



## Original article

## Sorafenib, rapamycin, and venetoclax attenuate doxorubicin-induced senescence and promote apoptosis in HCT116 cells

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## ABSTRACT

Emerging evidence has shown that the therapy-induced senescent growth arrest in cancer cells is of durable nature whereby a subset of cells can reinstate proliferative capacity. Promising new drugs named senolytics selectively target senescent cells and commit them into apoptosis. Accordingly, senolytics have been proposed as adjuvant cancer treatment to cull senescent tumor cells, and thus, screening for agents that exhibit senolytic properties is highly warranted. Our study aimed to investigate three agents, sorafenib, rapamycin, and venetoclax for their senolytic potential in doxorubicin-induced senescence in HCT116 cells. HCT116 cells were treated with one of the three agents, sorafenib (5  $\mu$ M), rapamycin (100 nM), or venetoclax (10  $\mu$ M), in the absence or presence of doxorubicin (1  $\mu$ M). Senescence was evaluated using microscopy-based and flow cytometry-based Senescence-associated- $\beta$ -galactosidase staining (SA- $\beta$ -gal), while apoptosis was assessed using annexin V-FITC/PI, and Muse caspase-3/-7 activity assays. We screened for potential genes through which the three drugs exerted senolytic-like action using the Human Cancer Pathway Finder PCR array. The three agents reduced doxorubicin-induced senescent cell subpopulations and significantly enhanced the apoptotic effect of doxorubicin compared with those treated only with doxorubicin. The senescence genes *IGFBP5* and *BMI1* and the apoptosis genes *CASP7* and *CASP9* emerged as candidate genes through which the three drugs exhibited senolytic-like properties. These results suggest that the attenuation of doxorubicin-induced senescence might have shifted HCT116 cells to apoptosis by exposure to the tested pharmacological agents. Our work argues for the use of senolytics to reduce senescence-mediated resistance in tumor cells and to enhance chemotherapy efficacy.

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## 1. Introduction

Colon cancer is the third most diagnosed malignancy and ranks third in cancer mortality among men globally (Sung et al., 2021). In Saudi Arabia, colon cancer is the most prevalent cancer among Saudi men, with a higher mortality rate relative to developed countries (Alsanea et al., 2015; Alqahtani et al., 2020). The majority of

colon cancer-related mortality is attributed to cancer recurrence which occurs months or years after the completion of therapy (Miller et al., 2016; SEER, 2020). Insufficient response to chemotherapy due to resistance is one of the underlying causes of cancer relapse, even in patients with favorable prognostic characteristics at diagnosis (de Divitiis et al., 2014; Kanwar, 2012). Therefore, establishing effective therapeutic strategies aimed at reducing risk for recurrence is greatly needed.

Senescence is a cellular mechanism that describes the inability of cells to proliferate (Collado et al., 2007; Gewirtz et al., 2008; Kuilman et al., 2010). In addition to growth arrest, senescent cells exhibit a broad spectrum of morphological, cellular, and molecular changes, such as cell enlargement, metabolic dysfunction, upregulation of inflammatory cascades, resistance to apoptosis, and genetic and epigenetic alterations (Gorgoulis et al., 2019;

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Hernandez-Segura et al., 2018; Tchkonja et al., 2013). Cancer cells undergo senescence in response to the exposure to conventional chemotherapy, targeted chemotherapy, and radiotherapy, frequently termed Therapy-Induced Senescence (TIS) (Saleh et al., 2020a). Although the senescent growth arrest has been recognized to be irreversible, emerging reports have shown that tumor cells can re-enter the cell cycle and become proliferatively active (Milanovic et al., 2018; Patel et al., 2016; Saleh et al., 2019). For instance, different senescent cancer cells induced by various therapy can regain proliferative capacity *in vitro* and account for tumor development *in vivo* (Alotaibi et al., 2016; Elmore et al., 2005; Saleh et al., 2020b). Moreover, senescent tumor cells overcoming the growth arrest are often more aggressive, cannibalistic and exhibit stem cell-like characteristics (Milanovic et al., 2018; Tonnessen-Murray et al., 2019; Yang et al., 2017a; 2017b). These findings suggest that cancer cells enter a transit senescence phase, evade treatment-induced cell death, and eventually contribute to cancer recurrence. This was best demonstrated in acute myeloid leukemia models where relapse is mediated by a senescence-like resilience phenotype induced by exposure to therapy (Duy et al., 2021). In addition to the precipitation of senescence in tumor cells, exposure to therapy also induces senescence in the tumor microenvironment which has also been implicated in cancer relapse (Fletcher-Sananikone et al., 2021). In this case, relapse is propagated through the non-cell-autonomous effect of senescence mediated through the senescence-associated secretory phenotype (Demaria et al., 2017). Collectively, the elimination of TIS cells, both malignant and non-malignant, appears to be a plausible approach to mitigate cancer recurrence.

Recent studies in the field of aging have identified several promising agents named senolytics that selectively eliminate senescent cells (Baker et al., 2011; Dörr et al., 2013; Xu et al., 2015; Zhu et al., 2015). For instance, ABT-263 (navitoclax), an established non-selective BCL-2 inhibitor, was shown to eradicate radiation-induced senescent cell populations and activate hematopoietic stem cells to replace dysfunctional bone marrow in mice (Chang et al., 2016). In other reports, ABT-263 cleared accumulating senescent cells in pathological states such as myocardial infarction and diabetes mellitus, resulting in improved health outcomes in animals (Aguayo-Mazzucato et al., 2019; Walaszczyk et al., 2019). These results encouraged the investigation of the senolytic activity of ABT-263 against therapy-induced senescent cancer cells (Short et al., 2019). We have demonstrated previously that the exposure of senescent MDA-MB-231 and A549 tumor cells to navitoclax results in their selective elimination, which is accompanied by a significant reduction in tumor growth in tumor-bearing mice (Saleh et al., 2020b). The ability of ABT-263, and other senolytics, to cull senescent tumor cells has been demonstrated in multiple TIS models including, melanoma cells induced into senescence by aurora kinase inhibitors, breast tumor cells induced into senescence by doxorubicin, radiation, or BET inhibitors, ovarian tumor cells induced into senescence by PARP inhibitors, and prostate tumor cells induced into senescence by androgen-deprivation (V. Carpenter et al., 2021; Gayle et al., 2019; Shahbandi et al., 2020; Wang et al., 2017a). These reports support the rationale of implementing senolytics as adjuvant therapy in cancer treatment to decrease the likelihood of recurrence and improve patient survival. Nevertheless, despite the senolytic efficacy of ABT-263, its utilization can be associated with several limitations (V. J. Carpenter et al., 2021). Thus, the screening for other compounds for their senolytic activity where they can be readily included within combinational cancer therapy is sought.

In this work, we examined the potential senolytic properties of three agents with distinct pharmacological targets, namely, sorafenib, rapamycin, and venetoclax, in a model of doxorubicin-induced senescence in HCT116 colorectal cancer cell line. Sorafenib is a

multi-protein kinase inhibitor that has been approved to treat several malignancies such as advanced hepatocellular carcinoma (HCC), advanced renal cell carcinoma, FLT3-ITD positive acute myeloid leukemia (AML), and advanced thyroid cancer (Bazarbachi et al., 2019; Fleeman et al., 2019; Guevremont et al., 2009; Marisi et al., 2018). It has been reported that sorafenib inhibited p21 activity and thereby improved the cytotoxic activity of DNA damaging agents such as doxorubicin (Inoue et al., 2011). p21 is an essential driver for promoting cellular therapy-induced senescence when cells are exposed to DNA damaging agents. (Chang et al., 2000; Chen et al., 2002; Shtutman et al., 2017; Yosef et al., 2017). Rapamycin is an immunosuppressant agent that has been shown to attenuate the mTOR signaling pathway (Ballou and Lin, 2008; Huang et al., 2003). mTOR overexpression is a marker of senescent cells. In fact, the interference with mTOR function by rapamycin treatment has been reported to inhibit cellular senescence (Liu et al., 2020). Lastly, venetoclax is a BCL-2 inhibitor that has been used in chronic lymphoblastic leukemia, AML, small lymphocytic lymphoma (Pollyea et al., 2019; Rogers et al., 2021; Samra et al., 2020). We have previously shown that BCL-2 is the target through which senolytics mediate their effects in TIS models (Saleh et al., 2020b). Several reports have shown that the three agents enhanced the anticancer efficacy of doxorubicin against cancer cell lines (Cervello et al., 2012; Chang and Wang, 2013; Du et al., 2013; Li et al., 2019; Mondesire et al., 2004; Wang et al., 2020b). We hypothesized that the three agents improve doxorubicin efficiency by interfering with doxorubicin-induced senescence and shifting cell fate towards apoptosis. We believe the present work opens a new therapeutic avenue to reduce senescence-mediated therapy resistance and improve chemotherapy efficiency.

## 2. Materials and methods

### 2.1. Cell lines and experimental design

The colon cancer cell line HCT116 (CCL-247<sup>TM</sup>) was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco<sup>®</sup>, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco<sup>®</sup>) and 1% antibiotic-antimycotic (Gibco<sup>®</sup>). Cells were grown in a 75 cm<sup>2</sup> flask at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

Eight experimental groups of HCT116 cells were constructed to assess the senolytic activity of sorafenib, rapamycin, and venetoclax. These eight groups were treated with drug-free (control), 1 μM doxorubicin (Goehe et al., 2012), 5 μM sorafenib (Gulhati et al., 2012), the combination of 1 μM doxorubicin and 5 μM sorafenib, 100 nM rapamycin (Leontieva and Blagosklonny, 2011), the combination of 1 μM doxorubicin and 100 nM rapamycin, 10 μM venetoclax (Lafontaine et al., 2021), and the combination of 1 μM doxorubicin and 10 μM venetoclax. Cells in each studied group were exposed to the treatment regimen for 24 h.

### 2.2. Senescence characterization

#### 2.2.1. Microscopic evaluation of β-galactosidase staining

Cells (250,000) were seeded in six-well plates. On the following day, cells were treated with the indicated treatments for 24 h. Then, the cells were washed once with phosphate-buffered saline (PBS), fixed with 2% formaldehyde/0.2% glutaraldehyde, and incubated for 5 min. Wells were washed twice with PBS and incubated overnight at 37 °C in a staining solution containing 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactosidase in dimethylformamide (Santa Cruz Biotechnology, Dallas, TX, USA), 100 mM potassium ferricyanide, 100 mM potassium ferrocyanide, 5 M sodium chlo-

ride, 1 M magnesium chloride, and 0.2 M citric acid/sodium phosphate. The next day, the cells were washed twice with PBS and left to dry. The stained cells were viewed, and representative microscopic fields were captured using an Olympus inverted microscope (Olympus, Tokyo, Japan) (Alotaibi et al., 2016).

### 2.2.2. Flow cytometric characterization of C<sub>12</sub>FDG intensity

Senescence manifestations were confirmed by flow cytometric quantification of  $\beta$ -galactosidase intensity using 5-dodecanoylaminofluorescein di- $\beta$ -D-galactopyranoside (C<sub>12</sub>FDG) antibody (Thermo Fisher, Waltham, MA, USA) (Goehe et al., 2012). A total of 250,000 cells were precultured in six-well plates and then treated with the indicated treatments for 24 h. The next day, the cells were washed with PBS and incubated for 1 h in fresh DMEM containing 100 nM bafilomycin A1. Then, C<sub>12</sub>FDG antibody was added to each well and incubated for 2 h. The cell culture medium was aspirated, and the cells were washed twice with PBS. Then, cells were harvested by trypsinization and centrifuged at 1,500 rpm for 5 min at 4 °C. The supernatant was discarded, and the cells were resuspended in ice-cold PBS and analyzed by Cytomics FC 500, Beckman Coulter flow cytometry (Life Sciences, Indianapolis, IN, USA) (Debacq-Chainiaux et al., 2009).

## 2.3. Apoptosis evaluation

### 2.3.1. Annexin V-FITC/PI flow cytometry assay

Annexin V-FITC and propidium iodide (PI) markers were used to examine the impact of treatment combinations on apoptosis induction. The assay was performed according to the manufacturer's instructions (BioLegend, San Diego, CA, USA) (Ranganathan et al., 2014). Cells were trypsinized and centrifuged after treatment. The cell pellet was washed with PBS and then suspended in 100  $\mu$ L of binding buffer. Five microliters of Annexin-V-FITC and 5  $\mu$ L of propidium iodide (10  $\mu$ g/mL) were added to the cell suspension and incubated at room temperature for 15 min in opaque 1.5-ml Eppendorf tubes. Finally, 400  $\mu$ L of Annexin V binding buffer was added to the tubes before loading on a Cytomics FC 500 Beckman Coulter flow cytometer (Life Sciences, Indianapolis, IN, USA). The assay quantifies four cell populations: nonapoptotic (viable) cells (Annexin V -/PI -), early apoptotic cells (Annexin V +/PI -), late apoptotic cells (Annexin V +/PI +), and necrotic (dead) cells (Annexin V -/PI +).

### 2.3.2. Caspase-3/7 flow cytometry assay

The expression of caspase-3 and caspase-7 proteins was evaluated to investigate their molecular involvement in inducing apoptosis using the Muse™ caspase-3/7 assay (Merck Millipore, Burlington, MA, USA) as previously published (Alhoshani et al., 2020). Briefly, treated cells were trypsinized and collected by centrifugation. The cells were resuspended in 50  $\mu$ L of assay buffer BA mixed with 5  $\mu$ L of Muse caspase-3/7 reagent and then incubated for 30 min in a 37 °C incubator supplied with 5% CO<sub>2</sub>. Then, the cells were stained with 7-aminoactinomycin D (7-AAD) for 5 min at room temperature in the dark to measure cell viability. Finally, cells were run on the Muse™ Cell Analyzer (Merck Millipore, Burlington, MA, USA). The assay quantifies four cell populations: live cells (caspase-3/7 - /7-AAD -), apoptotic cells (caspase-3/7 + /7-AAD -), dead apoptotic cells (caspase-3/7 + /7-AAD +), and dead cells (caspase-3/7 - /7-AAD +).

## 2.4. RT-PCR array

We screened for potential regulatory genes by which the combination treatments interfered with doxorubicin-induced senescence and might have shifted cancer cells to apoptosis using the RT<sup>2</sup> Profiler™ PCR Array, Human Cancer Pathway Finder™ (Qiagen,

Germantown, MD, USA) (Tilli et al., 2014). The array evaluated the expression of 84 genes involved in apoptosis, senescence, angiogenesis, the cell cycle, DNA repair, epithelial-mesenchymal transition, hypoxia, metabolism, and telomeres. Total RNA was harvested using the miRNeasy mini kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol after treatment (As Sobeai et al., 2021). Total RNA concentration and purity were measured using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific). Total RNA (500 ng) was converted to copy DNA transcripts (cDNAs) using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Twenty-five microliters of mixed PCR components (RT<sup>2</sup> SYBR Green Master Mix, 25 ng cDNA of treated sample, and RNase-free water) (Qiagen, Germantown, MD, USA) was added to each well of the array. The array was sealed with an adhesive cover and loaded on the QuantStudio® 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Five housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*) were used to normalize the gene expression data. Data were expressed as a fold change adopting the 2<sup>- $\Delta\Delta$ CT</sup> method (Livak and Schmittgen, 2001).

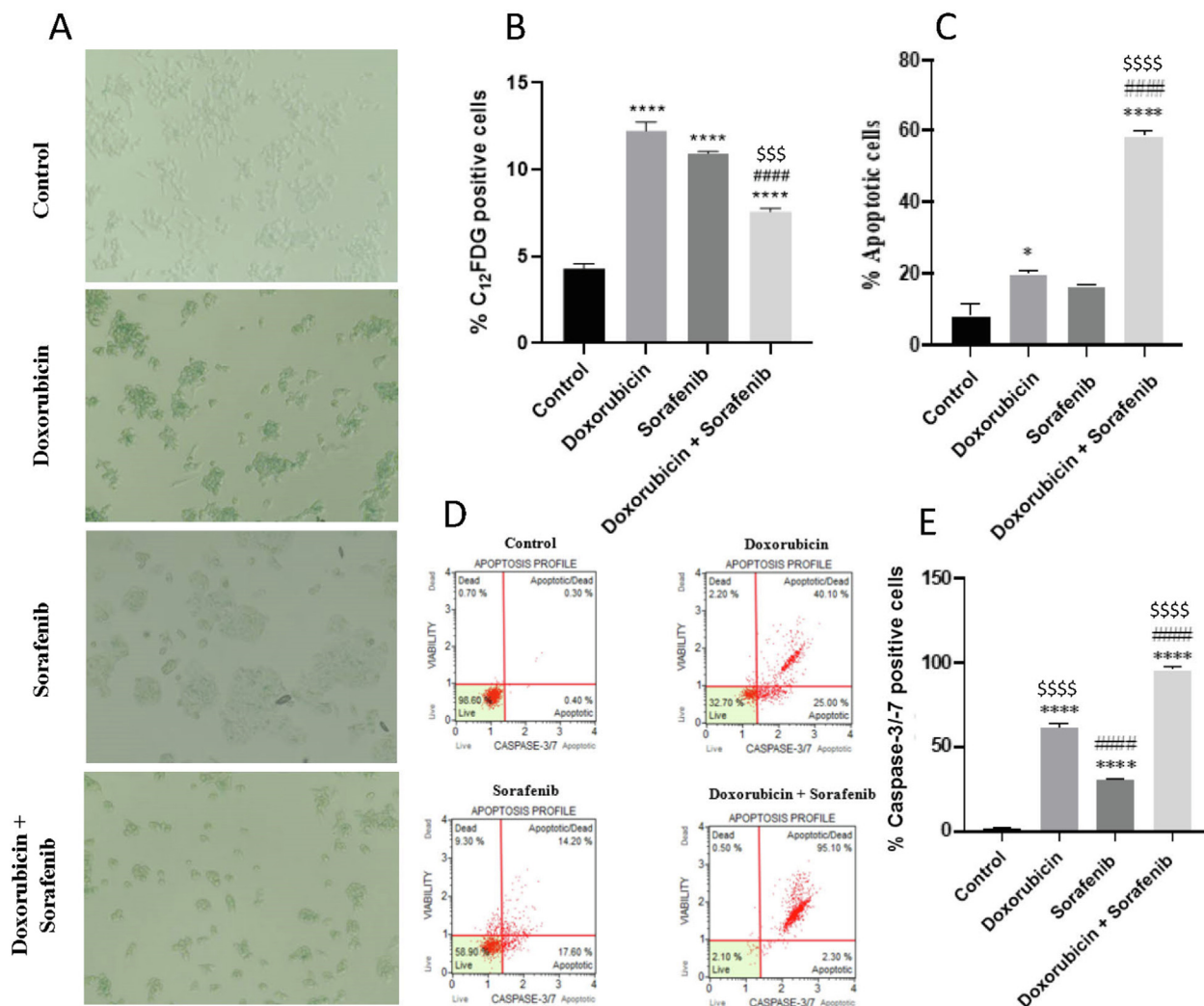
## 3. Results

### 3.1. Sorafenib decreases doxorubicin-induced senescent cell subpopulations and induces apoptosis in HCT116 cells

First, we wanted to confirm senescence induction HCT116 cells treated with doxorubicin alone showed more morphological changes, such as flattening, enlargement, and positive staining for SA- $\beta$ -galactosidase, than the drug-free treated control cells (Fig. 1A). Moreover, quantification of SA- $\beta$ -gal using the fluorogenic substrate C<sub>12</sub>FDG demonstrated a significant induction of senescence in HCT116 cells (Fig. 1B). Next, HCT116 cells were treated with sorafenib alone or with the combination of doxorubicin. Sorafenib in combination with doxorubicin showed fewer morphological changes and less SA- $\beta$ -galactosidase staining in comparison to doxorubicin alone (Fig. 1A). Moreover, quantification of the senescence marker C<sub>12</sub>FDG using flow cytometry showed that sorafenib, in combination with doxorubicin, significantly decreased the percentage of C<sub>12</sub>FDG-positive cells by approximately 38% relative to doxorubicin-treated cells (Fig. 1B).

To examine the senolytic potential of sorafenib, we measured the apoptotic cell populations of the experimental groups using an Annexin V/PI flow cytometry protocol (Supplementary Figure S1A). While doxorubicin has slightly increased the number of cells that underwent apoptosis compared to control samples ( $p = 0.015$ ), a significant increase in the percentage of apoptotic cells was recorded in cells treated with the combination of sorafenib and doxorubicin (from 19.61% to 58.5%) ( $p < 0.0001$ ) (Fig. 1C). Notably, treatment with sorafenib alone was not associated with apoptosis induction in HCT116 supporting its potential for killing senescent cells selectively (Fig. 1C).

The role of sorafenib in enhancing apoptosis induction after doxorubicin treatment was validated molecularly by assessing caspase-3/caspase-7 activation (Fig. 1D). Caspase-3 and caspase-7 are crucial proteins in the caspase cascade, which is the primary driver for the execution of apoptosis (Lamkanfi and Kanneganti, 2010; Porter and Jänicke, 1999; Shalini et al., 2015). Again, sorafenib, when combined with doxorubicin, significantly increased caspase-3/-7 activation by approximately two-fold relative to doxorubicin-treated cells, supporting the finding that sorafenib induces apoptosis in doxorubicin-induced senescent HCT116 cells (Fig. 1E). These findings provide supporting evidence that sorafenib has a promising potential to synergistically enhance doxoru-



**Fig. 1.** Impact of sorafenib on doxorubicin-induced senescence and apoptosis in HCT116 cells. HCT116 cells were treated with drug-free control, 1  $\mu$ M doxorubicin, 5  $\mu$ M sorafenib, the combination of 1  $\mu$ M doxorubicin and 5  $\mu$ M sorafenib for 24 h. A) Representative microscopic photos of HCT116 cells in the four experimental groups. Cells were stained with  $\beta$ -galactosidase. B) Quantification of C<sub>12</sub>FDG-expressing (senescent) cells in the experimental groups using a flow cytometry-based assay. C) Quantification of apoptotic cell populations in the experimental groups. D) Representative flow cytometry charts of caspase-3/7-expressing cells in the treated groups. The x-axis represents the intensity of cells expressing caspase-3/7 that underwent apoptosis, while the Y-axis represents the intensity of cells stained with PI, which indicates necrosis or late apoptosis. E) The percentages of caspase-3/-7-positive cell populations in the experimental groups. Values represent the mean  $\pm$  SEM of three independent experiments. ANOVA followed by Tukey's honest significance post hoc test was used to examine the statistical significance. \* and # indicate a significant difference compared with control, doxorubicin-treated cells, and sorafenib-treated cells, respectively ( $P < 0.05$ ).

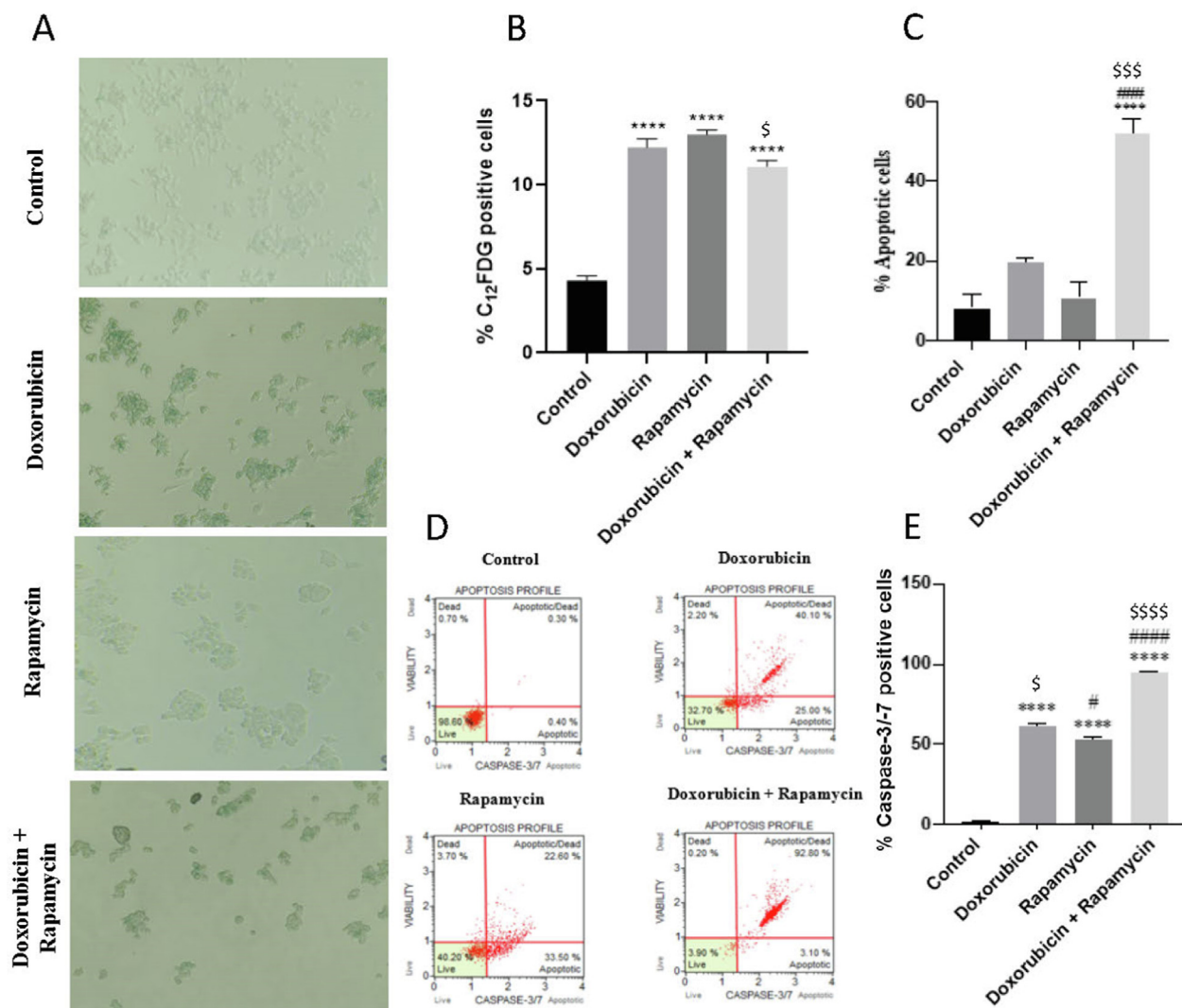
bicin antitumor effect by shifting its response from senescence to apoptosis.

### 3.2. Rapamycin attenuates doxorubicin-induced senescence and enhances apoptosis in HCT116 cells

Second, we tested for the senolytic potential of rapamycin in driving senescent HCT116 into apoptosis. Fig. 2A shows that treatment with rapamycin alone had no significant morphological changes in HCT116 and negative staining for SA- $\beta$ -galactosidase, in comparison to cells treated with doxorubicin. Furthermore, when cells were treated with doxorubicin and rapamycin, fewer morphological changes were detected accompanied by lower SA- $\beta$ -galactosidase staining compared to doxorubicin alone. In addition, the influence of rapamycin on chemotherapy-induced senescence was verified using C<sub>12</sub>FDG labeling examination (Fig. 2B). Rapamycin, when combined with doxorubicin, decreased the percentage of C<sub>12</sub>FDG-positive cells compared with doxorubicin

treatment alone, albeit not statistically significant ( $p = 0.14$ ). These data suggest that rapamycin might partially interfere with cells undergoing senescence when exposed to doxorubicin.

Treatment with rapamycin had no significant impact on apoptosis induction in HCT116 cells relative to controls. However, when it was combined with doxorubicin, as shown in Figures 1B and 2C, it resulted in a significant increase in the percentage of apoptotic cells from 10% and 19.6% to 52.3% compared to the control and doxorubicin alone, respectively. The effect of rapamycin and the doxorubicin combination on apoptosis induction was verified by measuring caspase-3/caspase-7 activation (Fig. 2D). Concurrent treatment with rapamycin and doxorubicin significantly increased caspase-3/-7 activation, from 61.5% to 94.5%, compared with doxorubicin treatment alone ( $p < 0.0001$ , Fig. 2E). These data indicate that rapamycin improves the ability of doxorubicin to induce apoptosis by partially suppressing HCT116 cells to undergo senescence.



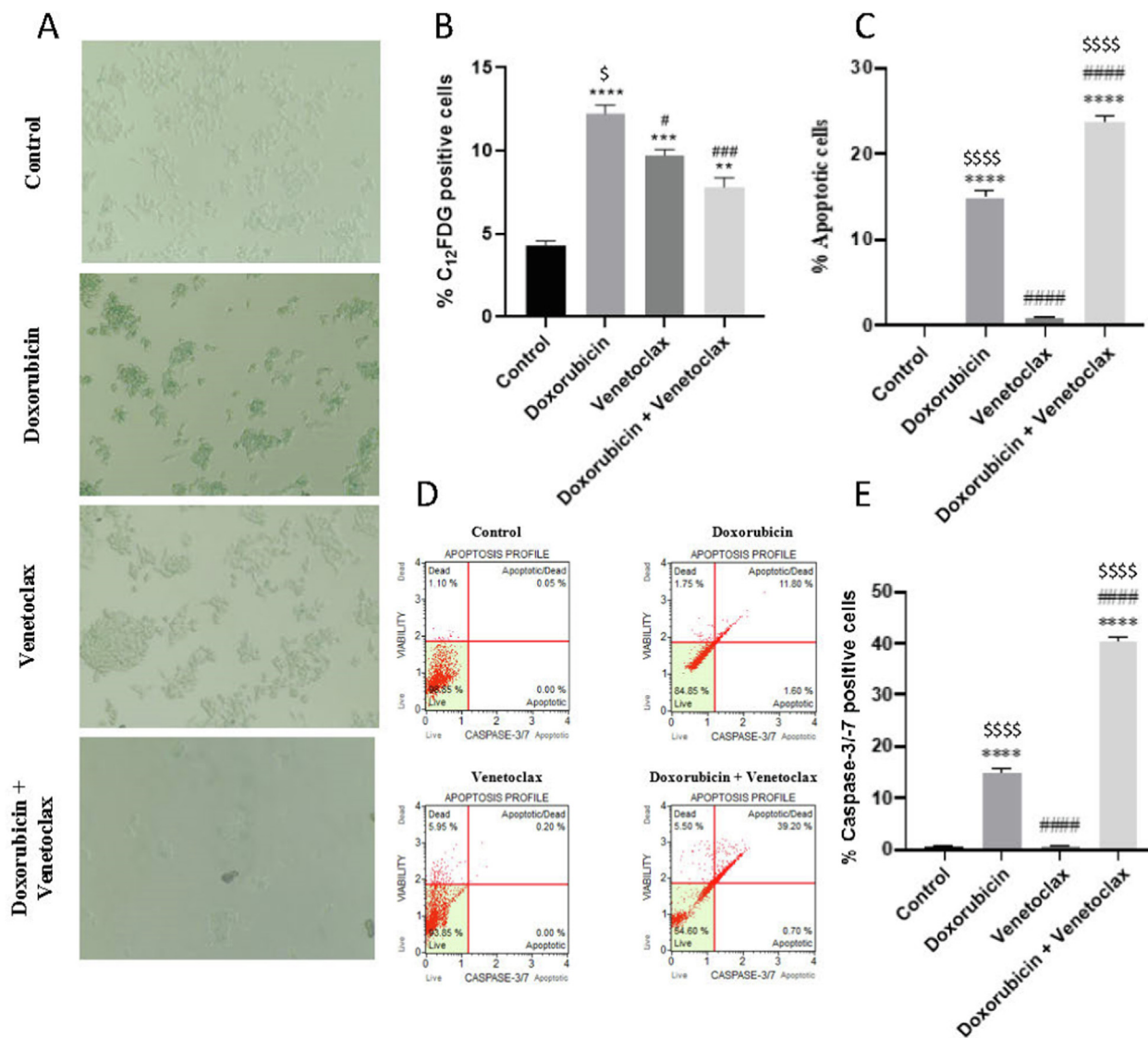
**Fig. 2. Impact of rapamycin on doxorubicin-induced senescence and apoptosis in HCT116 cells.** HCT116 cells were treated with drug-free control, 1  $\mu$ M doxorubicin, 100 nM rapamycin, and the combination of 1  $\mu$ M doxorubicin and 100 nM rapamycin for 24 h. A) Representative microscopic photos of HCT116 cells in the four experimental groups. Cells were stained with  $\beta$ -galactosidase. B) Quantification of C<sub>12</sub>FDG-expressing (senescent) cells in the experimental groups using a flow cytometry-based assay. C) Quantification of apoptotic cell populations in the experimental groups. D) Representative flow cytometry charts of caspase-3/-7-expressing cells in the treated groups. The x-axis represents the intensity of cells expressing caspase-3/-7 that underwent apoptosis, while the Y-axis represents the intensity of cells stained with PI, which indicates necrosis or late apoptosis. E) The percentages of caspase-3/-7-positive cell populations in the experimental groups. Values represent the mean  $\pm$  SEM of three independent experiments. ANOVA followed by Tukey's honest significance post hoc test was used to examine the statistical significance. \* and # indicate a significant difference compared with control, doxorubicin-treated cells, and rapamycin-treated cells, respectively ( $P < 0.05$ ).

### 3.3. Venetoclax reduces doxorubicin-induced senescent cell subpopulations and improves apoptosis in HCT116 cells

Venetoclax was the third drug that was investigated in our study. Similar to sorafenib and rapamycin, cells treated with venetoclax did not show morphological changes or an increase in SA- $\beta$ -gal staining (Fig. 3A). However, cells treated with the combination of venetoclax and doxorubicin manifested fewer senescence-associated morphological changes than cells treated with doxorubicin alone (Fig. 3A). We confirmed the suppressive effect of venetoclax on doxorubicin-induced senescence using flow cytometry-based detection of C<sub>12</sub>FDG staining. Venetoclax, when combined with doxorubicin, significantly reduced the C<sub>12</sub>FDG-positive cell population relative to doxorubicin-treated cells ( $p = 0.004$ , Fig. 3B). These findings indicate that venetoclax can reduce the number of doxorubicin-induced senescence.

We, then, explored the effect of venetoclax on apoptosis induction in doxorubicin-treated cells (Supplementary Figure S1C).

Interestingly, venetoclax did not increase the apoptotic cell population when used alone in comparison to the control baseline. Again, this suggests that venetoclax has limited cytotoxicity when given alone against proliferating colon cancer cells, despite targeting the antiapoptotic protein BCL-2. This phenomenon has been observed in several cancer cell lines, including HCT116 (Ko et al., 2014; Muenchow et al., 2020; Shi et al., 2021; Zhou et al., 2018). An extrinsic factor, such as chemotherapy treatment, that stimulates apoptosis might be required. Such stimulation can be synergistically enhanced by the antiBCL2 activity of venetoclax. Fig. 3C demonstrates that venetoclax, when given doxorubicin, significantly increased the percentage of apoptotic cells by 63.18% relative to cells treated with doxorubicin alone ( $p < 0.0001$ ). The synergistic induction of apoptosis in doxorubicin-treated cells by venetoclax was validated by caspase-3/-7 activation assay (Fig. 3D). Cells treated with venetoclax did not show an increase in caspase-3/caspase-7 activation compared to controls. However, when cells were treated with venetoclax and doxorubicin, a signif-



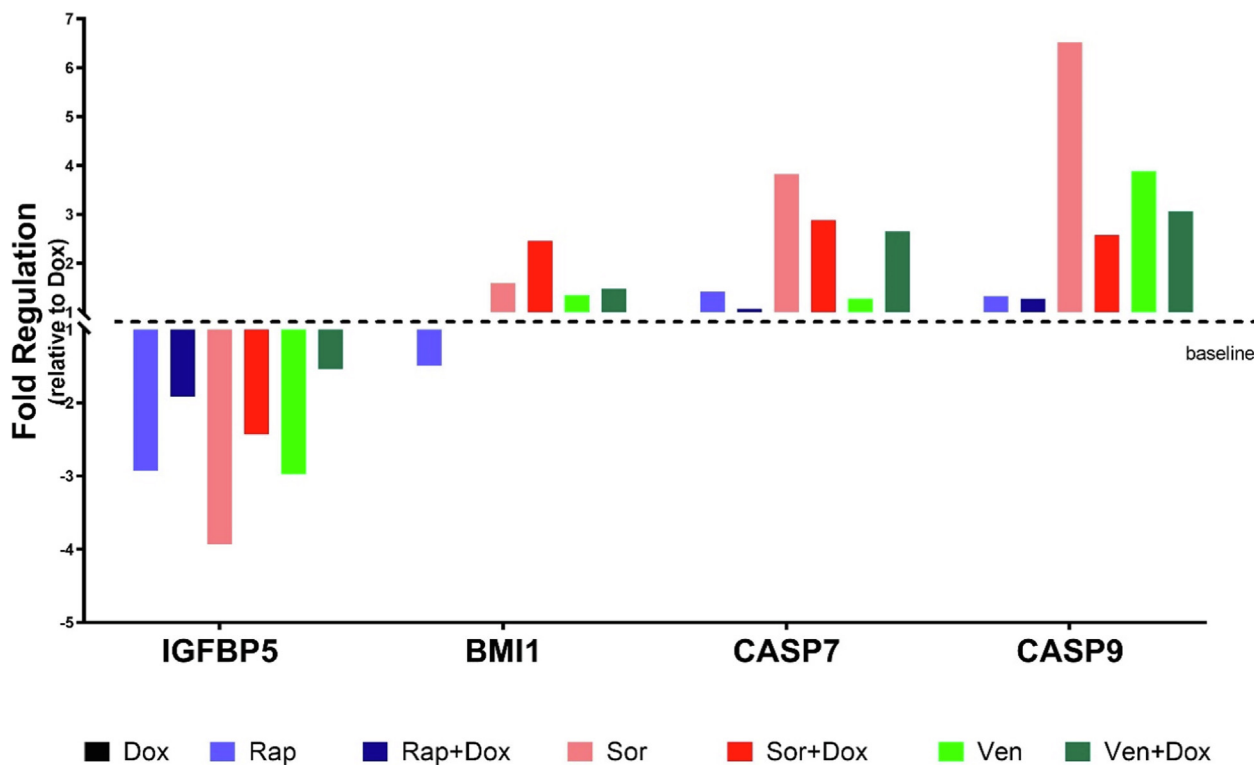
**Fig. 3. Impact of venetoclax on doxorubicin-induced senescence and apoptosis in HCT116 cells.** HCT116 cells were treated with drug-free control, 1  $\mu$ M doxorubicin, 10  $\mu$ M venetoclax, the combination of 1  $\mu$ M doxorubicin and 10  $\mu$ M venetoclax for 24 h. A) Representative microscopic photos of HCT116 cells in the four experimental groups. Cells were stained with  $\beta$ -galactosidase. B) Quantification of C<sub>12</sub>FDG-expressing (senescent) cells in the experimental groups using a flow cytometry-based assay. C) Quantification of apoptotic cell populations in the experimental groups. D) Representative flow cytometry charts of caspase-3/-7-expressing cells in the treated groups. The x-axis represents the intensity of cells expressing caspase-3/-7 that underwent apoptosis, while the Y-axis represents the intensity of cells stained with PI, which indicates necrosis or late apoptosis. E) The percentages of caspase-3/-7-positive cell populations in the experimental groups. Values represent the means  $\pm$  SEM of three independent experiments. ANOVA followed by Tukey's honest significance post hoc test was used to examine the statistical significance. \* and # indicate a significant difference compared with control, doxorubicin-treated cells, venetoclax-treated cells, respectively ( $P < 0.05$ ).

icant increase in caspase-3/caspase-7 activation was identified (from 14.93 to 40.3%, compared to doxorubicin treatment alone) ( $p < 0.0001$ , Fig. 3E). These data support the senolytic potential for venetoclax in doxorubicin-induced senescent HCT116 *in vitro*.

### 3.4. The attenuation of doxorubicin-induced senescence and the induction of apoptosis might be mediated by IGFBP5, BMI1, CASP7, and CASP9

We screened for underlying genes through which the three drugs interfere with doxorubicin-induced senescence. We examined a panel of 84 genes involved in senescence, apoptosis, DNA repair, hypoxia, the cell cycle, angiogenesis, epithelial-mesenchymal transition, telomere function, and metabolism. Genes and their functions are illustrated in **Supplementary Table S1**.

The pro-senescence gene *IGFBP5* and the anti-senescence gene *BMI1* emerged as potential underlying regulators of the inhibition of doxorubicin-induced senescence that was manifested in doxorubicin-treated HCT116 cells upon treatment with rapamycin, sorafenib, and venetoclax. (Fig. 4). *IGFBP5* was downregulated in all experimental groups, rapamycin, the combination of rapamycin and doxorubicin, sorafenib, the combination of sorafenib and doxorubicin, venetoclax, and the combination of venetoclax and doxorubicin, compared to the doxorubicin-treated group (Fig. 4). Treatment with either sorafenib or the combination of sorafenib and doxorubicin resulted in the upregulation of *BMI1* by 2.46- and 1.48-fold, respectively, relative to treatment with doxorubicin alone in HCT116 cells (Fig. 4). PCR amplification of the *BMI1* product in the rapamycin/doxorubicin-treated group failed in quality control tests. Thus, it was not reported. These data suggest that the inhibition *IGFBP5* and the activation of *BMI1* might mediate the



**Fig. 4. Gene Expression of *IGFBP5*, *BMI1*, *CASP7*, and *CASP9* in the experimental groups.** Gene expression was evaluated using the Human Cancer Pathway Finder™ PCR array. The experimental groups were doxorubicin (black), rapamycin (purple), rapamycin + doxorubicin (navy), sorafenib (pink), sorafenib + doxorubicin (red), venetoclax (bright green), and venetoclax + doxorubicin (dark green). Gene expression was reported as fold regulation relative to the expression of doxorubicin-treated cells.

interference of doxorubicin-induced senescence that was observed in cells treated with rapamycin, sorafenib, or venetoclax.

Among the ten apoptosis genes that were evaluated in the study, two caspases, *CASP7* and *CASP9*, emerged as candidate genes through which rapamycin, sorafenib, and venetoclax promoted apoptosis (Fig. 4). *CASP7* was upregulated in rapamycin + doxorubicin-, sorafenib + doxorubicin-, and venetoclax + doxorubicin-treated cells relative to doxorubicin-treated cells. Rapamycin, sorafenib, or venetoclax, when combined with doxorubicin, upregulated *CASP9* by 1.28-, 2.58-, or 3.07-fold compared with doxorubicin alone, respectively (Fig. 4). The gene expression data indicate that rapamycin, sorafenib, and venetoclax promote apoptosis through the activation of the intrinsic apoptosis pathway mediated by *CASP9* and *CASP7*. Threshold cycle ( $C_t$ ) values of *IGFBP5*, *BMI1*, *CASP7*, and *CASP9* in the experimental groups are in **Supplementary Table S2** and **Supplementary Table S3**. The expression of all analyzed genes involved in senescence, apoptosis, DNA repair, angiogenesis, the cell cycle, hypoxia, epithelial-mesenchymal transition, metabolism, and telomere function is illustrated in **Supplementary Figures S2-S9**.

#### 4. Discussion

TIS describes a form of senescence that develops in a variety of tumor cells in response to the exposure to a myriad of anticancer therapeutics (Saleh et al., 2020a). While TIS has been described as a favorable cell stress response that commits tumor cells into a stable growth arrest (Lee and Lee, 2019), preventing their progressive growth, accumulating evidence suggested that TIS has several disadvantageous effects that can be both cell-autonomous and cell-non-autonomous (Wang et al., 2020a). Cell-autonomous effects are largely based on the observation that a subpopulation of senescent tumor cells can overcome the irreversible growth

arrest and restore their ability to proliferate (Bojko et al., 2020; Pacifico et al., 2021). Moreover, cell variants that escape senescence acquire ominous traits such as becoming more aggressive (Yang et al., 2017a; 2017b), forming fast growing tumors in mice (Milanovic et al., 2018), developing stem cell-like characteristics and drug resistance (Pati and Weissman, 1990). On the other hand, cell-non-autonomous effects of senescence, which are largely attributed to the SASP, can propagate adverse effects of therapy (Demaria et al., 2017), tumor progression (Kim et al., 2016) and potential evasion of immunosurveillance (Muñoz et al., 2019). Accordingly, the elimination of senescent tumor cells using senolytics has been proposed to mitigate some of these unwanted effects of TIS and improve the efficacy of senescence-inducing cancer therapy (Wang and Bernards, 2018). Despite the promising potential of senolytics, several limitations have arisen which require further investigation of their use in cancer models (V. J. Carpenter et al., 2021). In particular, the screening of currently available compounds, preferably those approved for the treatment of cancer, for possible senolytic effects is required.

In this work, we investigated the senolytic potential of three compounds that are currently used for the treatment of cancer. Sorafenib and venetoclax, but not rapamycin, are approved as part of drug combinations for the treatment of different malignancies. Rapamycin, the prototypical inhibitor of mTOR is an established immunosuppressive that has been considered for cancer therapy (Hua et al., 2019). Rapamycin's ability of interfering with components of the senescent phenotype has been established. For example, rapamycin has been shown to interfere with the induction of the SASP marked by a reduction in the release of inflammatory cytokines from senescent mouse fibroblasts (Wang et al., 2017b) and human coronary artery endothelial cells (Sasaki et al., 2020). In cancer models, rapamycin was able to induce apoptosis in doxorubicin-treated human epidermoid squamous carcinoma cells

and reduce tumor size *in vivo* (Back et al., 2011). However, a similar effect of rapamycin in colon cancer models has not been demonstrated previously. In this work, we show that rapamycin was capable of inducing apoptosis in doxorubicin-exposed senescent HCT116 colorectal cancer cells marked by caspase activation (Fig. 2).

Sorafenib, a protein kinase inhibitor, is approved for the treatment of several forms of cancer (Bazarbachi et al., 2019; Fleeman et al., 2019; Guevremont et al., 2009; Marisi et al., 2018). Unlike rapamycin, little evidence is available on the association between sorafenib and senescence. Previously, it was shown that components of the SASP, such as interleukin-6 (IL-6), contribute to the development of resistance against sorafenib in HCC cells (Niu et al., 2018). Moreover, IL-6-producing hepatic tumor cells were less sensitive to sorafenib (Niu et al., 2018). Nevertheless, a senolytic effect of sorafenib in therapy-induced senescent cells has not been confirmed. In our study, sorafenib treatment of senescent HCT116 colon cancer cells induced by doxorubicin shifted the response towards apoptosis and reduced the number of SA- $\beta$ -gal positive cells, suggesting a potential senolytic activity of sorafenib in these cells (Fig. 1).

Lastly, venetoclax, a selective BCL-2 inhibitor, is indicated for a number of hematological malignancies (Scheffold et al., 2018). We have previously demonstrated that senolysis exerted by inhibition of members of the BCL-2 family in TIS models is largely dependent on the interference of BCL-X<sub>L</sub> rather than BCL-2 (Saleh et al., 2020b). This observation has been confirmed by others including in models of prostate cancer (Malaquin et al., 2020) and non-malignant, senescent IMR-90 human fibroblasts (Yosef et al., 2016). However, a recent report by Schwarzenbach et al. showed that venetoclax act as a senolytic agent in glioblastoma cells induced into senescence by temozolomide exposure, suggesting that senescent tumor cells dependence on BCL-X<sub>L</sub>, but not BCL-2, is not a universal phenomenon across senescence models (Schwarzenbach et al., 2021). In agreement with this observation, our data indicate that venetoclax, despite its selectivity against BCL-2, can still exert a senolytic activity in TIS models (Fig. 3). This can be explained, in part, by the heterogeneity of the senescent response especially in the context of TIS in cancer models where resistance to apoptosis can be mediated through several pathways or variable components of the same pathway (Bojko et al., 2019). Subsequently, the development of novel senolytics requires further analysis in the diversity of genetic and molecular signatures of senescence in different models in order to identify common targets.

When comparing the three agents against each other regarding their senolytic and apoptotic profiles, sorafenib and venetoclax eliminated a larger population of doxorubicin-induced senescent cells than rapamycin (Fig. 1B, 2B, and 3B). These results suggest that sorafenib and venetoclax exerted a greater senolytic potential compared with rapamycin. On the other hand, sorafenib and rapamycin had a stronger apoptotic effect against doxorubicin-treated cells than venetoclax (Fig. 1C, 2C, and 3C). Such an effect might be mediated by several mechanisms, not exclusively by the inhibition of senescence. In summary, our work argues to favor sorafenib, in terms of its combined senolytic and apoptotic properties, over venetoclax and rapamycin. However, confirmatory investigations need to be conducted beyond HCT116 cells to support our findings.

In order to understand some of the gene expression alterations that occur in the context of senolysis, we investigated changes in gene expression of 84 genes involved in senescence and apoptosis among other related process. Two genes, *IGFBP5* and *BMI1*, were identified as potential molecular players through which the three examined agents attenuate doxorubicin-induced senescence in HCT116 cells. *IGFBP5* is an insulin-like growth factor binding protein that is overexpressed during the induction of cellular

senescence (Sanada et al., 2018). Upregulation of *IGFBP5* has been shown to promote senescence in umbilical vein endothelial cells (HUVECs) (Rombouts et al., 2014), while knockdown of *IGFBP5* expression stalled cellular senescence in HUVECs (Kim et al., 2007). *IGFBP5* was downregulated in all the combination therapy groups compared to the doxorubicin-treated group (Fig. 4).

*BMI1* (B cell-specific Moloney murine leukemia virus integration site 1) belongs to the polycomb repressive complex that plays an important role in regulating self-renewal and senescence (Park et al., 2004). *BMI1*-knockout mice manifested premature senescence and a decline in lifespan (Lee et al., 2016). Prolonged interference with *BMI1* expression promoted cellular senescence in diffuse intrinsic pontine glioma cells, which eventually contributed to cancer recurrence (Balakrishnan et al., 2020; Balakrishnan et al., 2020). The combination treatment with either sorafenib and doxorubicin or venetoclax and doxorubicin upregulated *BMI1* relative to treatment with doxorubicin alone (Fig. 4). These data suggest that the inhibition of the pro-senescence gene *IGFBP5* and the activation of the anti-senescence gene *BMI1* might mediate the interference of doxorubicin-induced senescence that was observed in cells treated with rapamycin, sorafenib, or venetoclax.

On the other hand, *CASP7* and *CASP9* emerged as candidate genes through which rapamycin, sorafenib, and venetoclax promoted apoptosis in doxorubicin-induced senescent HCT116 cells. Caspase-7, encoded by *CASP7*, exhibits proteolytic activity, which upon activation leads to digestion of cell organelles and ultimately cell death (Lamkanfi and Kanneganti, 2010). Caspase-9, encoded by *CASP9*, initiates the caspase cascade as part of propagating the intrinsic pathway of apoptosis (Degterev et al., 2003). The activated form of caspase-9 stimulates executioner caspases including caspase-7 (Li et al., 2017). *CASP7* and *CASP9* have been reported to be underexpressed in cancer cells (Palmerini et al., 2001; Shen et al., 2010; Yoo et al., 2004). Such underexpression contributes to resistance to cell death, which is one of the well-established hallmarks of cancer (Olsson and Zhivotovsky, 2011; Shalini et al., 2015). In our analysis, *CASP7* was upregulated in rapamycin + doxorubicin-, sorafenib + doxorubicin-, and venetoclax + doxorubicin-treated cells relative to doxorubicin-treated cells (Fig. 4). These findings were consistent with the results of the impact of the combinations on apoptosis using the caspase-3/caspase-7 activation assay (Fig. 1E, 2E, and 3E). The gene expression data indicate that rapamycin, sorafenib, and venetoclax promote apoptosis through the activation of the intrinsic apoptosis pathway mediated by *CASP9* and *CASP7*. However, confirmatory gene and protein expression investigations of the identified senescence and apoptosis genes are needed.

## 5. Conclusion

Our present work showed that interference with doxorubicin-induced senescence might have shifted HCT116 cells to apoptosis. This phenomenon was demonstrated using pharmacological interventions with rapamycin, sorafenib, and venetoclax. Senescence-associated characteristics and markers were reduced by treatment with rapamycin, sorafenib, or venetoclax in combination with doxorubicin relative to treatment with doxorubicin alone. In addition, the combinations significantly increased the apoptotic cell population of HCT116 cells compared with doxorubicin alone. The senescence genes *IGFBP5* and *BMI1* and the apoptosis genes *CASP7* and *CASP9* emerged as candidate regulators through which the three drugs exert their anti-senescence and proapoptotic actions. These findings suggest that rapamycin, sorafenib, and venetoclax attenuate doxorubicin-induced senescence or have senolytic-like properties, which ultimately may drive cancer cells to apoptosis. We believe our work proposes a new therapeutic strategy to reduce



senescence-mediated resistance and improve chemotherapy efficiency. Our findings pave the way for future senescence mechanistic studies to validate whether the reduction in the senescent cell subpopulation in the combination-treated groups was due to the inhibition of cellular senescence fate. In addition, *in vivo* studies of the combinations are warranted to confirm the improvement of the efficacy over doxorubicin alone and to evaluate the impact of the proposed combinations on doxorubicin-induced cardiotoxicity.

### Authors contribution

Homood M. As Sobeai, Munirah Alohaydib, Ali R. Alhoshani, Khalid Alhazzani, and Mashal M. Almutairi performed, analyzed, and interpreted experiments and wrote the manuscript. Tareq Saleh designed, analyzed, and interpreted experiments, performed the statistical analysis, and wrote the manuscript; David A. Gewirtz and Moureq R. Alotiabi analyzed and interpreted data and wrote the manuscript and the grant proposals. All authors read and approved the final manuscript.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary material

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