy induction.

3.5. FoxO3A is involved in cell death induction

FoxO3A, a major tumor suppressor, is involved in the transcription of various genes involved in the cellular response to p38 inhibition [30]. Various kinases are known to be involved in FoxO3A regulation, and upon different insults FoxO3A has the ability to switch cellular responses toward different types of cell death mechanisms by reprogramming transcription of its target genes [31]. Furthermore, our previous studies showed that p38α inhibition triggers FoxO3A accumulation in the nuclei of CRC cells [27,32]. Due to its important role in tumor suppression, we studied the subcellular localization of FoxO3A in response to SB202190 and cisplatin co-administration. Following treatment, HCT116 and HT29 cells were subjected to a cellular fractionation protocol and cytoplasmic and nuclear fractions were probed for FoxO3A by immunoblot to detect the subcellular localization of endogenous FoxO3A. In HCT116 cells, FoxO3A nuclear localization did not show substantial changes after cisplatin treatment, but appeared significantly increased after SB202190 administration and dual treatment. On the other hand, in HT29 cells FoxO3A showed reduced nuclear levels in basal condition and failed to accumulate in the nucleus after cisplatin treatment, while SB202190 administration and co-treatment with cisplatin greatly enhanced nuclear localization (Fig. 4A). Similar results were obtained when HCT116 and HT29 cells were stained for FoxO3A, counterstained with propidium iodide and visualized by immunofluorescence to detect endogenous FoxO3A (Supplementary Fig. 5). Additionally, analysis of the immunoblot data relating to both cytoplasmic and nuclear fractions showed a slight increase in FoxO3A total levels in SB202190- and co-treated HT29 and HCT116 cells, while no significant change was detected in cells treated with cisplatin alone (Fig. 4A). This observation is in agreement with our previous

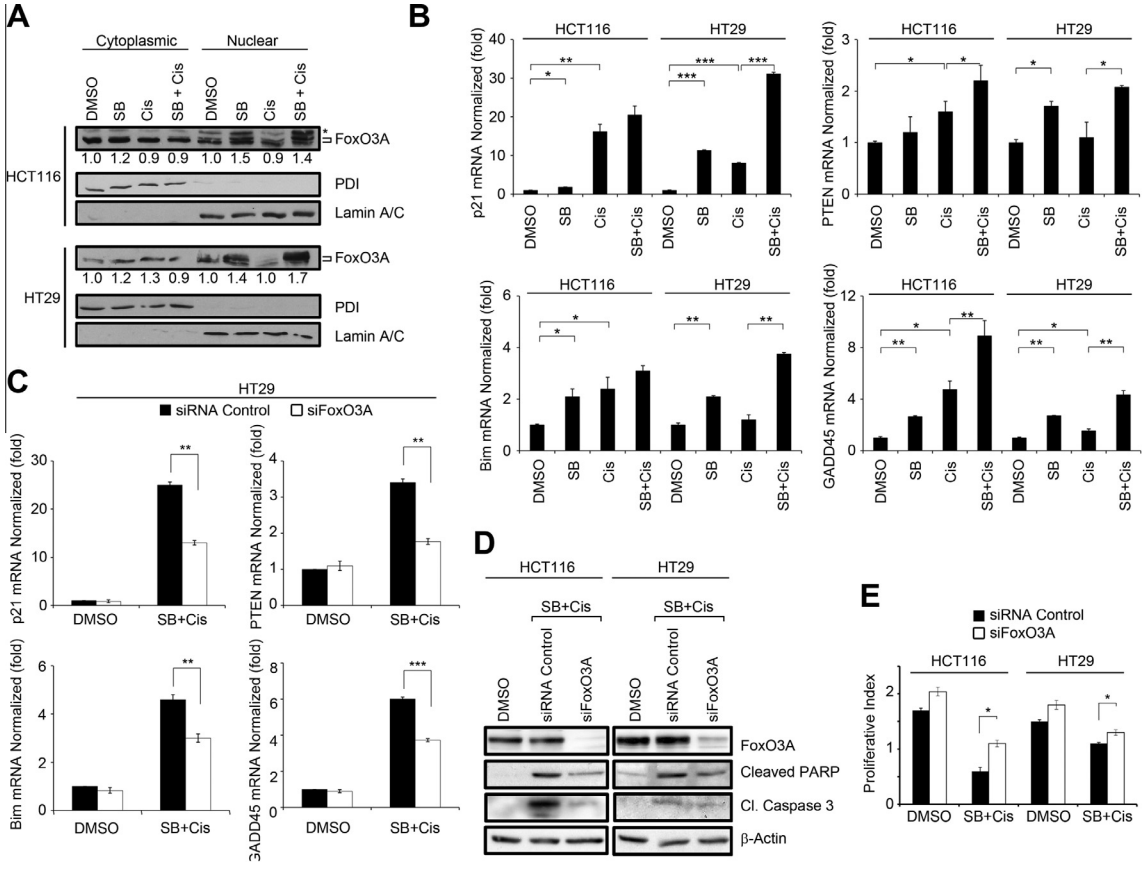

Fig. 4. FoxO3A activation is necessary to induce apoptosis in CRC cells. (A) SB202190 and cisplatin co-treatment triggers nuclear import of FoxO3A. Endogenous FoxO3A was measured by immunoblot in nuclear and cytoplasmic fractions of CRC cells treated with SB202190, cisplatin or a combination of both (asterisk: non-specific band). The values shown correspond to FoxO3A levels quantified by densitometric analysis and normalized to the loading controls (arbitrary units, DMSO 48 h = 1). The purity of each fraction was controlled with specific markers (nucleus: Lamin A/C; cytoplasm: Protein Disulfide Isomerase, PDI). SB: 10 μM SB202190, Cis: 30 μM cisplatin, SB + Cis: 10 μM SB202190 and 30 μM cisplatin co-treatment. (B) A strong induction of FoxO3A target gene transcription was detected in response to the dual treatment: real-time PCR analyses were performed for p21, PTEN, Bim and GADD45. β-Actin was used for normalization. SB; 10 μM SB202190, Cis; 30 μM cisplatin, SB + Cis: 10 μM SB202190 and 30 μM cisplatin co-treatment. (C–E) Silencing of FoxO3A abolishes apoptosis induction: cells transfected with FoxO3A-specific and non-silencing siRNAs were subjected to the dual treatment (SB202190 and cisplatin). Then, cells were analyzed by real-time PCR (C), immunoblotting carried out for FoxO3A, cleaved PARP and cleaved caspase 3 (D), and WST-1 assay to score the proliferation index. SB + Cis: 10 μM SB202190 and 30 μM cisplatin co-treatment. Statistical analysis was performed using Student's *t*-tail test; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

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studies showing increased overall FoxO3A protein levels upon p38α inhibition in CRC cell lines *in vitro* and xenografted tumors, and in neoplastic tissues of $APC^{min/+}$ mice [27]. Of note, we detected a double band for FoxO3A in nuclear fractions of both cell lines (Fig. 4A), which is likely due to post-translational modifications mediated by kinases (i.e. AMPK, JNK) and cofactors (p300, CBP, PCAF, ubiquitin-ligases) that regulate FoxO3A nuclear accumulation and activity [33]. The observed increase in the upper band of the doublet is in agreement with our previous results revealing that inhibition of p38α caused an increase in AMPK and JNK activity, as well as a reduction in Akt activity, thus also preventing FoxO3A export into the cytoplasm and its consequent degradation [27,34].

To evaluate the mRNA levels of FoxO3A target genes (p21, PTEN, Bim, GADD45), we performed real-time PCR analyses following the indicated treatments. In HT29 cells, SB202190 alone and cisplatin alone caused an about 10-fold and 8-fold increase in p21 mRNA levels, respectively, while an over 30-fold increase was observed in response to co-treatment. Similarly, concomitant administration of both compounds also induced increased mRNA levels of the other FoxO3A target genes analyzed, PTEN (2.2-fold), Bim (3.5-fold) and GADD45 (4.2-fold), while treatment with cisplatin alone did not change significantly their mRNA levels (Fig. 4B). Importantly, all genes were downregulated by genetic ablation of FoxO3A in SB202190 plus cisplatin-treated CRC cells (Fig. 4C). These results suggest that the nuclear localization of FoxO3A induced by the dual treatment causes elevated transcription of its target genes, which supports the involvement of FoxO3A in the cellular response observed in CRC cells.

To further evaluate whether the effects of the dual treatment were FoxO3A-dependent, we performed an RNA interference experiment by transiently transfecting FoxO3A-specific siRNAs into CRC cells. 36 h after transfection, cells were exposed to the dual treatment for another 36 h and total lysates were immunoblotted to evaluate changes in the expression of apoptosis indicators. As shown in Fig. 4D, silencing of FoxO3A significantly interferes with activation of caspase 3 and subsequent PARP cleavage in both cell lines. Moreover, evaluation of the proliferation index of these cells confirmed the role of FoxO3A in the cellular response to co-administration of SB202190 and cisplatin (Fig. 4E). These results indicate that co-treatment with SB202190 and cisplatin induces FoxO3A activation, which is necessary to induce apoptosis in both sensitive and chemoresistant CRC cells.

3.6. Pharmacological inhibition of p38 enhances the effect of cisplatin in chemoresistant CRC cells in vivo

According to our results, combined use of SB202190 and cisplatin *in vitro* induced a significant reduction in cancer cell growth by promoting apoptosis. To evaluate whether the co-treatment effect observed *in vitro* was also relevant to *in vivo* mouse models, we established xenografted tumors by injecting HT29 cells into athymic nude mice ($n = 28$). As soon as tumors reached a measurable size, mice were divided into four groups to be treated with the vehicle (DMSO), SB202190 and/or cisplatin. Drug treatments were administered intraperitoneally every day (for SB202190) or once every three days (for cisplatin) for 12 days, and tumor volume and body weight were recorded every 2–3 days. At the end of the treatments, xenografted tumors were explanted, weighed and subjected to immunohistochemical analysis. To evaluate the levels of active p38 (phospho-p38) in vehicle- or cisplatin-treated HT29-xenografted mice, colon tumors were stained with an anti-phospho-p38 antibody. In agreement with our *in vitro* results, the administration of cisplatin into xenografted mice induced the activation of p38 *in vivo* as shown in Fig. 5A. Since co-treatment of HT29 cells *in vitro* with SB202190 and cisplatin did overcome chemoresistance, mice bearing HT29-derived xenograft tumors were subjected to the dual therapy and tumor samples were further analyzed by phopsho-MK2 immunohistochemistry, by H&E (hematoxylin and eosin) staining to monitor tumor morphology, and by TUNEL assay to visualize apoptotic cells. The results confirmed also *in vivo* the efficacy of p38 inhibition by SB202190 (Fig. 5B); moreover, they showed clear tumor regression and an increased number of apoptotic cells in HT29-derived colorectal tumors exposed to the dual therapy (Fig. 5C). Finally, we scored relative tumor volumes in the different treatment groups. Xenografted tumors treated with SB202190 alone or in combination with cisplatin exhibited a significant volume decrease compared to controls and to tumors treated with cisplatin alone (Fig. 5D). These data were further corroborated by the end-point analysis of explanted tumor volume and weight (Fig. 5E). Importantly, no significant change in mice weight was reported along the treatment (Supplementary Fig. 6).

Overall, the results described above suggest that, *in vitro,* p38 inhibition by SB202190 notably sensitizes chemoresistant CRC cells to cisplatin treatment by inducing Bax-dependent apoptosis through FoxO3A activation. Importantly, this additive effect on chemoresistant CRC cells was also documented in HT29-xenografted mouse models *in vivo*, thus confirming that this dual therapy strategy is able to overcome chemoresistance by triggering apoptosis and thereby leading to tumor shrinkage.

4. Discussion

Cancer is becoming the leading cause of death in the western world. In particular, CRC is a major health concern, with 142,820 new cases and 50,830 deaths estimated in the United States in 2013 (National Cancer Institute). At present, CRC prognosis is only based on histological evaluation, and no molecular markers are internationally recognized as standard predictor factors. Actual therapies involve surgery, chemotherapy (5-FU) and radiation for locally advanced CRC, and FOLFIRI (5-FU or capecitabine and irinotecan) or FOLFOX (oxaliplatin and irinotecan) for metastatic CRC. However, despite the improvement in CRC progression-free and overall survival, the large majority of patients die within 5 years [35]. This is largely due to the fact that chemotherapy affects apoptosis by inducing DNA damage response, but gene mutations at apoptotic and/or anti-apoptotic loci cause the acquisition of chemoresistance. To circumvent these problems, molecular oncologists are searching for cancer-specific molecular targets to improve treatment efficacy and specificity. Indeed, Bevacizumab and Cetuximab, two monoclonal antibodies targeting VEGF and EGFR, respectively, moved from bench to bedside in CRC treatment and are now in use in clinical practice for advanced tumors. However, while they offer better survival responses when added to chemotherapeutic regimens, their use is restricted by the limited presence of the targeted antigen in cancer tissues and by mutations in downstream targets (e.g. KRAS), which impair their activity [35]. These evidences stimulated the search for new pharmacological targets able to circumvent drug resistance, increase effectiveness, guarantee specificity and reduce side effects.

Our previous studies highlighted the essential role of p38α in CRC biology and therapy. Indeed, we showed that this p38 MAPK isoform is required for proper CRC cell proliferation and cancer-specific metabolism in established cell lines and preclinical models [18,19,28,36]. p38α inhibition also sensitizes CRC cells to molecularly-targeted drugs such as MEK1/2 inhibitors, Lapatinib and Sorafenib *in vitro* and *in vivo* independently from KRAS or BRAF mutational status [32,37]. Moreover, other groups showed that p38α is an essential mediator of chemoresistance to FOLFIRI in CRC [20–22].

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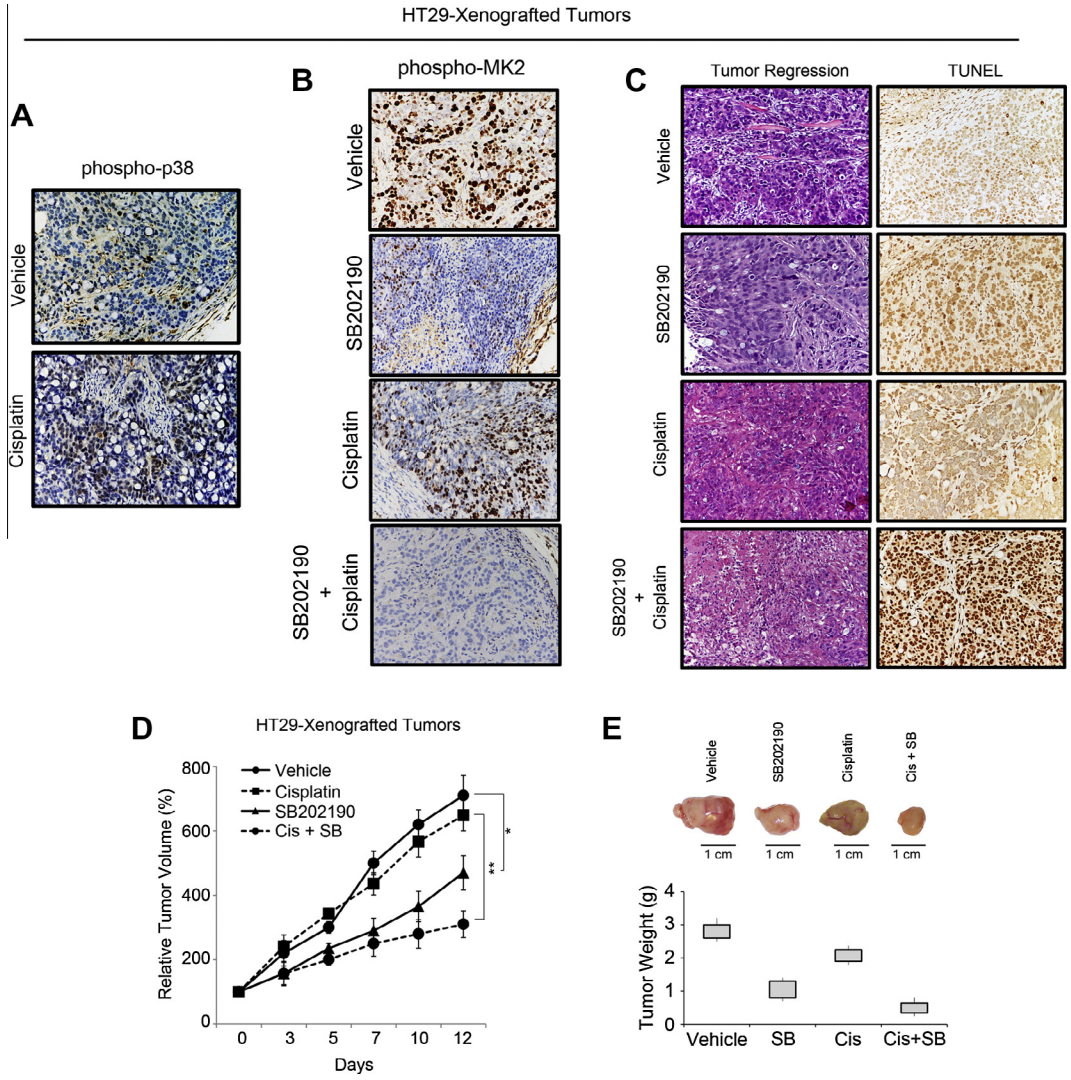

Fig. 5. Pharmacological inhibition of p38 overcomes chemoresistance to cisplatin *in vivo*. (A) Cisplatin causes p38 activation in HT29-xenografted tumors: immunohistochemistry was performed in two groups (vehicle and cisplatin treated animals) to detect active p38 (phospho-p38). (B and C) Dual therapy affects the direct p38α target p-MK2 and results in tumor regression by inducing apoptosis: untreated and treated tumors were analyzed by p-MK2 immunohistochemistry (B), H&E staining and TUNEL assay (C). (D and E) Dual therapy overcomes chemoresistance to cisplatin *in vivo* and decreases tumor volume and weight: HT29-xenografted tumors were extracted after the indicated treatments and time points and then measured (D) and weighted (E). Cisplatin (2 mg/kg); SB202190 (25 μg/kg); SB + Cis: SB202190 (25 μg/kg) plus cisplatin (2 mg/kg) dual treatment. Statistical analysis was performed using Student's *t*-tail test; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

Here we show that p38α signaling is activated in cisplatin-treated CRC cells, and that p38α genetic ablation or pharmacological blockade sensitizes HT29 chemoresistant cells to cisplatin. Furthermore, p38α inhibition showed an additive effect with cisplatin in HCT116 chemosensitive cells. At the molecular level, the co-treatment induced or increased Bax-dependent apoptosis in both sensitive and resistant cells *in vitro* and in xenograft models *in vivo*. Importantly, Bax-inactivating mutations have been described in more than 50% of CRCs characterized by a MIN phenotype, though these only account for 10–15% of all CRCs [38]. Thus, Bax status may potentially represent a predictive bio-marker for p38α-targeted therapy, as does KRAS for treatments directed against EGFR. This is also supported by data obtained by our and other groups [27,37], which showed that retention of one Bax wild-type allele (HCT116 $Bax^{+/-}$ cells) is still sufficient to transduce apoptotic signals, while inactivation of the second allele (HCT116 $Bax^{-/-}$ cells) produces apoptosis resistance.

Thus, our data suggest that several patients might potentially benefit from receiving p38α inhibitors together with molecularly-targeted drugs (anti-EGFR; MEK inhibitors; BRAF inhibitors, Sorafenib, etc.) and/or chemotherapeutics (cisplatin, 5-FU, irinotecan). However, the rationale of this intervention requires the presence of high levels of the enzymatically active form of p38 in tumor samples. Indeed, we found high levels of phospho-activated p38 in high grade human CRC specimens [32].

Pharmacological inhibition of p38α exerts its chemosensitizing effects through nuclear accumulation of the transcription factor FoxO3A and activation of its pro-apoptotic gene expression program. FoxO3A is a well-known tumor suppressor gene and emerges as a key downstream effector of various drugs used in tumor treatment. In addition to the above mentioned p38 inhibitors [28,36] and Cisplatin [39], FoxO3A is also involved in the cellular response to paclitaxel, doxorubicin, imatinib, PI3 K-Akt inhibitors, EGFR/HER2 inhibitors, and ionizing radiation [34].

Elucidation of the cellular players involved in resistance to chemotherapy and sensitization to cell death is a key issue for improving the efficacy of anti-cancer strategies, since response to treatment is often compromised by the development of chemoresistance. In this light, the new role described in this paper for the p38-FoxO3A axis in chemoresistance might prove of high importance for the design of new therapeutic strategies for CRC.

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5. Conflict of Interest

As corresponding author, I warrant that all authors have read and concur with the submission of this manuscript. I also warrant that the material submitted for publication has not been previously reported and is not under consideration for publication elsewhere. Furthermore, we declare no competing financial interests.

Acknowledgements

We thank Dr Francesco Paolo Jori for his helpful discussion during the preparation of the manuscript and editorial assistance. T.T. and A.P. are supported by Italian Foundation for Cancer Research (FIRC) fellowships. This study was partially supported by an 'Investigator Grant 2010' (IG10177) (to C.S.) from the Italian Association for Cancer Research (AIRC) and the Italian Fund for Basic Research (FIRB).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.10.035.

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