# Small molecule nonsense mediated mRNA decay and MDM2-p53 inhibitors synergistically induce apoptosis in cervical cancer cells

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**Research Article**

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## Abstract

# Purpose

Nonsense-mediated mRNA decay (NMD) pathway and p53 pathway, which play important roles in RNA quality control and cancer suppression respectively, are tightly related to tumorigenesis, and both are promising targets for tumor therapy. However, the impact of NMD inhibitor, p53-MDM2 inhibitor, and the combination of these two agents was poorly understood in cervical cancer.

# Methods

Cell apoptosis, cell cycle distribution, and the expression level of p53-target genes were evaluated the influence of NMD inhibitor and MDM2-p53 inhibitor treated alone or in combination on HPV-18 positive HeLa cells. Rescue experiments by E6* overexpression and RNA-seq were conducted to explore the molecular mechanism.

# Results

The novel MDM2-p53 inhibitor, XR-2, did not activate p53 and thereby induced apoptosis in HeLa cells, whereas the NMD inhibitor (SMG1i) repressed cell proliferation at high concentrations. More importantly, the combination of these two agents significantly inhibited cell proliferation, arrest cell cycle progression, and induced cell apoptosis. Moreover, the combination treatment rose the level of hypoxia and unfolded protein response, which further increased cellular stress and led to cell apoptosis. Mechanistically, MDM2-p53 inhibitor and NMD inhibitor may act synergistically through the truncated E6 protein.

# Conclusion

The universal synergistic effects between MDM2-p53 inhibitor and NMD inhibitor provide a potential candidate for the clinical treatment of HPV-infected tumors. However, further studies are required to elucidate the specific molecular mechanism.

## Introduction

Human papillomavirus (HPV) vaccines developed in recent decades have prevented cervical cancer(Clark and Trimble, 2020). However, cervical cancer remains the fourth most common cancer in women, causing substantial economic losses and adding to the healthcare burden worldwide(Vu et al., 2018). High-risk HPV infections, primarily of HPV type 16 and 18, are the most crucial risk factors for cervical cancer (Chan et al., 2019). The main treatment methods for cervical cancer are surgery, radiotherapy, chemotherapy, and immunotherapy(Ferrall et al., 2021). Nevertheless, for patients in the advanced or metastatic phase, treatments with high efficiency and fewer side effects are still lacking. Hence, there is

an urgent unmet medical need to identify safer and more effective drugs for the treatment of cervical cancer.

p53 is a critical tumor suppressor that controls cell proliferation and apoptosis, which is ubiquitinated and degraded by MDM2(Demir et al., 2021). MDM2-p53 inhibitors, such as RG7388, mainly target *TP53* wild-type tumor cells and block the interaction between MDM2 and p53, thereby activating p53 and inhibiting tumor growth(Liu et al., 2019). In addition to MDM2, oncoprotein E6 also participates in p53 degradation. HPV DNA integrates with human genomic DNA, thus continuously expressing E6, which format E6-E6AP-p53 complex and mediated ubiquitination and degradation of p53(Oyervides-Muñoz et al., 2018). Hence, p53 dysfunction via E6 is the leading cause of tumors caused by high-risk HPV infection.

NMD is an crucial pathway for the quality control of gene fidelity at the mRNA level, which mainly degrades mRNA containing premature terminal codons and regulates 10–20% of all transcripts(Kurosaki et al., 2019). NMD is involved in stress response, tumor development, and other aspects for its wide array of substrates(Supek et al., 2021). UPF1 phosphorylation by SMG1, the only kinase in the NMD pathway, is an essential step in NMD, making UPF1 and SMG1 the key factors in NMD(Nicholson et al., 2010). In the *TP53* wild-type and mutant cell lines, NMD inhibition by the SMG1 inhibitor compound 11j could activate the p53 pathway and induce apoptosis via p53 isoforms(Gudikote et al., 2021). Our previous study demonstrated that another NMD inhibitor (SMG1i) combined with the MDM2-p53 inhibitor exhibited a significant synergistic effect in inducing apoptosis in *TP53* wild-type tumor cells. However, the influence of MDM2-p53 or NMD inhibitors on HPV-positive cervical cancer HeLa cells is poorly understood.

Here, we investigated the effects of treatments with NMD and MDM2-p53 inhibitors alone and in combination in HeLa cells. The combination of these two agents achieved a significant synergistic effect in inducing apoptosis. These findings could help in developing novel therapeutic options for women with HPV-positive cervical cancer.

## Materials And Methods

# Cell culture and reagents

The HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS plus antibiotics (100-U/mL penicillin and 100-mg/mL streptomycin) and maintained in a humidified incubator with 5% CO2 and 37°C.

The NMD inhibitor (SMG1i) and MDM2-p53 inhibitor XR-2 were homemade. RG7388 was purchased from Selleck (S7205, Selleck Chemicals, TX, USA).

# Western blotting

Cells in 6-well culture plates were rinsed briefly with cold PBS and added 150 μL cell lysis buffer (Beyotime, China). Cells were lysed on ice and spined down at 12,000 g for 5 min. 2 μL of supernatant was used for protein concentration measurement with BCA kit (Beyotime, China) according to manufacturers' instruction. An adequate 5x loading buffer (Beyotime, China) was added to the supernatant. Same amounts of proteins of each sample were loaded and separated by 12% SDS-PAGE gel and then transferred to 0.45 μM polyvinylidene fluoride (PVDF) membranes. GAPDH was used as the loading control. The primary antibodies used in this study were p53 (CY2167, Abways, USA), γH2AX (ab81299, Abcam, UK), cleaved caspase-3 (asp175, Cell signaling, USA), PARP and cleaved PARP (sc-365315, Santa Cruis, USA), GAPDH and Flag-tag (M20006 and M20008, Abmart, China).

# RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated by TRIzol reagent (Thermo Fisher Scientific, USA). cDNA was synthesized by reverse-PCR kit (AGbio, China) using 500 ng total RNA. Quantitative real-time PCR was conducted on cDNA using 2× SYBR Green Mix (AGbio, China). Fold-change expressions were calculated by $2^{-\Delta\Delta Ct}$ method with GAPDH as an endogenous control. Sequences of primers were listed in Supplementary Table 1.

# Cell viability assay

1000 HeLa cells were seeded in 96-well plates and treated with different concentrations of XR-2 or SMG1i for 72 hours. Cell Counting Kit-8 (Beyotime, China) was used to measure cell viability. The magnitude of synergistic effect was calculated using SynergyFinder (Version 2.0, https://synergyfinder.fimm.fi/synergy/) with Bliss and Loewe model. If the synergy score is more than 10, the interaction between XR-2 and SMG1i in HeLa cells is likely to be synergistic. The higher the score, the greater the synergistic effect. Dose-response curves and dose-response matrix were generated using GraphPad Prism (Version 9, USA) and SynergyFinder, respectively.

# Cell apoptosis assay

Annexin V-FITC-PI Apoptosis Detection Kit (Meilunbio, China) was used to measure HeLa cell apoptosis after 24 hours incubation with the indicated concentrations of agents according to manufacturers' instruction. Samples were measured on a BD Flow Cytometer (BD Biosciences, USA), and the data were analyzed with FlowJo (Version 10, USA).

# Cell cycle analysis

HeLa cells were treated with two agents for 24 hours, fixed by 70% ethanol, digested RNA by 50μg/μL RNaseA at 37°C for 1 h, and stained DNA with 50 μg/μL PI. Cells were analyzed on a BD Flow Cytometer within 24 h after staining. The data were analyzed with Modfit software (Version 5, USA).

# Plasmid construction

A Flag-tag was added to the 5' end of the sequence of HPV18-E6*, and it's 3' was connected to mCherry. PCDH vector digested by XhoI and NdeI restriction endonuclease was ligated with the whole inserted sequence using Gibson Assembling kit (Thermo Fisher Scientific, USA). The sequence of the constructed plasmid was confirmed by Sanger sequencing.

# siRNA and plasmid transfection

siRNA was transfected using TransMate reagent (Sangon, China), and plasmid was transfected using lipo8000 reagent (Beyotime, China) according to manufacturers' instruction. The sequence of siUPF1 were sense (5'-3'): CCUGCGUGGUUUACUGUAAUATT, antisense (5'-3'): UAUUACAGUAAACCACGCAGGTT.

# RNA sequencing of transcriptomes

The row reads data were filtered by FastQC (https://github.com/s-andrews/FastQC), assembled by STAR (https://github.com/alexdobin/STAR), and analyzed differentially expressed genes (DEGs) by HTseq-count (https://pypi.org/project/HTSeq) and edgeR (https://bioconductor.org/packages/edgeR). Fold change > 2 or < 0.5 with $p < 0.05$ was regarded as DEGs. The enriched pathways were analyzed by MSigDB Hallmark database (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp).

# Statistical analyses

Statistical analyses were performed in GraphPad Prism. $p < 0.05$ was considered to indicate a significant difference.

# Results

### SMG1i and MDM2-p53 inhibitors exhibit synergistic inhibition of cell proliferation in HeLa cells

XR-2 is a novel small-molecule MDM2-p53 inhibitor synthesized in our laboratory. Similar to RG7388, XR-2 can activate p53 in MDM2-overexpressing *TP53* wild-type tumor cells, thereby promoting cell apoptosis. As shown in Fig. 1a, XR-2 activated the expression of p53 in the *TP53* wild-type HCT116 cells in a dose-dependent manner. HeLa cells express wild-type *TP53* gene; however, the majority of translated p53 protein is degraded by the E6-E6AP-p53 complex(Hengstermann et al., 2001). To investigate the effect of

XR-2 on p53 in HeLa cells, we treated HeLa cells with different concentrations of XR-2 for 24 hours. Treatments with up to 10 μM of XR-2 did not show any significant increase in the low basal p53 expression (Fig. 1b). Cheruiyot et al.(Cheruiyot et al., 2021) reported a novel SMG1 inhibitor, SMG1i, with high selectivity for SMG1 and effective inhibition of NMD. In this study, we used this agent to attenuate NMD activity. Previous studies by Gudikote et al. (Gudikote, et al., 2021) and our results shown in Fig. 1a indicated that inhibition of NMD could activate p53β/γ, two p53 isoforms generated from alternative splicing of full-length p53, in HCT116 cells. We found that SMG1i stimulated p53β/γ expression in HeLa cells as well (Fig. 1c). Furthermore, treatment with 3.3 μM or 10 μM of SMG1i for 24 hours induced apoptosis in HeLa cells, which was accompanied by significantly increased expression of the apoptosis markers cleaved-caspase-3 and cleaved-PARP (Fig. 1c). Our unpublished data showed that a co-treatment with XR-2 and SMG1i exhibits significant synergistic effects in inducing cell apoptosis and inhibiting cell cycle progression in *TP53* wild-type tumor cells. To determine the combined effects on HeLa cells, we treated HeLa cells with different concentrations of these two agents alone or in combination. Interestingly, as shown in Fig. 1d and Fig. 1e, although HeLa cells were insensitive to XR-2 and SMG1i, the combination of these two agents significantly inhibited cell proliferation. To further assess the magnitude of the synergistic effect, we calculated the synergy score using the Bliss independence (Fig. 1f) and the Loewe additivity models (Supplementary Fig. 1), which yielded synergy scores of 19.504 and 18.629, respectively. The synergy score in both models was greater than 10, indicating significant synergistic interactions between the two agents. These two models also showed the most significant synergistic area between 0.2–3.2 μM of these two agents.

### Combination of NMD inhibition and MDM2-p53 inhibitors synergistically induce apoptosis in HeLa cells

Next, we evaluated the effect of the combination of these two agents on apoptosis using western blotting. The results revealed that XR-2 or SMG1i monotherapy had a small effect on the apoptosis of HeLa cells at 3.3 μM (Fig. 2a). However, the combination treatment significantly increased the expression of apoptosis markers, cleaved-caspase-3 and cleaved-PARP and dramatically increased the DNA damage marker, γH2AX. These results suggested that combining these two agents results in genomic DNA instability and apoptosis. Another MDM2-p53 inhibitor, RG7388, also showed similar synergistic effects in inducing apoptosis in HeLa cells when combined with SMG1i (Fig. 2b), as evidenced by an increased expression of cleaved PARP. Furthermore, NMD inhibition via siRNA targeting UPF1 (Fig. 2c and Supplementary Fig. 2) exhibited significant synergistic effects when combined with the XR-2 treatment. These results indicated that the synergism observed between XR-2 and SMG1i is specific to the inhibition of MDM2-p53 and NMD, results in increased DNA damage, and induces cell apoptosis in HeLa cells.

Subsequently, we assessed apoptosis using flow cytometry. As shown in Fig. 2d and Fig. 2e, low concentrations (1 μM) of XR-2 or SMG1i had no effect on apoptosis, and higher concentrations (3.3 μM) of monotherapy slightly increased apoptosis in HeLa cells. However, combining of these two agents at 1 μM and 3.3 μM induced 20% and 50% of apoptotic HeLa cells, respectively, which indicated a dramatically synergistic effect of these two agents.

## XR-2 and SMG1i co-treatment activates p53 pathway in HeLa cells

Next, we performed cell cycle analysis on HeLa cells upon treatment with XR-2, SMG1i, or a combination of these two agents by flow cytometry. As depicted in Fig. 3a and Fig. 3b, XR-2 and SMG1i treatments alone did not show any effect on cell cycle with no significant changes in the proportion of cells in G1- and S-phases. However, the combined treatment of the two agents resulted in significant changes in the cell cycle distribution with the majority of the cells confined to the G1-phase, which indicated that the combination treatment arrested the cell cycle and inhibited cell proliferation. Collectively, these results showed that the combination of these two agents results in growth arrest and increased apoptosis. Hence, we hypothesized that the combination treatment activates the p53 pathway. Using quantitative real-time PCR (Fig. 3c), we studied changes in the expression of four well-known p53 target genes, *P21* and *GADD45A*, which are associated with cycle progression, and *BAX* and *PUMA*, which are linked to apoptosis. The treatment with XR-2 did not show any changes in the expression of these genes, whereas that with SMG1i showed a small increase in their expression. Consistent with increased apoptosis and cell cycle arrest, the combination treatment showed significant upregulation in the expression of these four genes, which was also accompanied by an increase in the *TP53* expression.

## MDM2-p53 inhibitor and NMD inhibitor act synergistically through truncated E6 protein

Taken together, these experiments suggested that the combined treatment with the MDM2-p53 and NMD inhibitors activated the p53 pathway. p53 is mainly degraded by the E6-E6AP-p53 complex in HeLa cells; thus, we hypothesized that the synergistic effects might be related to E6 expressed by the HPV genome. As shown in Fig. 4a, the E6 gene in HeLa cells was expressed in two forms. The full-length transcript (E6-FL) produced a protein with 158 amino acid residues. The other one underwent alternative splicing (E6*) and deficit of a 182 bp fragment, which resulted in the generation of a premature termination codon and yielded a protein of only 57 amino acid residues(Inagaki et al., 1988, Olmedo-Nieva et al., 2018). We designed a set of primers, as illustrated in Fig. 4a, to detect these isoforms. HeLa cells expressed the two isoforms simultaneously (Fig. 4b and Supplementary Fig. 3a), and the expression level of E6* was much higher than that of E6-FL. Subsequently, we constructed an E6*-overexpression plasmid using the PCDH backbone vector. E6* was overexpressed in HeLa cells, and the resulting cells were treated with these two agents alone or in combination. The results as shown in Fig. 4c and Supplementary Fig. 3b depicted that overexpression of E6* could rescue apoptosis caused by the combination treatment, indicating that the E6* protein is indeed involved in the observed synergistic effect.

We further performed RNA-Seq analysis on HeLa cells treated with XR-2, SMG1i, and the combination of these two drugs. As shown in Fig. 4d, monotherapy with XR-2 had no effect on the transcriptome, whereas SMG1i single treatment resulted in more than 2,000 of DEGs, and the number of DEGs was even higher in the combination treatment group. To identify the enriched signaling pathways, we chose the top 200 upregulated genes in the combination group and performed enrichment analysis using the MSigDB Hallmark database (Fig. 4e). TNF-α and p53 were the most enriched pathways, apoptosis, unfolded protein response, and hypoxia were also significantly enriched. In summary, the combined treatment

activated the p53 pathway along with an increased unfolded protein cellular stress response and hypoxia, eventually leading to cell apoptosis.

# Discussion

Although HPV vaccines can prevent cervical cancer, it remains a major cause of death in women in developing countries owing to the high cost and low vaccination rate(Bruni et al., 2016). Upon high-risk HPV infection, the genomic DNA of the virus gets integrated into the genome of the dormitory cells, stably expressing E6. E6 has the same function as MDM2, which can mediate the ubiquitination and degradation of p53, resulting in p53 dysfunction and tumor development(Martinez-Zapien et al., 2016). Studies have shown that in cervical cancer cell lines, such as HeLa and CaSki, the primary degradation of p53 is mediated by E6 rather than MDM2(Hengstermann, et al., 2001). Our results also showed that HeLa cells were insensitive to the MDM2-p53 inhibitor XR-2 as this drug could neither increase p53 protein levels nor induce cell apoptosis. Therefore, the impact of the MDM2-p53 inhibitor on HPV infection-induced tumor cell lines is not obvious. Hence, researchers have explored a series of methods that target E6(Hoppe-Seyler et al., 2018), including siRNA or shRNA targeting E6(Butz et al., 2003), small molecule inhibitors(Malecka et al., 2014), and monovalent or bivalent peptides(Dymalla et al., 2009). However, there are no E6-targeting agents applicable to clinical treatment yet.

NMD is also a promising target for tumor therapy(Lejeune, 2016). Inhibition of NMD can reduce tumor proliferation by increasing R-loop levels, causing genomic instability(Cheruiyot, et al., 2021), activating the p53 pathway through p53β/γ isoforms(Gudikote, et al., 2021), and enhancing the immune response of tumor cells(Pastor et al., 2010). This study also demonstrated that high concentrations of SMG1i could induce apoptosis and activate the expression of p53β/γ in HeLa cells. More importantly, the combination of XR-2 and SMG1i achieved an excellent synergistic effect, which significantly impeded cell cycle progression and induced apoptosis. Although XR-2 and SMG1i alone treatment slightly increased the proportion of cells in G1 phase, the differences were not statistically significant. However, most of the cells were arrested in G1 under combination treatment, make the cells were unable to enter S-phase for DNA replication, thus inhibiting cell proliferation.

Interestingly, we did not detect a significant increase in the protein level of p53α under the combined treatment, but increased p53 and p53 target genes expression levels were detected at mRNA levels. Moreover, pathway enrichment analysis of the upregulated DEGs of the combination treatment by transcriptome sequencing demonstrated that p53 pathway was significantly enriched, which suggested the activation of the p53 pathway. Considering that the expression level of p53 in HeLa is very low and out of regulation of MDM2, we hypothesized that the combination of the two agents may affect the interaction between E6 or E6* and p53 because the overexpression of E6* could rescue the apoptosis resulted from the combination treatment. It has been reported that an engineered virus containing only E6* cannot integrate into host cell genomes(Pastor, et al., 2010), but the E6* can regulate E6 function(Filippova et al., 2007, Pim and Banks, 1999, Rosenberger et al., 2010). However, there is no studies have investigated the effects of MDM2-p53 inhibitors and NMD inhibition on E6 and E6*. As an

inhibitor, XR-2 blocks the interaction between MDM2 and p53, but did not disrupt the interaction of p53 with E6 or E6*. SMG1i also did not affect the mRNA level of E6 or E6*, even the E6* carries a PTC in its sequence. Therefore, we believe that the synergistic effects of the combination of the two agents is more likely to affect the expression of one or more intermediate genes, thus affecting the function of E6. However, further studies are required to elucidate the specific molecular mechanisms involved.

## Conclusion

In conclusion, our study demonstrated that treatment with either XR-2 or SMG1i alone had a weak inhibitory effect on HPV18-positive HeLa cells. However, the combination of XR-2 and SMG1i had synergistic effects, which significantly arrested the cell cycle, induced cell stress, and increased cell apoptosis. This approach could be a potential candidate for the clinical treatment of HPV-infected tumors.

## Declarations

# Funding

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# Competing interest

All the authors declare no conflict of interest.

# Author contributions

F.X. conceived the study and designed the experiments. Y.L., LL.Z., HX.L., JY.C., L.W., LX.Z., GY.S., W.H., and ZH.Z. performed the experiments. Y.L., LH.Z., and F.S. analyzed the data. Y.L., F.X., and M.T. wrote the manuscript. All authors read and approved the final manuscript.

# Data availability statement

The sequencing data are available from the corresponding author on reasonable request.

# Ethics approval

The present study did not require approval from an ethics committee because this study did not involve any human participants or patient material.

# Consent to participate

The present study did not require consent to participate because this study did not involve any human participants.

# Consent to publish

The present study did not require consent to publish because this study did not involve any human participants.

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# Figures

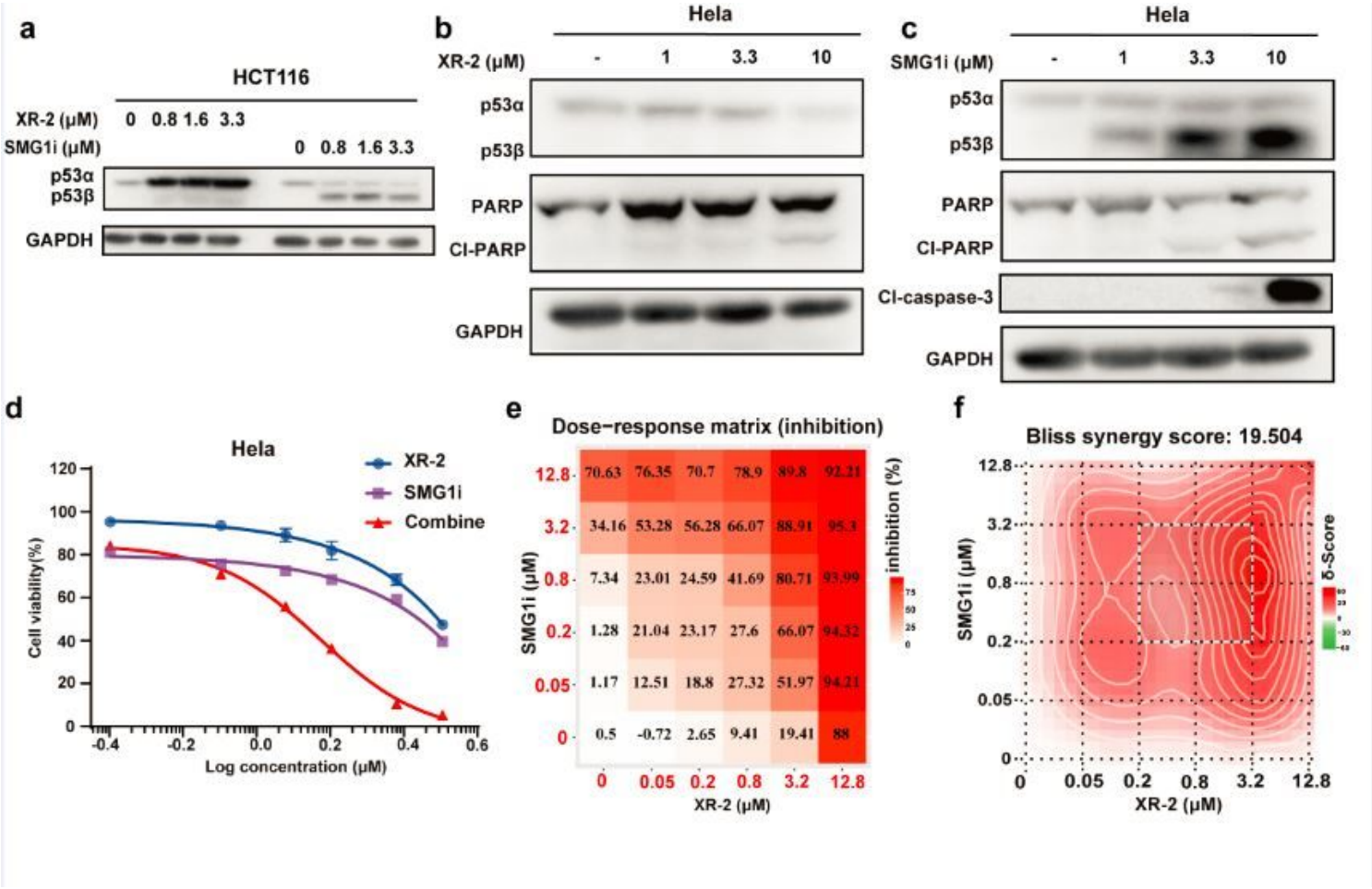
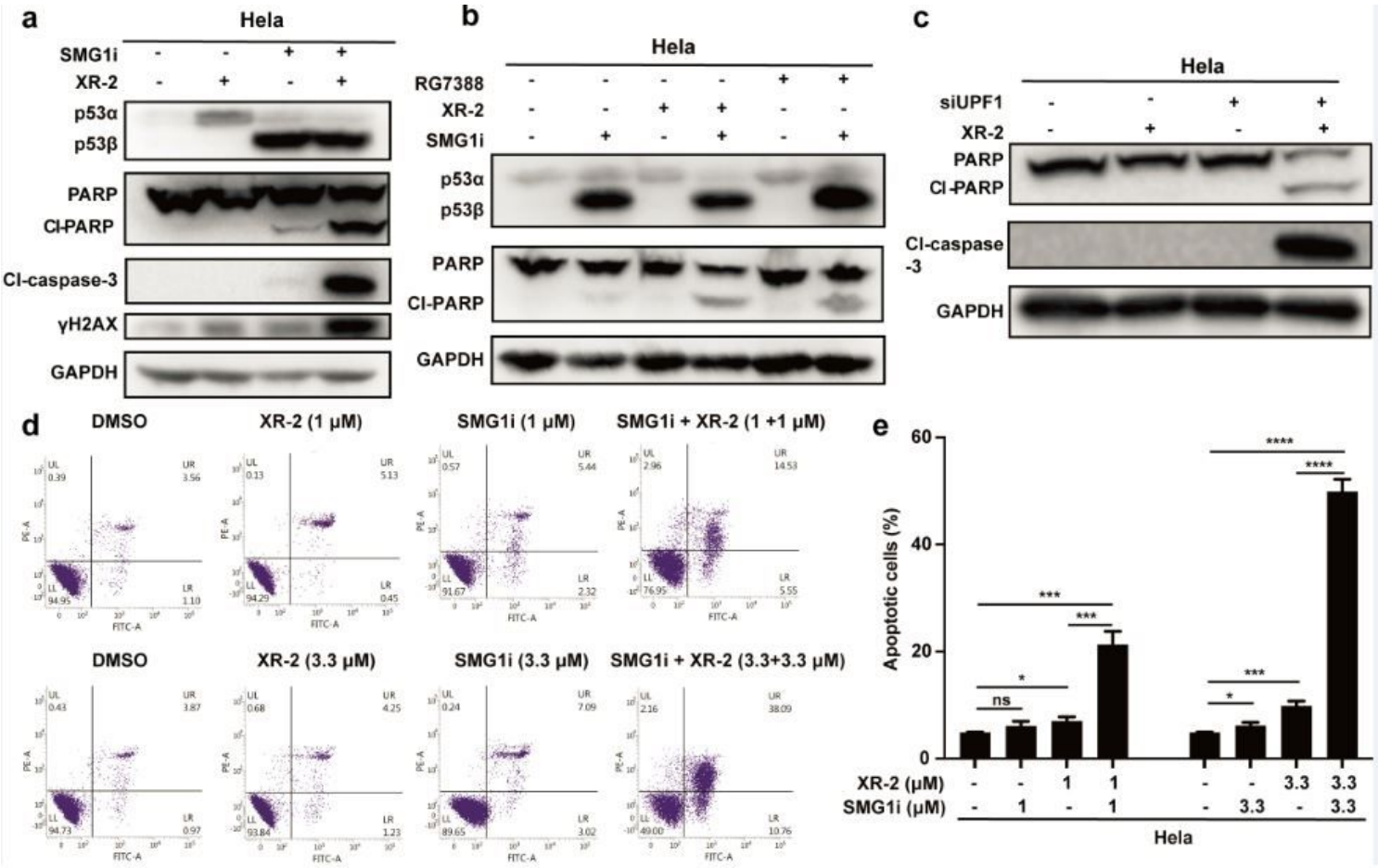



**Figure 1**

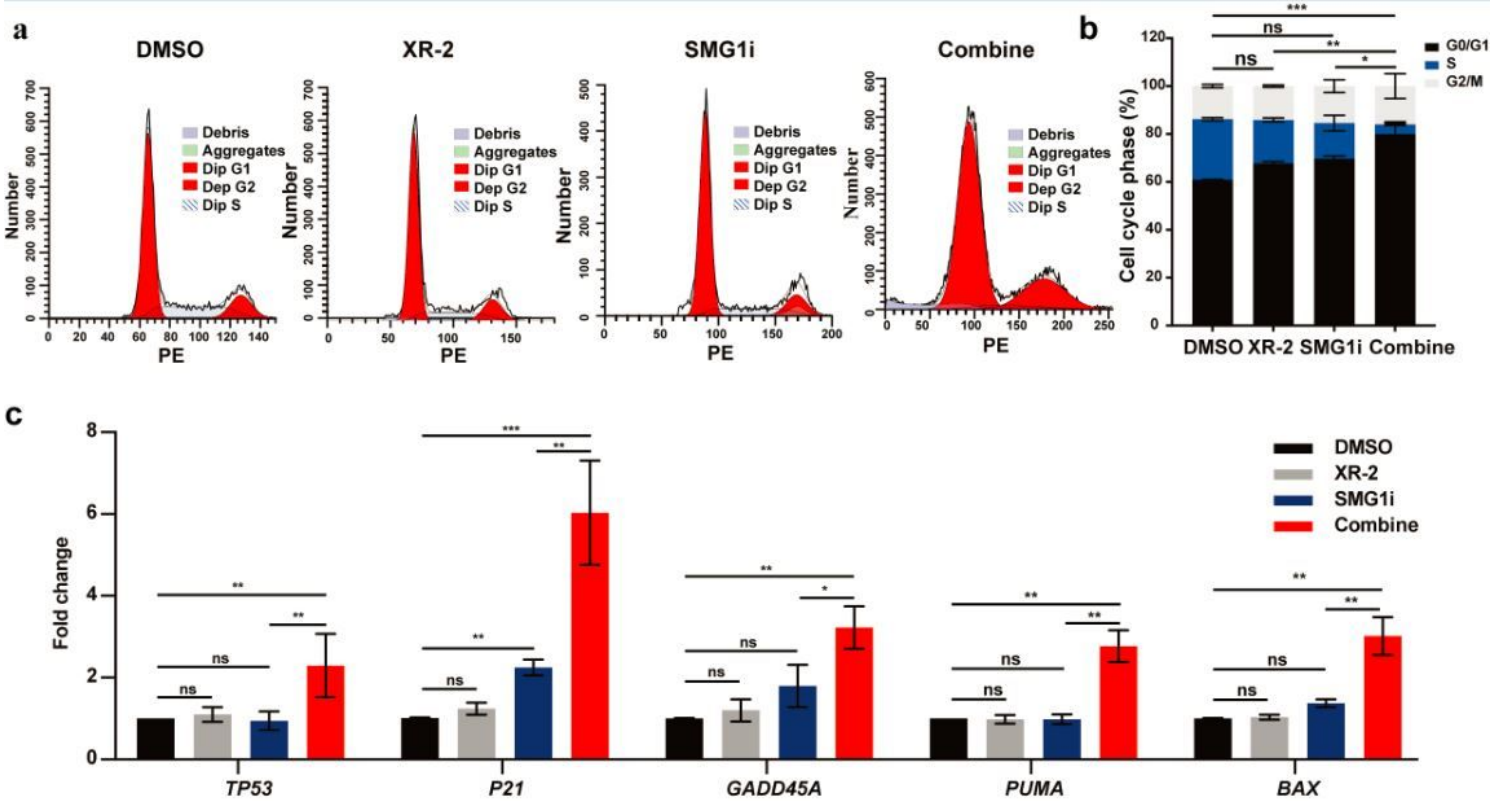
Effect of SMG1i and XR-2 treatment alone or in combination in HeLa cells. **(a)** Western blotting results of p53 protein in HCT116 cells treated with different concentrations of XR-2 and SMG1i. **(b,c)** Western blotting analysis of p53 and apoptosis markers in HeLa cells treated with indicated concentrations of XR-2 and SMG1i. **(d,e)** Dose-response curve and matrix of SMG1i and XR-2 treatment alone or in

combination in HeLa cells. Cl-PARP: cleaved PARP. Cl-caspase-3: cleaved caspase-3. **(f)** Synergy score of XR-2 combined with SMG1i using indicated concentrations via Bliss model




## Figure 2

The combination of XR-2 and SMG1i in HeLa cells induces apoptosis. Cells were treated with 3.3 μM of SMG1i, XR-2, and RG7388 alone or in combination for 24 hours. **(a)** Western blotting analysis of p53, apoptosis marker, and γH2AX in HeLa cells treated with SMG1i and XR-2 alone or in combination. **(b)** RG7388 has the same synergistic effect as XR-2 when combined with SMG1i by western blotting. **(c)** Knockdown UPF1 synergized with XR-2 to increase apoptosis biomarker in HeLa cell via western blotting analysis. **(d,e)** HeLa cells were treated with the indicated concentration of XR-2 or SMG1i alone or in combination for 24 hours, and the cell apoptosis was measured with Annexin V-FITC/PI staining and flow cytometry. Data are presented the mean ± standard deviation of three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; ns, not significant. An ungrouped t-test was used to calculate the p-values




## Figure 3

Effects of XR-2 combined with SMG1i on cell cycle and p53 target genes in HeLa cell. Cells were treated with 3.3 μM of SMG1i and XR-2 alone or in combination for 24 hours. **(a)** The pattern of the cell cycle was analyzed by PI staining and flow cytometry on HeLa cells treated with XR-2 and SMG1i alone or in combination. **(b)** Analysis of the distribution of cell cycle stage on HeLa cells under the indicated treatment. The cell stage distribution of the DMSO treated group was used as control, p-value was calculated by chi-square test. **(c)** mRNA level expression analysis of p53 and p53 targets via quantitative real-time PCR. Data are presented the mean ± standard deviation of three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; ns, not significant. An ungrouped t-test was used to calculate the p-values

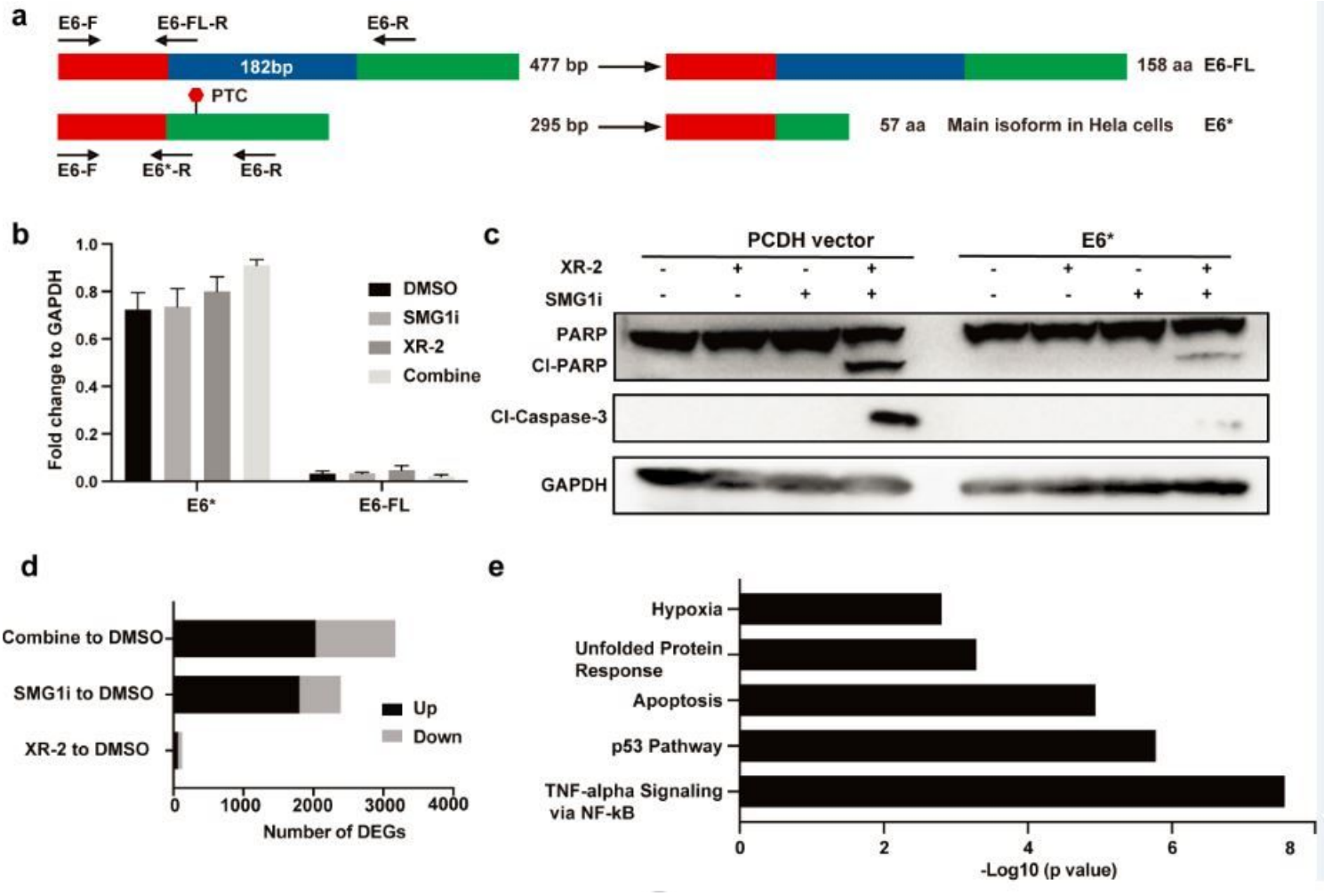



**Figure 4**

Rescue experiment by E6* overexpression and RNA-seq reveal the mechanisms under synergistic effects between XR-2 and SMG1i. Cells were treated with 3.3 μM of SMG1i and XR-2 alone or in combination for 24 hours. **(a)** Illustration of two HPV18 E6 isoforms in HeLa cells. E6, full-length E6; E6*, short length E6 isoform arise from alternative splicing. **(b)** The relative expression level of E6 and E6* adjusted to GAPDH in HeLa cells treated with XR-2 and SMG1i alone or in combination via quantitative real-time PCR. **(c)** Western blotting results demonstrated that overexpression of E6* could rescue the apoptosis resulting from XR-2 and SMG1i combination treatment. **(d)** The number of differentially expressed genes in SMG1i treated group, XR-2 treated group, and combined treated group compared to DMSO treated group. **(e)** The top five significantly enriched pathways gathered from the top 200 up-regulated differentially expressed genes of combined treated group

# Supplementary Files

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- SupplementaryFig1.tif

- SupplementaryFig2.tif
- Supplementaryfig3.tif
- Supplementarytable1.docx