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Sigma 1 Receptor antagonist potentiates the anti-cancer effect of p53 by regulating ER stress, ROS production, Bax levels, and caspase-3 activation

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ABSTRACT

Over the last years, many improvements have been made in the treatment of breast cancer; however, novel and less toxic therapies are still needed, especially for relapsing and chemo-resistant patients. Here, we analyzed the therapeutic potential of p53 and Rimcazole, a Sigma 1 Receptor antagonist. Rimcazole and p53 are being evaluated in preclinical and clinical trials, respectively. While p53 is a promising antitumor therapeutic agent, antagonists of Sigma 1 Receptor also inhibit tumor cell survival and induce apoptosis. Our current study demonstrates for the first time the synergistic effect of p53 in combination with the Sigma 1 Receptor antagonist Rimcazole. Furthermore, we show that shRNA knockdown of Sigma 1 Receptor in combination with p53, lead to a similar synergistic effect, and that this synergistic effect, in breast cancer growth suppression occurs independent of p53 status. Furthermore, this combination treatment induced ER stress, p38 MAPK activation, ROS production, and proteins involved in apoptosis (caspases-3, Bax) in breast cancer cells. Combining these therapeutic anti-cancer molecules provides an innovative approach for potentially treating human breast cancer.

1. Introduction

The tumor suppressor gene p53 is able to induce apoptosis specifically in cancer cells [1]. Poor prognosis and resistance to chemotherapy and radiotherapy has been associated in several human tumors where mutations, including deletions, in p53 are present [2,3]. Adenovirus-mediated p53 gene therapy (Ad.p53) has shown promise as a safe and effective antitumor treatment in preclinical and clinical experiments on p53-inactivated tumors [4–9]. Despite the results of these pre- and clinical trials, the questions still remains as to whether, particular subsets of cancer cells might display inherent or acquired resistance to p53 gene therapy [4].

Sigma 1 Receptor (Sig-1R) is a ligand-regulated protein chaperone subject of an evolving research area that could lead to therapeutic developments for many diseases [10]. Sig-1R has also been shown to modulate endothelial cell proliferation and can control angiogenesis, which makes it a promising target for oncology applications [11,12]. Sig-1R was recently identified as a receptor chaperone whose activity can be activated/deactivated by specific ligands. Manipulation of Sig-1R can yield either cytoprotective or cytotoxic actions. Sig-1R agonists promote cellular survival by preventing oxidative stress caused by ischemia, diabetes, inflammation, and amyloid toxicity. Conversely, antagonists of the Sig-1R inhibit tumor cell survival and induce apoptosis. Sig-1R antagonist-mediated cell death is inhibited by the prototypic Sig-1R agonists (+)-SKF-10047 [13–15]. Furthermore, systemic administration of Sig-1R antagonists significantly inhibits the growth of mammary carcinoma xenografts, prostate tumors, and lung carcinoma in the absence of side effects [12]. On the other hand, several normal cell types such as fibroblasts, epithelial cells, and even Sig-1R receptor-rich neurons are resistant to the apoptotic effects of Sig-1R antagonists [16]. Cellular susceptibility appears to correlate with differences in Sig-1R coupling rather than levels of expression. Although Sig-1R is over-expressed in tumors and upregulated in rapidly dividing normal tissue, their antagonists induce cell death only in tumor tissue [12,17]. Recently, we have shown that Sig-1R interacts with Interleukin-24 (IL-24) and that interaction mediates and is critical for apoptosis induction by IL-24 [18]. We and others have shown that in cancer cells, Sig-1R antagonists evoke ER stress response that is inhibited by Sig-1R agonists [18–21].

In the present study, we demonstrate that Ad.p53, in combination with Rimcazole, a specific Sig-1R antagonist, induce apoptosis in breast cancer cells. Moreover, we reveal that Ad.p53 in

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combination with Rimcazole have a synergistic effect in growth suppression that occurs independent of p53 status. Furthermore, shRNA knockdown of Sig-1R in combination with p53, lead to similar synergistic effect, confirming that the observed growth suppression was indeed related to diminished Sig-1R function. The role of Sig-1R in breast cancer and the mechanisms by which its ligand, Rimcazole, leads to apoptosis in combination with p53 are unclear. We therefore set out to investigate the expression of ER stress genes (BiP/GRP78, CHOP, phosphor-eIF2α), ROS production and proteins involved in apoptosis (caspases-3, Bax). Whereas no significant changes occurred in the expression of p53, p21 and Sig-1R proteins in breast cancer cells after treatment with p53 and Rimcazole, marked changes were observed in ER stress markers and ROS production. Moreover we show that with the combination treatment Bax levels increased in breast cancer cells concomitantly with cleavage of caspases 3. These provocative findings suggest that this combinatorial strategy might provide a platform for developing effective treatments for therapy-resistant cancers.

2. Materials and methods

2.1. Cell culture and reagents

Human breast MCF-7, MDA-MB-231, MDA-MB-157 and T47D cell lines were obtained from American Type Culture Collection (ATCC). All cell lines were grown in RPMI 1640 with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin/streptomycin and 1 μM sodium pyruvate, in humidified atmosphere at 37 °C with 5% CO_2 and media was replaced every alternate day. Rimcazole was purchased from Fisher Scientific.

2.2. Virus infection

The p53-expressing, replication defective Ad.p53 and corresponding control adenovirus vector lacking exogenous gene, (Ad.vector) were custom engineered by Vector Biolabs, Inc. (Philadelphia, PA).

2.3. MTT assay

Cells were platedin 96-well dishes (1×10^3 cells/well) in RPMI 1640 containing 10% FBS and allowed to attach for 12 h prior to treatment(s). Inhibitors were added 4 h after infection with adenovirus. Cell growth and viable cell numbers were monitored by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as previously described [18].

2.4. Annexin V binding assays

Cells were trypsinized, washed once with complete medium and PBS, resuspended in 0.5 ml of binding buffer containing 2.5 mmol/L $CaCl_2$, and stained with allophycocyanin-labeled Annexin V (Becton Dickinson Biosciences, Palo Alto, CA) and propidium iodide (PI) for 15 min at room temperature. Flow cytometry assays were performed as previously described [18].

2.5. Assessment of reactive oxygen species (ROS) generation

MCF-7 and MDA-MB-231 cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well and were treated accordingly for 12 h. The cell cultures were treated with 10 μM 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma–Aldrich, St. Louis, MO) in PBS for 30 min. After incubation, the media was discarded, and the cells were washed with PBS. The fluorescence intensity was determined using a fluorescence plate reader at 485 nm for excitation and 530 nm for emission.

2.6. Western blot analysis

Protein extracts were prepared with RIPA buffer containing a mixture of protease inhibitors as described. Fifty micrograms of protein were run in a 12% SDS/PAGE and transferred to nitrocellulose membranes. The membranes were probed with polyclonal or monoclonal antibodies to Sig-1R, phospho-p38 MAPK, total p38 MAPK, phospho-eIF2α, BiP, CHOP, BiP, cleaved caspase 3, p53, p21, Bax, and β-actin. Membranes were imaged using a LiCor Odyssey scanner.

2.7. RNA interference

The shRNA vector containing Sig-1R oligonucleotide sequence for optimal suppression of Sig-1R was purchased from Vector Biolabs. Knockdown of Sig-1R shRNA in breast cancer cells was performed according to the manufacturer's recommendations. Scrambled shRNA was used as a control. Western blots were performed to verify the efficiency of the knockdown.

3. Results

3.1. Combinational treatment with Ad.p53 and Rimcazole, an antagonist of Sigma 1 Receptor, induces growth inhibition in breast cancer cells

We employed breast cancer cells to investigate growth inhibitory properties of combinational treatment of Ad.p53 and Rimcazole. To evaluate the efficacy of the combination treatment, we first determined the minimal cell death-inducing doses of Ad.p53 and Rimcazole, respectively. Exposing MCF-7 (wild type p53), T47D (mutated p53), MDA-MB-231 (mutated p53), and MDA-MB-157 (null p53) breast cancer cells to increasing concentration of the Ad.p53 resulted in a dose-dependent increase in cell death (Fig. 1A). Rimcazole was applied to MCF-7, T47D, MDA-MB-157 and MDA-MB-231 breast cancer cells, and dose-dependent growth inhibition was observed after 5 days (Fig. 1B). Low doses of Rimcazole (4 μM) did not significantly affect cellular growth. Above a certain threshold concentration (10 μM), a reduction in cell number became apparent (Fig. 1B). Virtually complete cell death was observed after 5 days of treatment with 40 μM Rimcazole in all cell lines (data not shown). Based on the results of single treatments shown in Fig. 1A and B, 10 pfu/cell of Ad.p53 and 4 μM of Rimcazole were chosen as the concentrations to assess the efficacy of the combination treatment. Combining Ad.p53 with Rimcazole significantly increased cell death of MCF-7, T47D, MDA-MB-157 and MDA-MB-231 human breast cancer cells (Fig. 1C). Because the different breast cancer cell lines contain either wild-type p53 (MCF-7), mutant p53 (MDA-MB-231, and T47D), or null p53 (MDA-MB-157), these results document that the growth-inhibitory activity of Ad.p53 in combination with Rimcazole occurs independently of the mode of action of p53, that is frequently altered in human cancers. Annexin V staining was used to monitor apoptosis induction by Ad.p53 in combination with Rimcazole. Induction of apoptosis was significant in all breast cancer cells treated with this combination treatment (Fig. 1D).

3.2. Sigma 1 Receptor is essential to the growth-inhibitory activity of Ad.p53 in combination with Rimcazole

It has been shown that several Sig-1R antagonists, including Rimcazole, inhibit tumor cell survival and induce apoptosis

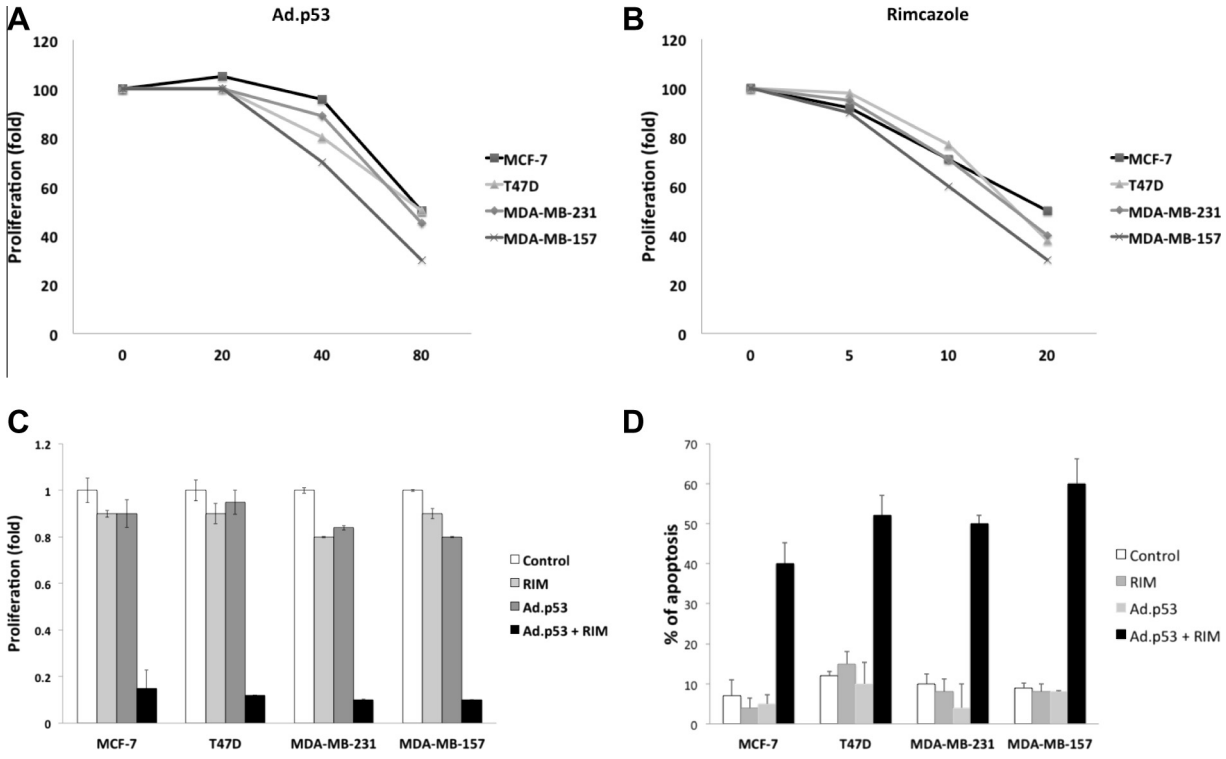

Fig. 1. Combination of Ad.p53 and Rimcazole induces apoptosis in breast cancer cells. Infection with Ad.p53 or Rimcazole results in a decrease in cell viability in breast cancer cells. The indicated cell type was infected with either Ad.vector (control), or Ad.p53 and either untreated or treated with the indicated concentrations of Rimcazole. Changes in cell viability were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT absorbance of untreated control cells was set at 1 to determine relative number of viable cells. Each data set was obtained from a representative experiment performed at least three times. Data points represent mean values (±SD) from wells in quadruplicate. (A) Cells were infected with different pfu/cell of Ad.vector (control) or Ad.p53 for 5 days. (B) Cells were treated with different concentrations of Sig-1R antagonist (Rimcazole) for 5 days. (C) Combination of Ad.p53 with Rimcazole efficiently inhibits *in vitro* growth of indicated breast cancer cell lines. Breast cancer cells were infected with Ad.vector (control) orAd.p53 at low doses (10 pfu/cell), with or without low concentration of Rimcazole (4 μM). Cells were treated with Rimcazole after 2 h of the indicated Adenovirus infection. MTT assays were measured 5 days after treatment. (D) Combination of Ad.p53 with Rimcazole induces apoptosis of breast cancer cell lines. Cells were infected with either Ad.vector, or Ad.p53 at an m.o.i of 10 pfu/cell and either untreated or treated with 4 μM of Rimcazole. An Annexin V binding assay was performed at 48 h post-treatment as described in Section 2.

[12,16,18,22,23]. Also, Sig-1R antagonist-mediated cell death is inhibited by the prototypic Sig-1R agonist (+)-SKF10047 [13–15]. To investigate the role of Sig-1R on the growth-inhibitory activity of Ad.p53 in combination with Rimcazole, cells were treated in the presence or absence of the specific Sig-1R agonist, (+)-SKF-10047. Sig-1R agonist (+)-SKF-10047 inhibited combination Ad.p53 plus Rimcazole-mediated killing in breast cancer cells (Fig. 2A). (+)-SKF-10047 treatment alone did not alter tumor growth. Therefore, Sig-1R agonist (+)-SKF-10047 substantially reduces the growth-inhibitory activity of Ad.p53 in combination with Rimcazole, confirming that this effect is mediated at least by antagonism of Sig-1R sites.

Previous studies have shown that Sig-1R agonists and up-regulation of this protein protect cells against apoptosis triggered by Sig-1R antagonist [13–15,18]. We examined whether overexpression of Sig-1R *per se* may affect the growth-inhibitory activity of Ad.p53 in combination with Rimcazole in breast cancer cells. Ad.p53 in combination with Rimcazole was used to treat breast cancer cells in the presence or absence of ectopic expression of Sig-1R, and cell viability was measured. Ectopic expression of Sig-1R inhibited combination Ad.p53 plus Rimcazole-mediated killing in breast cancer cells (Fig. 2B). Sig-1R agonist (+)-SKF-10047 or ectopic expression of Sig-1R did not cause detectable effects alone or in the presence of either Ad.p53 or Rimcazole in breast cancer cells (data not shown). Rimcazole acted in a Sig-1R-dependent manner as demonstrated by reversal of their tumor growth-promoting activity by ectopic expression of Sig-1R (Fig. 2A).

3.3. Knockdown of Sig-1R, in combination with Ad.p53, mimicked the action of Rimcazole

Pervious research has shown that similarly to Sig-1R antagonist, knockdown of Sig-1R causes decreased survival and induction of apoptosis in cancer cells [24,25]. Here, the importance of Sig-1R on the growth-inhibitory activity of Ad.p53 was investigated using shRNA knockdown of Sig-1R in breast cancer cells. Breast cancer cells were transfected with control shRNA, or Sig-1R shRNA (Fig. 2B). Cells transfected with control shRNA showed no effect alone or in combination with Ad.p53 in viability assay, whereas cells transfected with Sig-1R shRNA in combination with Ad.p53 significantly increased cell death of human breast cancer cells. Sig-1R shRNA had no effect on viability (Fig. 2B). The knockdown efficiency was confirmed by Western blot analysis in breast cancer cells treated with the same shRNAs (data not shown). Thus, knockdown of Sig-1R, in combination with Ad.p53, mimicked the action of Rimcazole, demonstrating the involvement of Sig-1R in the synergistic killing effect.

3.4. ER stress, ROS production and p38MAPK activity is promoted by the combination of Ad.p53 plus Rimcazole

Sig-1R is a ligand-regulated membrane protein chaperone involved in BiP regulation and the ER stress response [15,26,27]. In the present study we investigated the effects of combination of Ad.p53 (10 pfu/cell) and Rimcazole (4 μM) on the ER stress-signaling

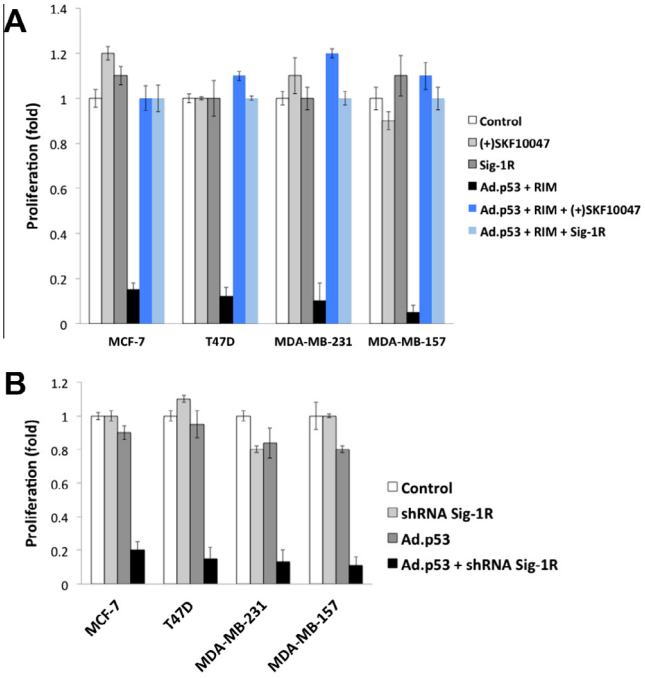

Fig. 2. Sigma 1 Receptor mediates cancer-specific apoptosis induced by combination treatment with p53 plus Rimcazole. (A) Breast cancer cells were infected with Ad.vector (control), Ad.p53 at low doses and Rimcazole, with or without ectopic expression of Sig-1R or with or without (+)-SKF-10047. An MTT assay was performed 5 days after treatment. An average of three independent experiments is shown ± SD. (B) shRNA knockdown of Sig-1R mimics Rimcazole. Breast cancer cells were transfected with Sig-1R shRNA, with or without Ad.p53 or Ad.vector. An MTT assay was performed 5 days after treatment. Data points represent mean values (±SD) from wells in quadruplicate. Each data set was obtained from a representative experiment performed at least three times.

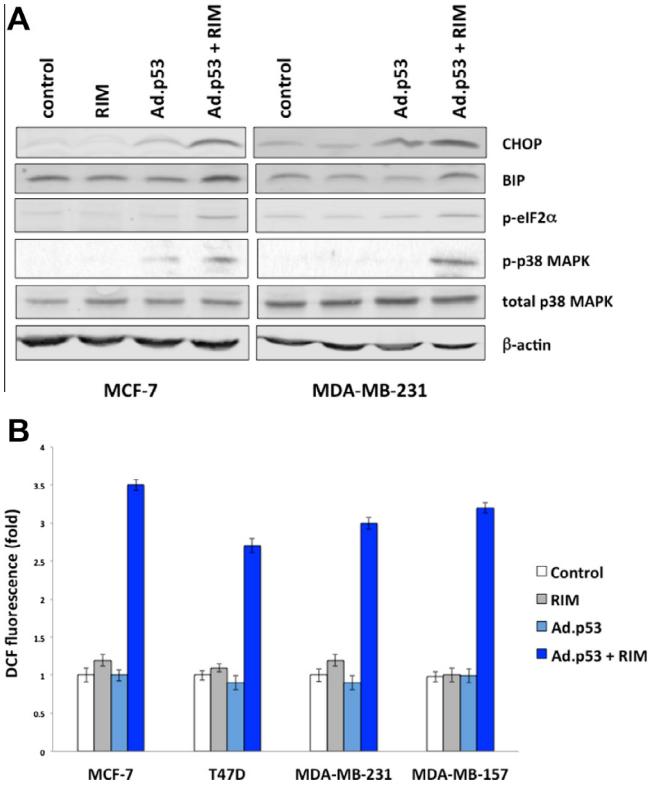

Fig. 3. Ad.p53 in combination with Rimcazole is critically involved in induction of ER stress, p38 MAPK and ROS production. MCF-7 and MDA-MB-231 cells were infected with 10 pfu/cell of Ad.vector or Ad.p53, and treated with or without 4 μM Rimcazole for indicated times. (A) Changes in BiP, CHOP, phospho-p38 MAPK and p-eIF2α proteins were evaluated by Western blot analysis 48 h after indicated treatments. (B) MCF-7 and MDA-MB-231 cells were infected with Ad.vector or with Ad.p53 and treated with or without 4 μM Rimcazole for 24 h. Intracellular ROS levels were measured with 10 μM DCF-DA 30 min after treatments. The results are expressed as the mean ± SD of three independent experiments.

pathway. For this purpose, we chose to investigate two well-characterized breast cell lines: strongly invasive breast cancer MDA-MB-231 cells (mutated p53); and noninvasive breast cancer MCF-7 cells (wild type p53). As shown in Fig. 3, single treatment with Rimcazole or Ad.p53 (applied at low, nontoxic doses) showed minimal effect on ER stress in all cell lines (Fig. 3A). Up-regulation of several ER stress markers, including p-eIF2α, CHOP, and BiP, were significantly induced by treatment with Ad.p53 in combination with Rimcazole (Fig. 3A).

Recent research has shown that mitogen-activated protein kinase (MAPK) signaling pathways have a role in the response to ER stress [28]. Therefore, activation of p38 MAPK has been recognized as key molecule on ER stress. We determined if p38 MAPK activation also plays a role in Ad.p53 plus Rimcazole-induced killing in breast cancer cells. MCF-7 and MDA-MB-231 cells were either uninfected or infected with Ad.p53 in combination with Rimcazole and analyzed by SDS–PAGE followed by Western blotting with anti-phospho-p38 MAPK, anti-total p38 MAPK, and β actin antibodies. Treatment with Ad.p53 in combination with Rimcazole promoted p38 MAPK phosphorylation in MCF-7 and MDA-MB-231 cells, whereas it did not affect total p38 MAPK (Fig. 3A).

Previous research has shown that knockdown of Sig-1R promotes apoptosis induced by ER stress and by ROS [15,29]. We observed that low-dose Rimcazole treatment (4 μM) or Ad.p53 (10 pfu/cell) did not cause detectable intracellular ROS production in breast cancer cells, whereas Ad.p53 infection in combination with Rimcazole increased production of various (superoxide as well as peroxide and/or nitric oxide) ROS species in breast cancer cells, as detected by DCFH-DA staining (Fig. 3B).

3.5. Combinational treatment with Ad.p53 and Rimcazole induces activation of caspase-3 and the upregulation of Bax

Caspases, a unique class of aspartate-specific proteases, are the central components of the apoptotic response. The release of cytochrome c from mitochondria into the cytosol leads to the activation of the initiator caspase-9, which in turn activates the effector caspase-3. Once activated, caspase-3 is responsible for the proteolytic cleavage of a broad spectrum of cellular targets, which ultimately leads to cell death [30]. As we showed in Fig. 1, combinational treatment with Ad.p53 and Rimcazole induced apoptotic breast cancer cell death. We next investigated activation of caspase 3 in breast cancer cells treated with Ad.p53 in combination with Rimcazole. Activation of caspase-3 was observed after incubation with Ad.p53 plus Rimcazole for 48 h (Fig. 4A). Increases in Bax expression may lead to mitochondrial depolarization and cytochrome c release, resulting in the downstream activation of executioner caspase to augment apoptosis. Combination treatment with Ad.p53 and Rimcazole of MCF-7 and MDA-MB-231 cells led to an increase in Bax protein expression as compared to control (Fig. 4). The combination of Ad.p53 and Rimcazole significantly increased Bax and activated caspase-3 in breast cancer cells, without any changes in total p53, p21 and Sig-1R protein levels.

4. Discussion

In the present study, we used a novel strategy of combining a gene therapy approach, Ad.p53, with Rimcazole, and evaluated

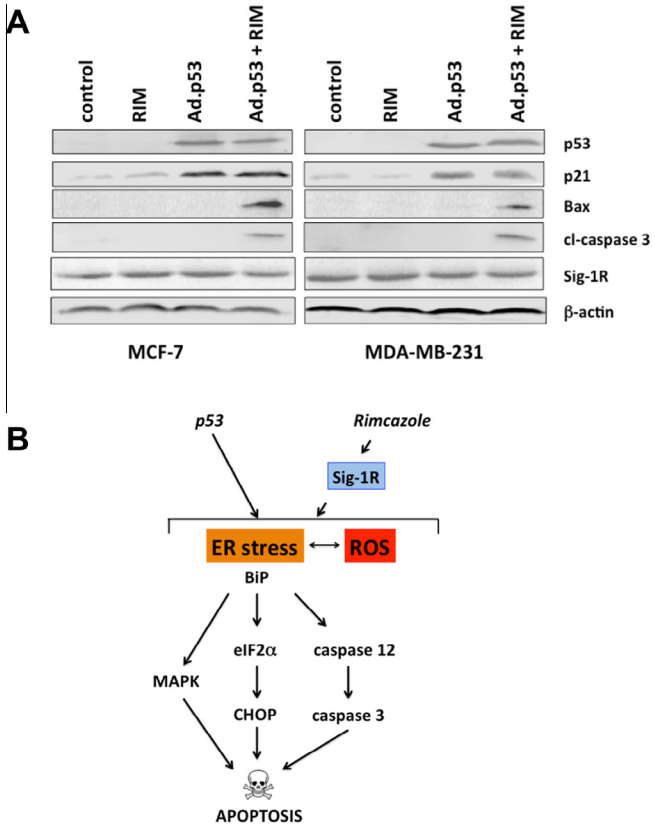

Fig. 4. Combination of Ad.p53 and Rimcazole induces Bax and caspase 3 activities. (A). MCF-7 and MDA-MB-231 cells were infected with either Ad.vector (control) or Ad.p53 at low doses and either untreated or treated with Rimcazole. Western Blot analysis was performed with antibodies for p53, p21, Sig-1R, phospho-p38 MAPK, cleavage-caspase 3, Bax, and β-actin. Each data set was obtained from a representative experiment performed at least three times. (B). Proposed model of the molecular mechanisms underlying combination Ad.p53 plus Rimcazole-induced apoptosis of breast cancer cells, involving ER stress, ROS generation, p38 MAPK activation, and caspase cascades. *Abbreviations:* Sig-1R, Sigma 1 Receptor; ROS, reactive oxygen species; ER, endoplasmic reticulum; CHOP, C/EBP homologous protein; MAPK, mitogen-activated protein kinases; eIF2α, α-subunit of eukaryotic initiation factor 2.

its antitumor effects against breast cancer cells. The tumor suppressor p53 has the ability of inducing apoptosis in diverse cancer cells without harming normal cells or tissues [1]. However, as with most treatment modalities, particular subsets of cancer cells might display inherent or acquired resistance to p53 gene therapy [4]. Additionally, the effective therapeutic window for many protocols can be very narrow, frequently resulting in toxicity [4]. Our current study highlights a novel strategy for enhancing therapy for breast cancer that employs a combination of p53 with Rimcazole. When employed at sub-optimal concentrations the combination of agents synergistically enhanced growth suppression and promoted apoptosis induction in a series of breast cancer cell lines with different p53 mutational alterations and with relative sensitivity or resistance to either agent employed alone. This therapeutic effect correlated with an activation of p38 MAPK signaling, ER stress and ROS production. These results suggest that the combinatorial effect of Ad.p53 with Rimcazole can bring about significant changes in specific cell signaling pathways that elicit an enhanced anti-proliferative and pro-apoptotic effect in breast cancer cells. Therefore, the combination of Ad.p53 and Rimcazole, at sub-optimal apoptosis-inducing concentrations synergistically enhanced growth inhibition and apoptosis induction over that observed with either agent alone independently from their p53 status. Also, our results demonstrate that shRNA knockdown of Sig-1R mimics the effect of Rimcazole in inducing apoptosis in combination with Ad.p53, these results not only suggest that Sig-1R is essential to the growth-inhibitory activity of Ad.p53 in combination with Rimcazole, but also highlights a new strategy using Ad.p53 in combination with shRNA of Sig-1R. Furthermore, our data suggest that the growth-inhibitory activity of Ad.p53 in combination with Rimcazole in breast cancer cells is achieved by antagonizing Sig-1R and the subsequent signaling of apoptotic events including increasing of Bax expression levels, as well as activating caspase-3. Taken together, these data suggest that Ad.p53 in combination with Rimcazole exerts growth-inhibitory effects on breast cancer cells via Sig-1R.

Sig-1R antagonists have been tested in human subjects for other indications [31,32], which could assist the translation of such drugs into human cancer trials. Further studies are required to determine the effect of this combinatorial approach in *in vivo* tumor models, which if successful, could serve as an entry point for translation into the clinic for defining safety and ultimately efficacy in patients with breast cancer.

Acknowledgments

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