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Original Research

poly(I:C) synergizes with proteasome inhibitors to induce apoptosis in cervical cancer cells

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ABSTRACT

Cervical cancer is one of the most common malignancies in women, with a poor survival rate. Thus, there is a need to define effective combination strategies to improve therapy. In this study, we report that dsRNA poly(I:C) up-regulated the expression of IFNβ and apoptosis-associated genes in cervical cancer cells, activating both intrinsic and extrinsic apoptotic pathways, and eventually inducing cell death. Similarly, proteasome inhibitors also effectively induced cervical cancer cell apoptosis, probably through prevention of p53 degradation, inhibiting NF-κB signal activation and decreasing BCL-2 expression. Importantly, the combination of poly(I:C) with proteasome inhibitors enhanced caspase-8 and caspase-9 activation, and synergistically induced cervical cancer cell apoptosis. Both activated p38 signals and increased ROS levels, and their combination extended these effects. Collectively, we show that the activation of multiple pro-apoptotic pathways by poly(I:C) and proteasome inhibitors underpin a synergistic effect on inducing cervical cancer cell death, suggesting a potential therapeutic combination with clinical relevance.

Introduction

Cervical cancer is one of the most common gynecological malignancies. There are approximately 600,000 new cases and over 340,000 deaths from cervical cancer worldwide in 2020 [44]. Moreover, the onset age of cervical cancer is becoming lower [11]. Multiple factors are associated with cervical cancer development, including persistent infection with high-risk human papillomavirus (HPV) [10]. Currently, traditional chemotherapy and targeted therapy usually associate with drug resistance and side effects, which greatly undermines their efficiency. Therefore, new strategies in cervical cancer therapy are greatly needed. poly(I:C), double-stranded RNA (dsRNA) is able to activate multiple biological events, including broad-spectrum antiviral responses and immune modulation [1, 12]. poly(I:C) induces downstream signaling cascades by engaging Toll-like receptors (TLR3) and/or the intracellular RIG-Like receptor (RLR) family members, RIG-I and MDA5 [32]. Induction of type I interferon (IFN) and the expression of various IFN-stimulated genes (ISG) is thought to be a major mechanism that mediates poly(I:C) biological functions [4, 8]. Recent studies have demonstrated that poly(I:C) can directly trigger cell apoptosis in colon, lung and cervical cancer [8, 45, 48]. In addition, poly(I:C) has the potential to help overcome the resistance of malignant cells to radiotherapy and chemotherapy [28, 34, 41]. Currently, an increasing number of clinical trials of poly(I:C) combined with various treatments, such as vaccines, adjuvants and monoclonal antibodies are undergoing.

Proteasome inhibitors have been approved by the USA Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for treating recurrent (refractory) multiple myeloma and mantle cell lymphoma [23, 30, 40]. Due to their success in hematological malignancies, proteasome inhibitors have been extensively studied for the treatment of various solid tumors including lung, colon, pancreas, breast and head and neck cancer [9, 23, 38]. Proteasome inhibitors can be combined with other drugs to induce cancer cell death [20, 43]. In cervical cancer, proteasome inhibitor Delanzomib sensitizes cells to doxorubicin-induced apoptosis [14]. Moreover, bortezomib combined with an HDAC inhibitor shows a synergistic effect on HPV-positive cervical cancer cells [21]. In this study, we report that proteasome inhibitors combined with poly(I:C) synergistically activate intrinsic and

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extrinsic apoptotic pathways and effectively promote cervical cancer cell death. This study suggests a potential application of poly(I:C) and proteasome inhibitors in cervical cancer therapy.

Materials and methods

Cell culture and reagents

Cervical cancer HeLa (HPV-18+), SiHa (HPV-16+) and C33A (HPV-) cell lines, lung cancer A549 cell line and colon cancer HCT116 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) serum (ABW) and 2 mmol/ L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, at 37 °C in an incubator containing 5% CO_2. MG132, Bortezomib and Lxazomib were products of MCE, Sorafenib, IMD-0354 and CUDC-907 were purchased from Selleckchem.

poly (I:C) transfection

For poly(I:C) transfection, 2×10^5 cervical cancer cells were seeded in 24-well plate and cultured to 80% confluence. Lipofectamine™ 3000 (Invitrogen) was used for transfection of poly(I:C) (Sigma-Aldrich Co. Ltd.) at a ratio of 2 μL of Lipofectamine™ 3000 to 1 μg poly(I:C). The treatment of 2 μL/ml Lipofectamine 3000 has no obvious cytotoxicity to cervical cancer cells compared to the control 0 μL/ml Lipfectamin 3000. Transfection mixture was prepared in pre-warmed Opti-MEM medium to make a total volume of 100 μL per ml culture volume. After incubation for 20 min at room temperature the mixtures were gently pipetted onto the cells in normal growth medium and gently mixed.

Western blotting

The whole cell protein was lysed with SDS sample buffer consisting of 4% SDS (sodium dodecylsulfate), 20% glycerol and 50 mM Tris.HCl (PH6.8), proteins were separated by SDS-PAGE, nitrocellulose membranes proteins were incubated overnight at 4 °C with specific primary antibodies. Caspase-8, caspase-9, PARP, p-IκBα(Ser32/36), p38 and p-p38(Thr180/Tyr182) were products of Cell Signaling; IκBα were bought from Santa Cruz Biotechnology; BCL-2 was bought from Dako; p53, MX1, ISG15, ISG54, BAK and TRAIL antibodies were purchased from Solarbio. Anti-rabbit and anti-mouse secondary antibodies and β-actin were bought from Servicebio. Protein bands were visualized with the Odyssey system (Pierce, Waltham, MA, USA).

Quantitative real-time PCR (qPCR)

Total RNA was obtained using Eastep TM Super Total RNA Extraction Kit (Promega), cDNA was obtained using cDNA Synthesis SuperMix (Novoprotein). qPCR was performed on ABI-7500 using SYBR-Green qPCR Master Mix (MCE) following the manufacturer's instructions. qPCR primers used in this study refer to previous publications [7, 27]. Relative gene expression was calculated based on the threshold cycle (Ct) values and normalization of internal control expression using the $2^{-\Delta\Delta Ct}$ method [22]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was used as an internal control in this study. Experiments were performed in triplicate and repeated three times.

Flow cytometry

Cell death was determined by flow cytometry. Briefly, cervical cancer cells were cultured in 24-well plate. Following by poly(I:C) transfection with Lipofectamine for 24 hours or/and inhibitors treatment for 48 hours at various concentrations as described, cells were released from the well by trypsin. Cells were washed and finally resuspended with PBS. Cells were stained with propidium iodide (PI) (50 μg/ml) at 4 °C in the dark. Percentages of cells death (PI-positive cells) were determined by flow cytometry (BD Biosciences). Intracellular ROS were measured using the oxidation-sensitive fluorescent probe DCFH-DA following the manufacturer's instructions. The protocol was performed according to the Reactive Oxygen Species Assay Kit (Beyotime, Shanghai, China). Briefly, after treating with poly(I:C) and/or proteasome inhibitor at the described time, cervical cancer cells were collected and incubated with DCFH-DA at 37C for 20 min. The cells were then washed three times with no-serum culture medium and analyzed using flow cytometry. The ROS level was expressed as mean fluorescence intensity (MFI) and normalized to the control, namely untreated cells by poly(I:C) and proteasome inhibitors. Assays were performed in duplicate and repeated at least two times.

Statistical analysis

Significance of the different treatments was assessed using the Student's *t*-test or two-way ANOVA analysis. Differences were considered statistically significance at a *P*-value of <0.05.

Results

poly(I:C) induces cervical cancer cell apoptosis

In order to define improved therapies for cervical cancer, we first investigated the effects of poly(I:C) on cervical cancer cell death. As shown in Fig 1, poly(I:C) effectively induced cell death in a dose-dependent pattern in cervical cancer HeLa, SiHa and C33A cell lines (Fig. 1A-C), at comparable levels to that induced in lung cancer A549 cells (Fig. 1D). Interestingly, poly(I:C) was not effective at inducing death in colon cancer HCT116 cells, even at high concentrations (Fig. 1E). The nature of the induced cell death was further investigated by measuring caspase activation. We found that poly(I:C) not only activated caspase-8 but also caspase-9 (Fig. 1F). Moreover, this was accompanied with a cleavage of PARP. These results together suggest poly(I:C) can induce cervical cancer cell death through activation of both mitochondrial- and death receptor-mediated apoptotic pathways. We also found that poly(I:C) stimulation up-regulated the expression of IFNβ and other apoptosis-associated genes, such as ISG15, ISG54, TRAIL, TNFα, OAS1, MX1, PUMA and BAK, in a time- and concentration-dependent model (Fig. 2 and Supplementary Fig. 1). In contrast, poly (I:C) had no effect on the expression of anti-apoptotic protein BCL-2 (Fig. 2 and supplementary Fig. 2). The induction of ISGs expression was further confirmed at the protein level by WB (Supplementary Fig. 2). These results show that poly(I:C) induces cervical cancer cell death by activation of the intrinsic and extrinsic apoptotic pathways, with upregulation of IFNβ signaling.

Proteasome inhibitors induce cervical cancer cell apoptosis

The success of proteasome inhibitors in hematological malignancies therapy makes them an attractive candidate to treat solid tumors. We therefore investigated the efficiency of proteasome inhibitors in cervical cancer, including MG132, bortezomib and lxazomib. As shown in Fig. 3, these inhibitors effectively induced cell death in HeLa and SiHa (Fig. 3A-F). Surprisingly, proteasome inhibitors had no effect on A549 but killed HCT116 (Fig. 3G-H), opposite to what was found with poly(I:C). We next investigated the possible mechanisms of proteasome inhibitor-induced cervical cancer cell apoptosis. We found MG132 promoted p53 protein accumulation (Fig. 4), consistent with previous results [37, 38]. Moreover, MG132 increased phosphorylation of IκBα, a natural inhibitor of NF-κB, suggesting a blockage on NF-κB pathway activation (Fig. 4A). MG132 also significantly inhibited BCL-2 expression, suggesting an unbalance of pro- and anti-apoptotic signals. In contrast, in A549 lung cancer cells, MG132 had no effect on BCL-2 expression and a very little effect on p-IκBα, and this was the opposite in HeLa cells (Fig 4B). MG132

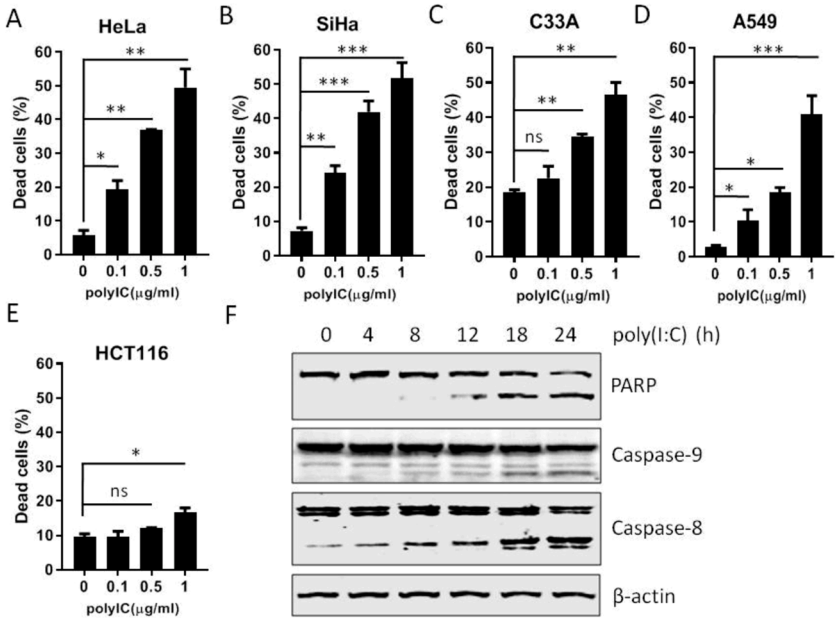

Fig. 1. poly(I:C) induces cervical cancer cell apoptosis Induction of (A) HeLa, (B) SiHa, (C) C33A, (D) A549 and (E) HCT116 cell death measured by PI Staining. Cells were transfected with poly(I:C) at various concentrations for 24 hours. Graphics show mean percentages of PI positive (dead) cells; error bars represent standard deviation (SD). Experiments were performed in duplicates and repeated at least 3 times; (F) Representative Western blots showing Caspase-8, caspase-9 and PARP protein expression in lysates of HeLa cells treated with 1 μg/ml poly(I:C) at different time points. β-actin was used as a loading control. Statistical significance was determined using the Student *t*-test. Statistical significance vs control is indicated by $^{*}p<0.05$, $^{**}p<0.01$, and *** $p<0.001$. Nonsignificant results are denoted by ns.

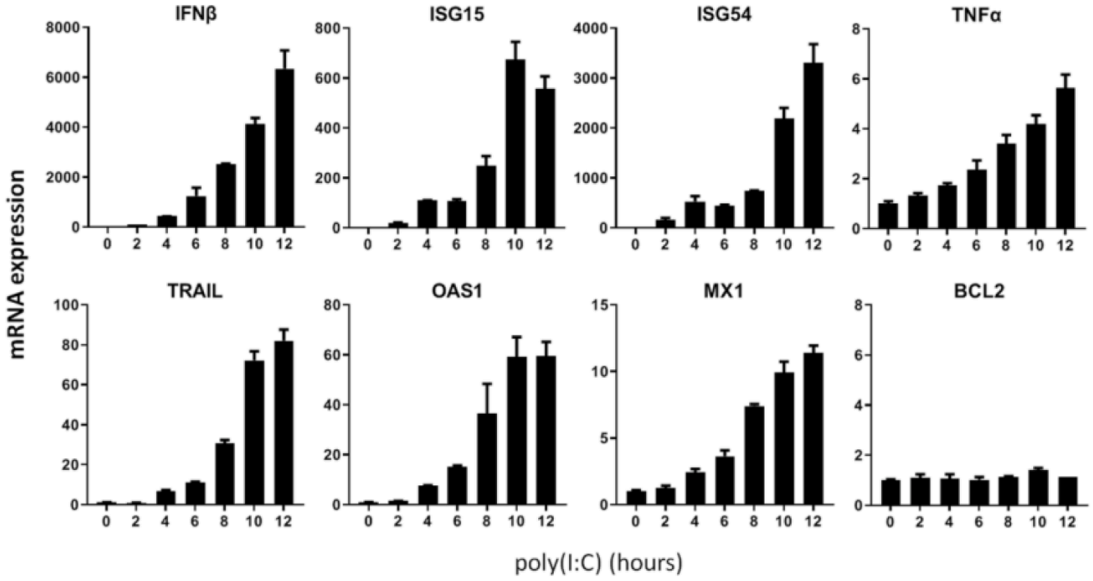

Fig. 2. poly(I:C) activates pro-apoptotic signals in cervical cancer cells mRNA expression of IFNβ, ISG15, ISG54, TNFα, TRAIL, OAS1, MX1 and BCL2, as measured by qRT-PCR in HeLa cells stimulated with 1 μg/ml poly(I:C) at various time points. The expression of target genes was normalized to internal control GAPDH. Data were expressed relative to control (no poly(I:C) stimulation), error bars represent SD. Experiments were performed in triplicates and repeated at least 3 times.

also activated caspase-8 and caspase-9 (Fig. 4A), which is consistent with the induction of multiple pro-apoptotic pathways and cervical cancer cell death.

poly(I:C) synergizes with proteasome inhibitors to induce apoptosis

The effects of poly(I:C) and proteasome inhibitors on cervical cancer cells led us to investigate the combined effects of both compounds. We found that poly(I:C) enhanced the efficacy of proteasome inhibitors to induce apoptosis in HeLa, and demonstrated a synergistic effect (Fig. 5A-B and Table S1). In contrast, the combination of poly(I:C) with Sorafenib, a RAF kinase inhibitor, and IMD-0354, an IKKβ/NF-κB pathway inhibitor, had inhibitory effects(Fig. 5C-D). poly(I:C) had a modest effect on the ability of CUDC907, a PI3K and HDAC inhibitor, to induce cell death (Fig. 5E). We compared this to the effects of the combination of poly(I:C) with proteasome inhibitors on other cancer cells and found that poly(I:C) only slightly increased the induction of cell death by MG132 in HTC116 (Supplementary Fig. 3A). The combination reduced apoptosis in A549, showing an antagonistic effect (Supplementary Fig. 3B). Consistently, this combination enhanced caspase-8 and caspase-9 activation and promoted PARP cleavage in HeLa, while inhibited caspases activation in A549 (Supplementary Fig. 3C-D).

Activation of the p38 and reactive oxygen species (ROS) production are important factors in inducing cancer cell apoptosis [4, 17, 49]. We found that both poly(I:C) and proteasome inhibitors can activate the p38 signaling pathway (Fig. 6A). Of note, the combination significantly extended the duration of p38 pathway activation (Fig. 6A). In addition, we also observed that the intracellular levels of ROS were significantly increased by the combination (Fig. 6B-C). Taken together, these results demonstrated poly(I:C) can synergize with proteasome inhibitor to

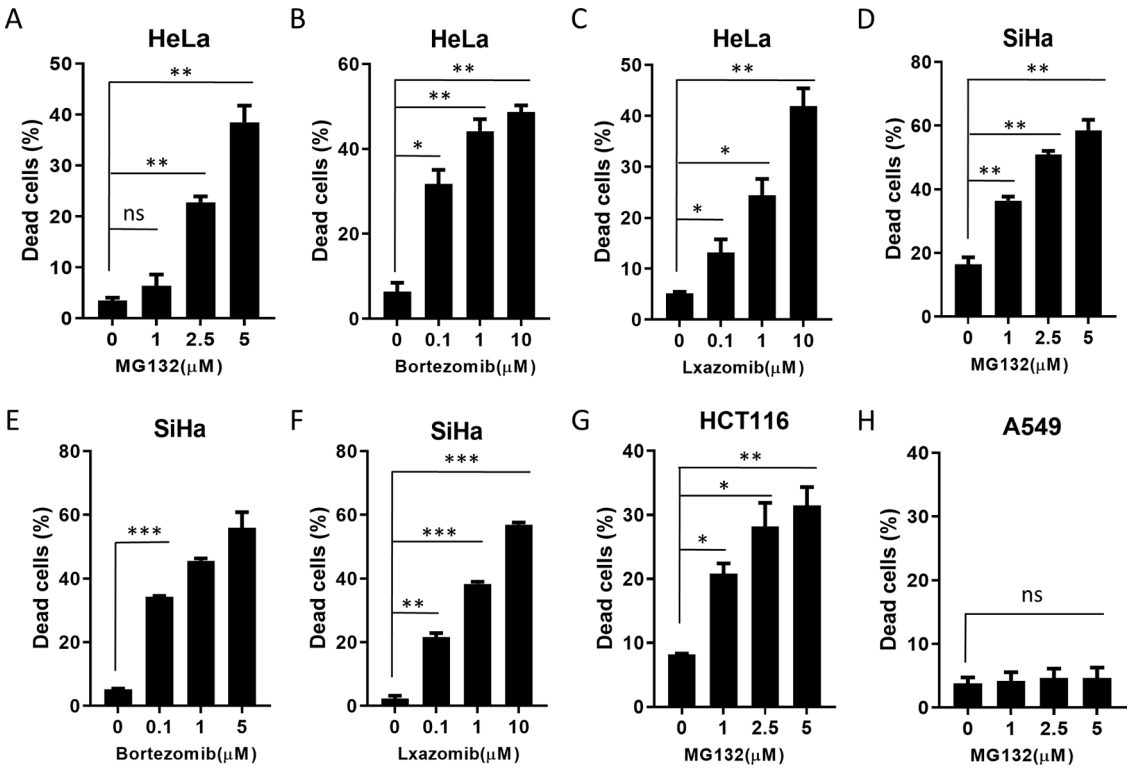

Fig. 3. Proteasome inhibitors induce cervical cancer cell apoptosis Cell death in (A-C) HeLa, (D-F) SiHa, (G) HCT116 and (H) A549 was measured by FACS analysis of PI-stained cells. Cells were treated with MG132, Bortezomib or Lxazomib at described concentrations for 48 hours. Experiments were performed in duplicates and repeated at least 3 times; Graphics show the mean percentage of PI positive (dead) cells, error bars represent SD. Statistical significance was determined using the Student *t*-test. Statistical significance vs no treatment/control is indicated by $*p<0.05$, $**p<0.01$, and $***$ $p<0.001$. Nonsignificant results are denoted by ns.

induce cervical cancer cells apoptosis and suggest a potential mechanism based on p38/ROS induction.

Discussion

Although HPV vaccination has greatly reduced the incidence of cervical cancer [3, 42], cervical cancer remains an important health problem in women, particularly due to the lack of effective treatments in almost half of the cases [31]. In the present study, we report a potential new combination treatment using poly(I:C) and proteasome inhibitors. Each of the drugs on their own induced cell death in cervical cancer cell lines. poly(I:C) significantly increased the expression of apoptosis-associated genes including IFNβ, OAS1, MX1, ISG15, ISG54, TNFα, PUMA and TRAIL, together with the cleavage of caspases and PARP. This suggests that poly(I:C) induces apoptosis simultaneously through different pathways in these cells. In contrast, poly(I:C) had less effect on the colon cancer HCT116 cell line which is consistent with previous report [45]. This could probably be because cervical cancer cells are HPV-positive and therefore sensitive to poly(I:C), an effective inducer of type I IFNs, while HCT116 is not a virus-infection-mediated cancer cell type and thus less sensitive to poly(I:C)-induced apoptosis.

We also found that proteasome inhibitors are very effective in inducing cervical cancer cell apoptosis. MG132 inhibited NF-κB activation, prevented p53 degradation and inhibited BCL-2 expression while activating caspases-8 and -9 [9, 38]. This may collectively contribute to its effects in cervical cancer cells, since activation of the NF-κB signaling pathway and high levels of BCL-2 expression are normally related to cancer cell survival and drug resistance [18, 47]. The pro-apoptotic protein p53 is often inactivated in cancer cells, and although the majority of cervical cancer cells have a wild-type p53 gene, the protein levels are strongly decreased due to HPV E6 protein [25, 26, 37]. Proteasome inhibitors could thus restore wild-type p53 protein levels in these cells by countering the E6 effect through a blockage in p53 degradation. In contrast, proteasome inhibitor MG132 had no effect on A549 lung cancer cells, despite these cells also having wild-type p53. The mechanisms of this discrepancy remain unknown, but we found significantly low levels of BCL-2 and p-IκBα in A549, and MG132 had no effect on BCL-2 expression and a very little effect on p-IκBα (Fig 4B) which is completely different from that in HeLa cells. A previous study showed that MG132 can induce A549 apoptosis but at a concentration of more than 10 μM or 30 μM, nonetheless such concentrations are out of clinical relevance. Conversely, lower concentrations of MG132 have the potential to promote A549 cell growth [16]. New generation proteasome inhibitor Bortezomib has a modest effect on non-small cell lung cancer (NSCLC) cells, and a greater effect on wild-type p53 cells than p53 mutant cells [13, 19]. Collectively, proteasome inhibitors have less effect on lung cancer cells compared to cervical cancer cells.

Currently, many clinical trials of dsRNA poly(I:C) combined with different reagents are being conducted including CpG, oligodeoxynucleotides(ODN) and an anti-CD40 antibody [1]. Proteasome inhibitors have also been studied in combination with various drugs to improve cancer treatments. For example, the combination of MLN2238 (Ixazomib) with IFNα has been found to enhance melanoma cell death [43]; Delanzomib treatment sensitizes cervical cancer cells to doxorubicin-induced apoptosis [14]; the combination of Bortezomib and HDAC inhibitors shows a synergistic killing effect on HPV-positive cervical cancer cells [21]; and MG132 combined with TRAIL promotes human osteosarcoma cells apoptosis [20]. In this study, we reported that poly(I:C) combined with proteasome inhibitors increase cervical cancer cells apoptosis, which was better than in other combinations, such as with CUDC907 (PI3K and HDAC inhibitor) [6], Sorafenib (RAF signaling pathway inhibitor), or IMD-0354 (NF-κB signal pathway inhibitor). The latter even showed an antagonistic effect, although the mechanisms remain unknown. In addition, it is also notable that no synergy was observed in lung cancer cells, but, instead, an inhibitory effect was found. This suggests that the synergistic effects may be tissue specific

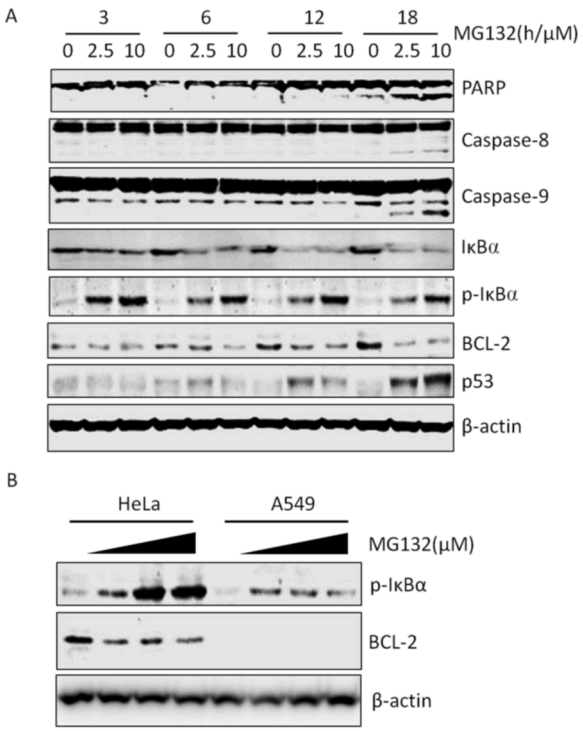

Fig. 4. Proteasome inhibitor MG132 affects pro-apoptotic signals in cervical cancer cells (A) Representative Western blots showing Caspase-8, caspase-9, PARP, IκBα, p-IκBα, BCL-2 and p53 protein expression in lysates of HeLa cells treated with MG132 at 0, 2.5 or 10 μM concentrations for different times. (B) Representative Western blots showing p-IκBα and BCL-2 protein expression in HeLa and A549 after treatment with MG132 for 12 hours. β-actin was used as loading control.

and highlight the relevance of this combination in the context of cervical cancer treatment.

Multiple pro-apoptotic mechanisms activated by poly(I:C) and proteasome inhibitors may contribute to the observed effects on cervical cancer cells. Increasing p53 protein levels may be an essential one in tumors in which the protein is wild type. In HeLa, the HPV-encoded E6 protein promotes the rapid degradation of p53 protein through changing E3 ubiquitination ligase activity [39, 46]. This can be prevented by the action of the proteasome inhibitor. Moreover, poly(I:C) may also promote p53 expression and activation through phosphorylation [2, 17, 33], and the combination of both drugs may restore p53 functions [14, 15, 21, 29]. Moreover, our results suggest that activating p38 signaling and inducing ROS production may also play an important role in this combination strategy [4, 17, 49]. Both proteasome inhibitors and poly(I:C) have been shown to induce apoptotic cell death through the formation of reactive oxygen species (ROS) [5, 24, 35, 36]. Importantly, the combination of both reagents extended the duration of p38 signal activation and further increased ROS production. Taken together, our results suggest that multiple factors activated by poly(I:C) and/or proteasome inhibitors may collaboratively induce cervical cancer cell apoptosis (Fig. 7), which provides a mechanistic explanation for a synergistic effect and suggests a potential application in clinical therapy of cervical cancer.

Authors' contributions

Xueqiong Meng contributed to the conception and design of the work, acquired the majority of the data and drafted the manuscript.
Xiaoxi Cui contributed to the acquirement of some data.
Xiaoya Shao contributed to the acquirement of some data.
Yanqi Liu contributed to the acquirement of some data.
Yihao Xing contributed to the acquirement of some data.
Victoria Smith contributed to the acquirement of some data.
Shiqiu Xiong contributed to the acquirement of some data.
Salvador Macip contributed to the design of the work and to

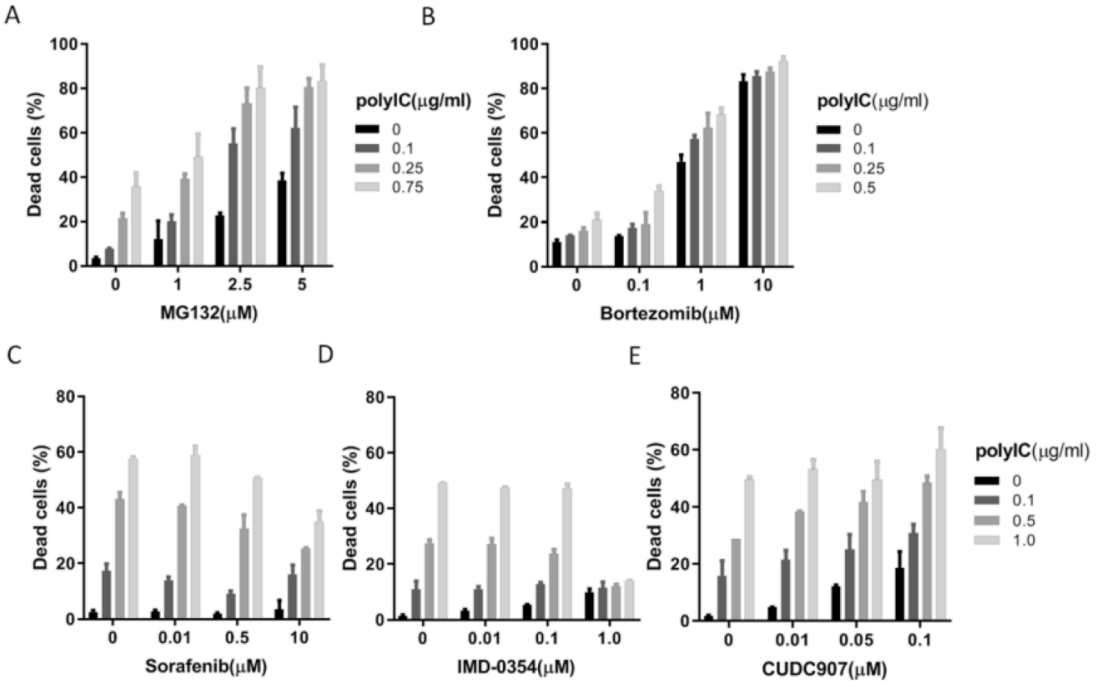

Fig. 5. poly(I:C) synergizes with proteasome inhibitors to induce cell death in HeLa cell Percentage of cell death measured by FACS analysis of PI-staining cells treated with different concentrations of poly(I:C) for 24 hours, alone or in combination with (A) MG132, (B) Bortezomib, (C) Sorafenib, (D) IMD-0354 and (E) CUDC907. Experiments were performed in duplicates and repeated at least 3 times; Graphics show the mean percentage of PI positive (dead) cells, error bars represent SD.

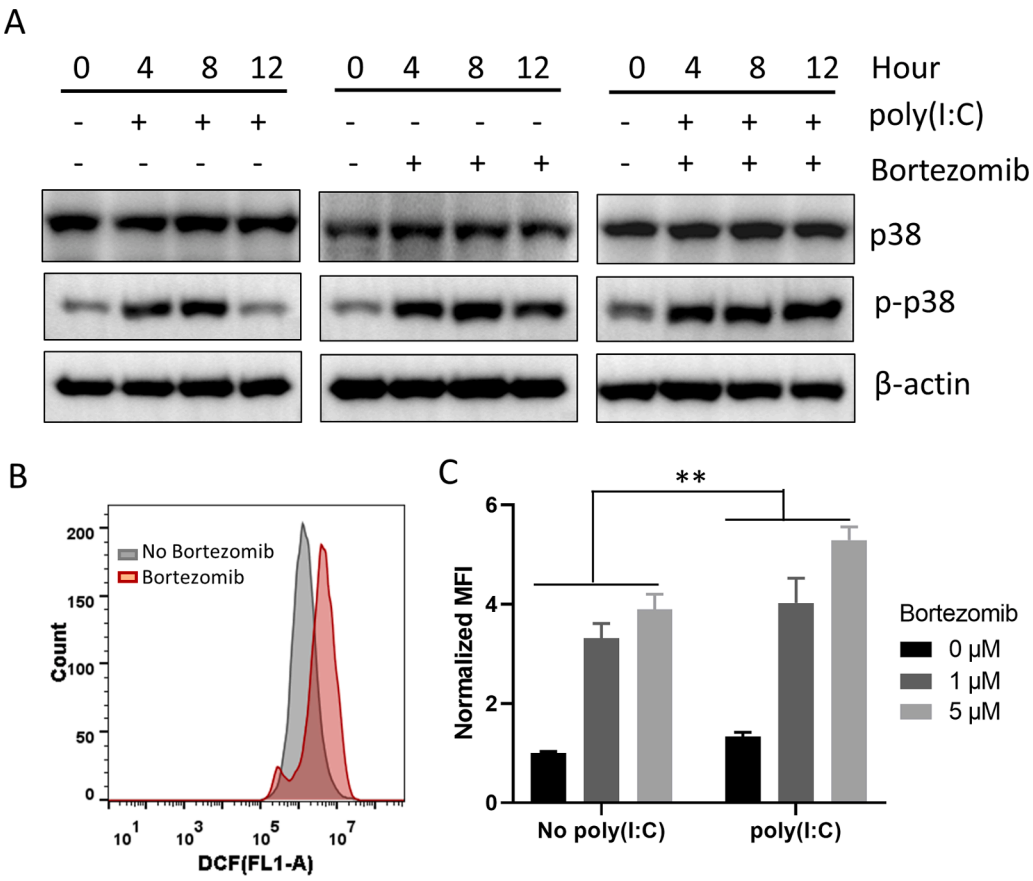

Fig. 6. poly(I:C) and proteasome inhibitors activate p38 and promote ROS production. (A) Representative Western blots showing total p38 and p-p38 protein expression in SiHa lysates. Cells were treated with 1 μg/ml poly(I:C) and/or 5 μM proteasome inhibitor Bortezomib and collected at described time points. β-actin was used as a loading control. (B) ROS levels as measured by DCFH-DA staining of HeLa cells treated with 0.1 μM Bortezomib for 40 hours (C) Same, in HeLa cells treated with Bortezomib 12 hours prior to poly(I:C) treatment. Data is expressed as mean fluorescence intensity (MFI) and normalized to control, namely, no poly (I:C) and no Bortezomib treated cells. Statistical significance was determined using 2-way ANOVA analysis. Statistical significance vs no poly(I:C) group is indicated by ** $p<0.01$.

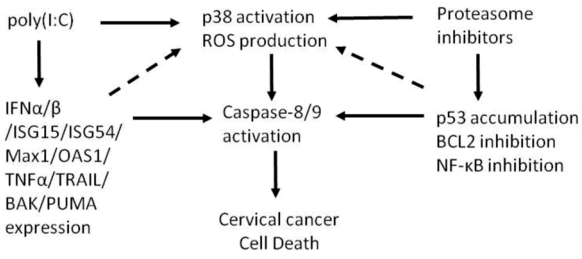

Fig. 7. Cervical cancer cell death induced by the combination of poly(I:C) and proteasome inhibitors. The model shows multiple pro-apoptotic mechanisms activated by poly(I:C) and proteasome inhibitors, which may explain how they collaboratively to induce cervical cancer cell death.

interpretation of data, and revised the manuscript.

Yixiang Chen contributed to the design of the work, to interpretation of data and substantively revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interests

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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Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101362.

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