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TLR3 induction by anticancer drugs potentiates poly I:C-induced tumor cell apoptosis

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Toll-like receptor 3 (TLR3) has gained recognition as a novel molecular target for cancer therapy because TLR3 activation by its synthetic ligand poly I:C directly causes tumor cell death. Recently, we reported that tumor suppressor p53 increases the expression of TLR3 in several tumor cell lines. Another study also showed that interferon-α (IFN-α) up-regulates TLR3 expression. We thus hypothesized that various anticancer drugs such as p53-activating reagents and IFNs may potentiate poly I:C-induced tumor cell death through the up-regulation of TLR3 expression. Here, we screened several anticancer drugs that, together with poly I:C, effectively cause tumor cell death in colon carcinoma HCT116 cells. We found that the DNA-damaging reagent 5-fluorouracil (5-FU) increased TLR3 mRNA expression and potentiated poly I:C-induced apoptosis in HCT116 $p53^{+/+}$ cells but had only minimal effect in $p53^{-/-}$ cells, indicating a p53-dependent pathway. On the other hand, IFN-α increased poly I:C-induced apoptosis and the TLR3 mRNA level in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell lines. Furthermore, the combination of poly I:C, 5-FU and IFN-α induced the highest apoptosis in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. Taken together, these data suggest that the anticancer drugs increased TLR3 expression and subsequently potentiated poly I:C-induced apoptosis likely via p53-dependent and -independent pathways. Considering that the p53 status in malignant cells is heterogeneous, this combination approach may provide a highly effective tumor therapy. (*Cancer Sci* 2010; 101: 1610–1617)

In recent years, diverse combinations of drugs targeting multiple pathways have been used for cancer treatment. One of the agents for tumor chemotherapy that has been assessed with favorable outcome in clinical trials is the synthetic double-stranded RNA (dsRNA; e.g. polyinosine-cytosine, poly I:C; polyadenylic-polyuridylic, poly A:U).[1,2] The ability of dsRNA to directly stimulate toll-like receptor 3 (TLR3) and produce type I interferons (IFNs) was primarily the rationale for its clinical use in cancer patients.[3] More recently, several studies have demonstrated that the activation of TLR3 by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells.[4–6] In view of these promising effects, the use of dsRNA-derived compounds in combination with anticancer agents for chemo-immunotherapy warrants robust investigation.

Chemotherapeutic drugs that induce DNA damage have been used to treat cancer patients. The effects of these agents are sensed by the DNA-damage sensors, which activate p53 and induce cell cycle arrest and/or apoptosis.[7,8] We recently reported that p53 up-regulates the transcription of TLR3 through the direct binding of p53 at the promoter region of TLR3.[9] But whether the up-regulation of TLR3 expression by p53 leads to enhanced tumor cell apoptosis was not investigated.[9]

The interferons, especially type I IFNs (such as IFN-α, and -β), are also some of the most investigated cancer therapeutic agents.[10,11] Interferon-α (IFN-α) is used for the treatment of renal cell carcinoma, leukemia, and malignant melanoma.[12,13] Interferons (IFNs) are cytokines that exhibit immunomodulatory, antiviral, antiproliferative, and apoptotic effects by activating signal transduction cascade through the classic JAK/signal transducers and activators of transcription (STAT) pathway.[14,15] It has been reported that IFN-α up-regulates the expression of TLR3 in A549 cells and HUVECs.[16] However, whether the IFN-α-induced TLR3 expression can enhance tumor cell death remains unexplored.

Because TLR3 can directly trigger tumor cell apoptosis,[6] we hypothesized that the induction of TLR3 expression either by p53-activating drugs or by IFNs may increase TLR3-mediated tumor cell killing. Thus, we investigated here the effects of DNA-damaging agents and IFNs together with poly I:C, a synthetic TLR3 ligand, on the apoptosis of colorectal cancer cells HCT116 $p53^{+/+}$ and $p53^{-/-}$. The DNA-damaging drug 5-fluorouracil (5-FU) with poly I:C enhanced the TLR3-mediated apoptosis most efficiently in HCT116 $p53^{+/+}$ cells while IFN-α enhanced poly I:C-induced apoptosis in $p53^{+/+}$ and $p53^{-/-}$ cells. Moreover, the combination of poly I:C, 5-FU, and IFN-α induced a statistically significant cell death in comparison with these drugs alone or their dual combination in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, but had low toxicity in primary murine embryonic fibroblasts or normal human embryonic kidney cells. These findings may contribute to the improvement of poly I:C-based tumor therapy.

Materials and Methods

Reagents and antibodies. Poly I:C was from InvivoGen (San Diego, CA, USA). 5-Fluorouracil (5-FU) was from Wako (Osaka, Japan). Cisplatin was provided by Nippon Kayaku (Tokyo, Japan). Etoposide was a kind gift from Dr Akinobu Hamada (Kumamoto University, Japan). Clinical grade IFN-α (Advaferon) was provided by Astellas Pharma (Tokyo, Japan). Interferon-β (IFN-β) was provided by Toray Industries (Chiba, Japan). Interferon-γ (IFN-γ) and caspase inhibitor Q-VD-OPh were from R&D System (Minneapolis, MN, USA). Janus-activated kinase (JAK) inhibitor I (sc-204021) and anti-p53 antibody (DO-1) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-STAT-1 (42H3), anti-phospho-STAT-1 (58D6), anti-phospho-p53 (9284), and anti-cleaved caspase-3 (5A1E) were from Cell Signaling Technology (Danvers, MA, USA). Anti-TLR3 antibody (IMG-315A) was from Imgenex (San Diego, CA, USA). Anti-Hsc70 antibody (SPA-815) was from Stressgen Bioreagents (Ann Arbor, MI, USA). HRP-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

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Cell culture, treatment, and transfection. Human colorectal cancer cell line HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were kindly provided by Dr Bert Vogelstein from Johns Hopkins University (Baltimore, MD, USA). Lung adenocarcinoma (A549 and Calu-3), hepatoma (HepG2), and embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (Manassas, VA, USA). These cell lines were maintained as described previously.[9,17] Mouse embryonic fibroblasts (MEF) were isolated from mouse embryos (12.5–13.5 dpc) and cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin.

For cell viability assays, cells were treated with the indicated concentration of anticancer drugs and/or poly I:C (5 μg/mL) for 24 h. For IFN treatment, cells were pre-treated with 1000 IU/mL IFN-α, -β, or -γ for 24 h. Medium was replaced with fresh medium with or without poly I:C as previously reported.[18] For caspase inhibitor treatment, cells were untreated or co-treated with 10 μM Q-VD-OPh. Cells were re-incubated for another 24 h before harvesting. For JAK inhibitor treatment, confluent cells were treated with 5 μM JAK inhibitor I reagent for 2 h, then stimulated with IFN-α for 3 h, as reported previously,[19] then recovered for Q-PCR analysis. For analysis of cleaved caspase-3 protein expression, cells were treated in the same manner as above except that 5-FU treatment was carried out for 6 h. For PCR analyses and immunoblotting of p53, STAT-1, and their phosphorylated forms, cells were treated with 5-FU, cisplatin, or etoposide for 8 h or treated with IFN-α, -β, -γ for 3 h. For co-treatment with 5-FU and IFN-α, cells were pre-treated with IFN-α for 3 h, then re-incubated in medium with or without 5-FU for 8 h before protein isolation. For small-interfering RNA (siRNA) transfection, 50 nM of si-TLR3 or si-GL2-luciferase (si-GL2; control) was transfected into HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells using Trans-IT TKO (Mirus, Madison, WI, USA) according to the recommended protocol. The target sequences of si-TLR3 and si-GL2 are listed in reference.[9] Twenty-four hours post transfection, cells were treated with 5-FU, IFN-α, and/or poly I:C and assayed for LDH release.

Lactate dehydrogenase (LDH) assay. Cells were assayed for LDH release according to the protocol as described previously.[20] Lactate dehydrogenase (LDH) release was expressed as percentage of LDH in the medium over the total LDH (medium and lysate). Values are means ± SE of triplicate testing for a representative experiment. At least two independent experiments were performed, and results obtained were similar.

Reverse transcription–polymerase chain reaction (RT–PCR) analysis. Polymerase chain reaction (PCR) analyses were performed using total RNAs isolated from cells with TRizol reagent (Invitrogen, Carlsbard, CA, USA) according to the recommended protocol. Real-time quantitative RT-PCR was performed as described previously.[9] The oligonucleotide primers for TLR3 and GAPDH used in this study have been listed previously.[9]

Immunoblotting. For the detection of p53, STAT-1, and their phosphorylated forms, cells were lysed on ice for 30 min with immunoprecipitation lysis buffer[21] containing 1% protease inhibitor (PI) cocktail. Samples were diluted with dilution buffer,[21] mildly sonicated, and passed through a syringe. Samples were centrifuged and lysates were isolated. For analysis of cleaved caspase-3 expression, cells were snap-frozen in liquid nitrogen and then scraped with lysis buffer[22] containing 1% PI. Samples were centrifuged and supernatants were recovered. Protein lysates were subjected to SDS-PAGE and western blotting. Blots were probed with the indicated antibodies, and visualized using SuperSignal (Pierce, Rockford, IL, USA).

Flow cytometry. To determine TLR3 protein, cells were untreated or treated with 400 μM 5-FU and/or IFN-α. Twenty-four hours after treatment, cells were trypsinized and washed twice with cold phosphate-buffered saline (PBS). Fixing and permeabilization of cells were performed using a cell fixation/permeabilization kit (BD Biosciences, San Jose, CA, USA) according to the recommended protocol. Cells were stained with anti-TLR3 monoclonal antibody and Alexa Fluor 488-labeled secondary antibody (Invitrogen). Fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences) using 10 000 gated cells.

Statistical analysis. Significance of the difference between groups was assessed with Student's *t*-test or one-way ANOVA with Tukey–Kramer's multiple comparison test (JMP software; SAS Institute, Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Anticancer drugs with poly I:C increased cell death in HCT116 $p53^{+/+}$ cells. To investigate whether poly I:C-induced tumor cell death is enhanced by co-treatment with anticancer reagents, we screened some anticancer drugs together with poly I:C using HCT116 $p53^{+/+}$ cells. Treatment with either an anticancer drug or poly I:C alone slightly induced cell death as measured by LDH release (Fig. 1a–f). However, the combination of poly I:C and an anticancer drug increased cell death in a statistically significant manner compared with either treatment alone (Fig. 1a–f). While the genotoxic reagents 5-FU, cisplatin, and etoposide activated p53, IFN-α, -β, and -γ activated the STAT-1 molecule (Fig. 1g), indicating that these anticancer reagents utilized different signaling pathways.

Effect of 5-FU or IFN-α with poly I:C in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. Because about 50% of tumors has non-functional p53, we examined the involvement of p53 in the cell death induced by these reagents by comparing the effects of poly I:C + 5-FU or IFN-α on LDH release in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. 5-Fluorouracil (5-FU) was used as a representative genotoxic drug while IFN-α was used to represent the interferons. 5-Fluorouracil (5-FU), which activates p53, slightly increased cell death in $p53^{+/+}$ but not in $p53^{-/-}$ cells. Notably, 5-FU + poly I:C induced cell death in $p53^{+/+}$ cells that was approximately three-fold higher than that in 5-FU + poly I:C-treated $p53^{-/-}$ cells (Fig. 2a). These results suggested that 5-FU enhances poly I:C-induced cell death mostly in a p53-dependent manner. In contrast, IFN-α + poly I:C induced cell death in both $p53^{+/+}$ and $p53^{-/-}$ cells at relatively similar levels (Fig. 2b), indicating that IFN-α potentiates poly I:C tumor cell killing independently of p53. To determine whether these treatments caused apoptotic cell death, we examined the expression of cleaved caspase-3, which is a marker of apoptosis. 5-Fluorouracil (5-FU) + poly I:C generated higher expression of cleaved caspase-3 compared with each treatment alone in $p53^{+/+}$ cells (Fig. 2c, left panel) while cleaved caspase-3 expression was barely observed in $p53^{-/-}$ cells (Fig. 2c, right panel). Interferon-α (IFN-α) + poly I:C up-regulated the expression of cleaved caspase-3 in both $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 2d). These data are consistent with the cell death assay (Fig. 2a,b), and suggest that these treatments induced apoptotic cell death in HCT116 cells.

Effect of 5-FU or IFN-α on TLR3 mRNA expression in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. Toll-like receptor 3 (TLR3) has been shown to induce apoptosis in human cancer cells.[6] Because we have previously reported that TLR3 is transcriptionally up-regulated by p53,[9] we next investigated whether 5-FU increases TLR3 mRNA expression. 5-Fluorouracil (5-FU) dose-dependently up-regulated TLR3 mRNA in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells (Fig. 3a), suggesting that the effect of 5-FU on TLR3 expression is dependent on p53. Interferon-α (IFN-α) at 1000 IU/mL increased TLR3 mRNA level to 2.5-fold in $p53^{+/+}$ cells and to 3-fold in $p53^{-/-}$ cells compared with their respective control (Fig. 3b). These results indicated that the effect of

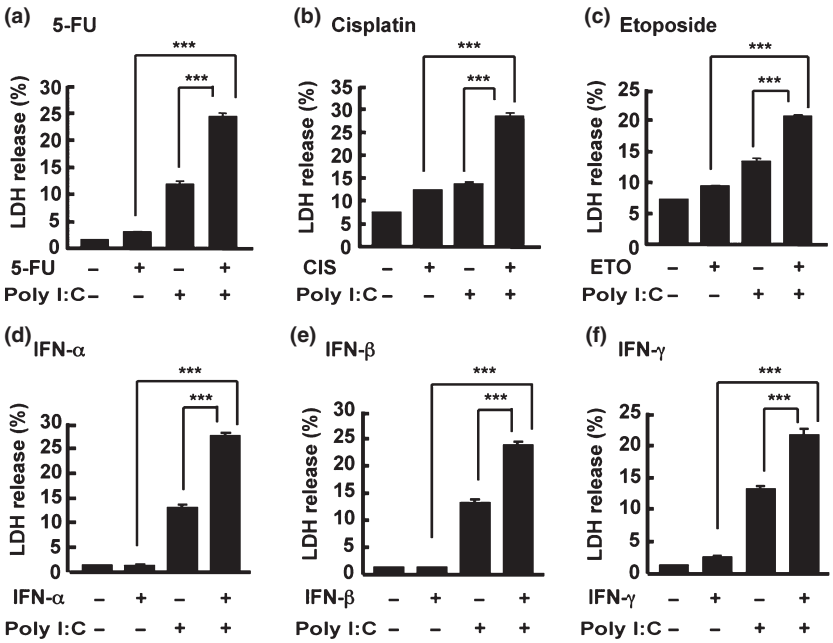

Fig. 1. Anticancer drugs + poly I:C increased cell death in HCT116 $p53^{+/+}$ cells. (a–f) Cells were untreated or treated with various anticancer drugs (5-fluorouracil [5-FU], 400 μM; cisplatin [CIS], etoposide [ETO], 50 μM) or 1000 IU/mL interferons (IFNs) and/or 5 μg/mL poly I:C for 24 h. Cell death was measured by LDH assay, and % LDH release was determined. Values are expressed as mean ± SE from triplicate tests. ***$P < 0.001$, assessed by ANOVA with Tukey–Kramer. (g) Cells were treated with the indicated anticancer drug for 8 h or with IFNs for 3 h using the same concentration as in (a–f). The cell lysates were analyzed for p53, phosphorylated STAT-1 (p-STAT-1), and basal STAT-1 expression by western blotting. Hsc70 was used as internal control.

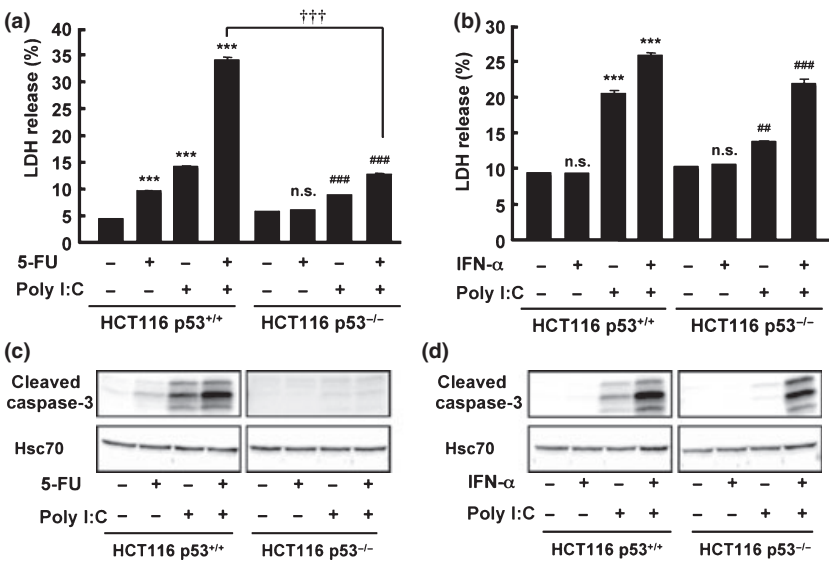

Fig. 2. Effect of 5-fluorouracil (5-FU) or interferon-α (IFN-α) with poly I:C in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. (a,b) Cells were untreated or treated with 5-FU (a) or IFN-α (b) with or without poly I:C. % LDH release was determined. Values are expressed as mean ± SE from triplicate tests. ***$P < 0.001$ *vs* untreated $p53^{+/+}$ cells. ##$P < 0.01$; ###$P < 0.001$ *vs* untreated $p53^{-/-}$ cells, assessed by ANOVA with Tukey–Kramer. n.s., not significant. †††$P < 0.001$, assessed by Student's *t*-test. (c,d) Cells were untreated or treated with 5-FU (c) or IFN-α (d) and/or poly I:C as described in the Materials and Methods. The lysates were analyzed for cleaved caspase-3 expression by western blotting. Hsc70 was used as internal control.

IFN-α on TLR3 transcription is not dependent on p53. Regardless of the robust increase of TLR3 by IFN-α in $p53^{-/-}$ cells, we observed that the level of TLR3 mRNA in IFN-α-treated $p53^{-/-}$ cells was not similar to that in $p53^{+/+}$ cells (Fig. 3b). This is due to the inherently low basal TLR3 expression in $p53^{-/-}$ cells.[9] Having found that the effect of IFN-α on TLR3 transcription was independent of p53 (Fig. 3b) and that STAT-1 was activated by treatment with IFNs (Fig. 1g), we hypothesize that TLR3 up-regulation by IFN-α is mediated by the JAK/STAT pathway. To validate this hypothesis, we stimulated HCT116 $p53^{-/-}$ cells with IFN-α untreated or pre-treated with JAK inhibitor. Blocking the JAK/STAT pathway consequently inhibited the IFN-α-induced increase of TLR3 (Fig. 3c). These results substantiate our findings that IFN-α affects TLR3 transcription, in part, through JAK/STAT signaling and independent of p53. We also observed that the treatment of HCT116 $p53^{+/+}$ cells with other anticancer drugs cisplatin, etoposide, IFN-β, or IFN-γ up-regulated the mRNA expression of TLR3 (Fig. S1).

To examine the importance of TLR3 in mediating cell apoptosis, we knocked down TLR3 using si-TLR3 oligonucleotide (Fig. S2). Depletion of TLR3 reduced the cell killing activity of

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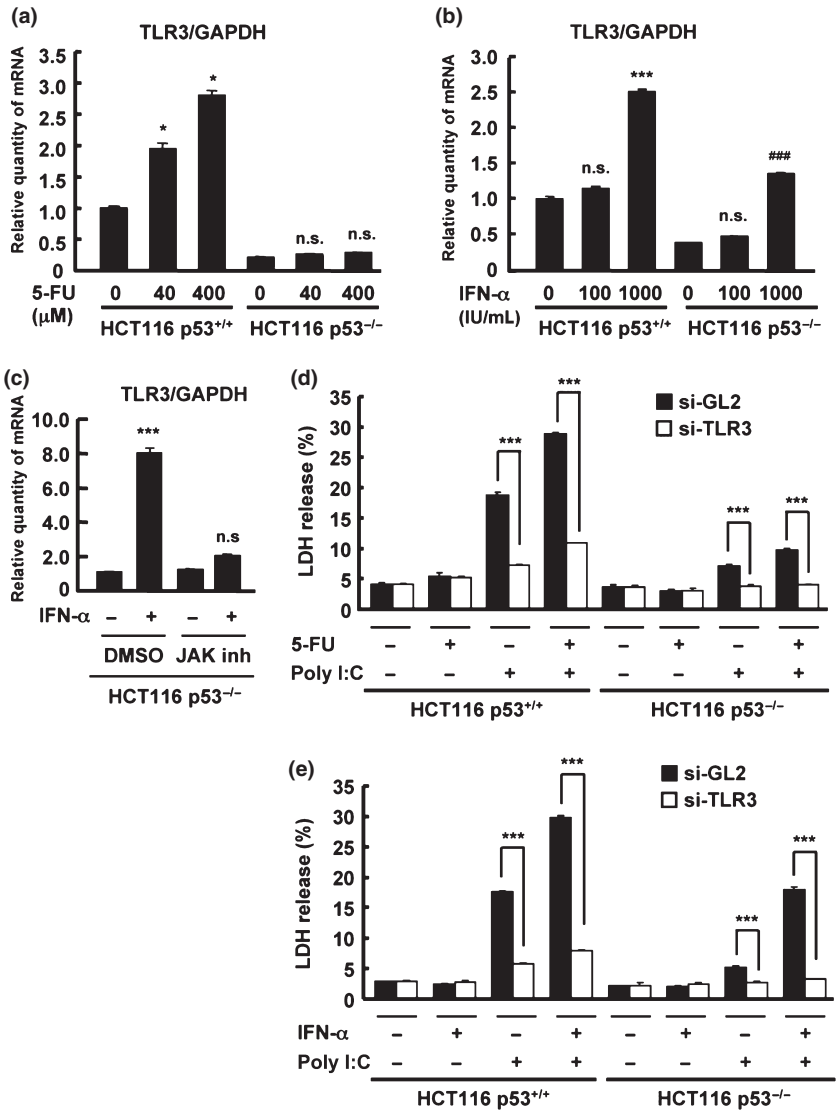

Fig. 3. Effect of 5-fluorouracil (5-FU) or interferon-α (IFN-α) on toll-like receptor 3 (TLR3) mRNA in HCT116 $p53^{+/+}$ and $p53^{-/-}$-cells. (a,b) Cells were treated with the indicated dose of 5-FU for 8 h (a) or IFN-α for 3 h (b). (c) HCT116 $p53^{-/-}$ cells were treated with 5 μM JAK inhibitor for 2 h and then stimulated with IFN-α for 3 h. For (a–c), toll-like receptor 3 (TLR3) mRNA was determined by real-time PCR. Toll-like receptor 3 (TLR3) mRNA level was normalized to GAPDH (internal control). Values are mean ± SD of triplicate measurements. $*P < 0.05$, $***P < 0.001$ *vs* untreated $p53^{+/+}$ cells; $\#\#\#P < 0.001$ *vs* untreated $p53^{-/-}$ cells, assessed by ANOVA with Tukey–Kramer. (d,e) Cells were transfected with si-TLR3 or si-GL2 (control). Twenty-four hours after transfection, cells were untreated or treated with 5-FU (d) or IFN-α (e) and/or poly I:C. % LDH release was determined. Values are expressed as mean ± SE from triplicate tests. $***P < 0.001$ *vs* the indicated si-GL2-transfected group, assessed by Student's *t*-test.

5-FU + poly I:C in HCT116 $p53^{+/+}$ cells (Fig. 3d; white bars). si-TLR3 also inhibited the low level of apoptosis induced by poly I:C in $p53^{-/-}$ cells (Fig. 3d; white bars). si-TLR3 suppressed the apoptosis induced by IFN-α + poly I:C in $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 3e; white bars). These data demonstrated that TLR3 is necessary for the apoptotic effects of these treatments.

Poly I:C + 5-FU + IFN-α induced the highest apoptosis in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. We next evaluated the effects of poly I:C + 5-FU + IFN-α on the apoptosis of HCT116 cells. Consistent with the results in Figure 1(a), 5-FU + poly I:C notably increased the LDH release in $p53^{+/+}$ cells, but not in $p53^{-/-}$ cells (Fig. 4a). Interferon-α (IFN-α) + poly I:C induced relatively similar levels of LDH release in both $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 4a). Interestingly, 5-FU + IFN-α + poly I:C significantly increased the cell death compared with dual treatments in both $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 4a). The LDH release induced by these drugs in both cell lines was abrogated by the addition of caspase inhibitor Q-VD-OPh (Fig. 4a). Furthermore, the expression level of cleaved caspase-3 was up-regulated in the presence of poly I:C + 5-fluorouracil (5-FU) + IFN-α in $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 4b). Taken together, our observations suggest that these treatments resulted in apoptotic cell death mediated most likely by caspase-3.

5-Fluorouracil (5-FU) + IFN-α enhanced the up-regulation of TLR3 mRNA in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. To determine the effects of 5-FU and IFN-α on the expression of TLR3, we performed quantitative RT-PCR in HCT116 cells untreated or treated with 5-FU and/or IFN-α. 5-Fluorouracil (5-FU) + IFN-α induced further increase of TLR3 expression over those of single treatments in $p53^{+/+}$ cells (Fig. 5a). Consistent with the data in Figure 3(a), 5-FU did not contribute to the up-regulation of TLR3 in $p53^{-/-}$ cells. As noted above, the basal level of TLR3 is much lower in $p53^{-/-}$ cells than in $p53^{+/+}$ cells (Fig. 5a, untreated $p53^{-/-}$), which corroborated our recent study showing that p53 is a transcriptional activator of TLR3.[9] Notwithstanding the low basal level of TLR3 in $p53^{-/-}$ cells, treatment with IFN-α (with or without 5-FU) notably increased the expression of TLR3 in these cells compared with control (Fig. 5a), confirming the above results that TLR3 expression is regulated by IFN-α through a p53-independent manner. Consistent with the increase in the amount of TLR3 mRNA, the protein level was also up-regulated by these treatments as determined by flow cytometry analysis of intracellular TLR3 (Fig. 5b). Furthermore, we confirmed that 5-FU triggered the phosphorylation of p53 in $p53^{+/+}$ cells while IFN-α induced the phosphorylation of STAT-1 in $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 5c). 5-Fluorouracil (5-FU) did not affect STAT-1 activation, and IFN-α did not affect p53

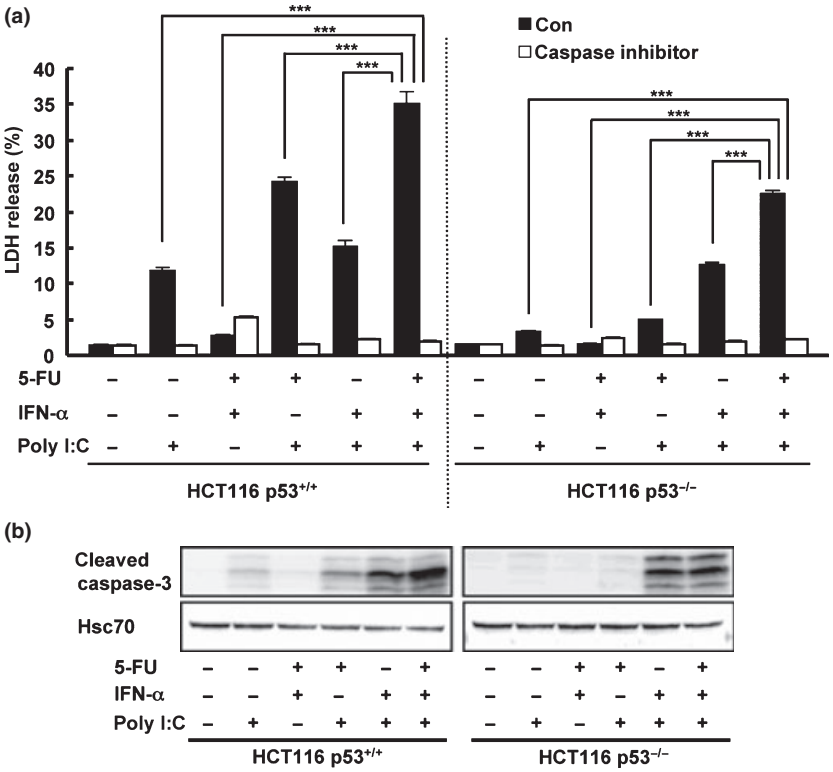

Fig. 4. 5-Fluorouracil (5-FU) + interferon-α (IFN-α) + poly I:C produced the highest increase of apoptosis in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. (a) Cells were untreated or treated with the indicated combination of 5-FU, IFN-α, and poly I:C in the absence or presence of 10 μM caspase inhibitor (Q-VD-OPh). % LDH release was determined. Values are expressed as mean ± SE from triplicate tests. ***$P < 0.001$ *vs* the indicated group, assessed by ANOVA with Tukey–Kramer. (b) Cells were treated with the indicated combination of reagents. Cell lysates were analyzed for cleaved caspase-3 expression by western blotting. Hsc70 served as internal control.

activation. Collectively, these data implied that 5-FU and IFN-α affected TLR3 expression through upstream pathways that are independent of each other.

Poly I:C + 5-FU + IFN-α induced cell death in other tumor cell lines. To examine the effect of these treatments on cell death in other cancer cell lines, we analyzed the LDH release in A549, HepG2, and Calu-3 cells treated with the indicated reagents (Fig. 6a–c). In A549 cells, 5-FU + IFN-α + poly I:C induced the highest LDH release compared with single and dual treatments (Fig. 6a). In the HepG2 cell line, 5-FU + poly I:C produced a comparable effect on LDH release with that of triple combination treatment (Fig. 6b). In Calu-3 cells, IFN-α either in combination with poly I:C or 5-FU had a pronounced effect on LDH release (Fig. 6c). Although the different cancer cell lines exhibited varying levels of response to single and dual treatments, all cancer cell lines treated with 5-FU + IFN-α + poly I:C tended to have the relatively highest LDH release (Fig. 6a–c). Next, we asked whether these treatments are toxic to normal cells. Treatment with 5-FU, IFN-α, or poly I:C alone did not increase the LDH release in HEK293 and MEF (Fig. 6d,e). The dual and triple combinations of 5-FU, IFN-α, and poly I:C slightly induced cell death in these cells but with the highest LDH release of only <2% in HEK293 cells and <7% in MEF, suggesting a relatively specific toxicity for cancer cells.

Discussion

In the present study, we screened for antitumor drugs that could potentiate the cell-killing activity of poly I:C. We showed here that 5-FU, cisplatin, etoposide, and the interferons increased poly I:C-induced cell death in HCT116 cells (Fig. 1a–f). To decipher the molecular mechanisms involved in the effects of these reagents, we focused on TLR3, which is the receptor of poly I:C and was reported to possess apoptotic function in cancer cells.[5,6]

Our previous investigation revealed that p53 transcriptionally activates TLR3,[9] which we confirmed here by showing that 5-FU increased TLR3 mRNA in HCT116 $p53^{+/+}$ cells but not in $p53^{-/-}$ cells (Fig. 3a). Moreover, treatment of HCT116 $p53^{+/+}$ cells with nutlin-3, a specific activator of p53,[23] enhanced the TLR3 mRNA level and increased poly I:C-induced LDH release (Fig. S3a,b). Thus, we propose that activation of p53 increases the expression of TLR3, which is then activated by its ligand poly I:C resulting in enhanced apoptosis in $p53^{+/+}$ cells.

Interferon-α (IFN-α) by itself did not induce significant cell apoptosis but it additively increased the apoptotic activity of poly I:C and also increased TLR3 mRNA level not only in HCT116 $p53^{+/+}$ but also in $p53^{-/-}$ cells (Figs 2b,3b). Importantly, we present evidence that the effect of IFN-α on TLR3 transcription relies not so much on p53 but on the JAK/STAT pathway (Fig. 3c). It was previously noted that the TLR3 promoter contains an ISRE (interferon-stimulated response element) and STAT binding site.[24] These consensus sites might regulate the response of TLR3 to IFN-α. Our data showing that inhibiting the JAK/STAT pathway diminished the induction of TLR3 by IFN-α firstly highlights the importance of JAK/STAT signaling on IFN-α-induced TLR3 transcription. The induction of TLR3 expression through p53-dependent and -independent mechanisms has important implication with regards to the therapeutic potential of poly I:C together with 5-FU and IFN-α. In some cases, poly I:C with 5-FU or with IFN-α may be sufficient to induce apoptosis: HepG2, which expresses wild-type p53, was responsive to poly I:C + 5-FU (Fig. 6b), whereas Calu-3, which harbors mutant p53, was sensitive to poly I:C + IFN-α (Fig. 6c). However, A549, which possesses wild-type p53, was most responsive to poly I:C + 5-FU + IFN-α (Fig. 6a). Thus, triple combination treatment may be more advantageous for certain types of tumor, and is relevant considering the heterogeneity of tumor tissues in terms of p53 functionality.

It is notable that the knockdown of TLR3 suppressed the cell killing activities of the drugs (Fig. 3c,d). How TLR3 mediates cell death in cancer cells is yet unclear, but previous studies[6,25] and our observations have suggested the involvement of caspase-3 because caspase-3 was up-regulated by these treatments

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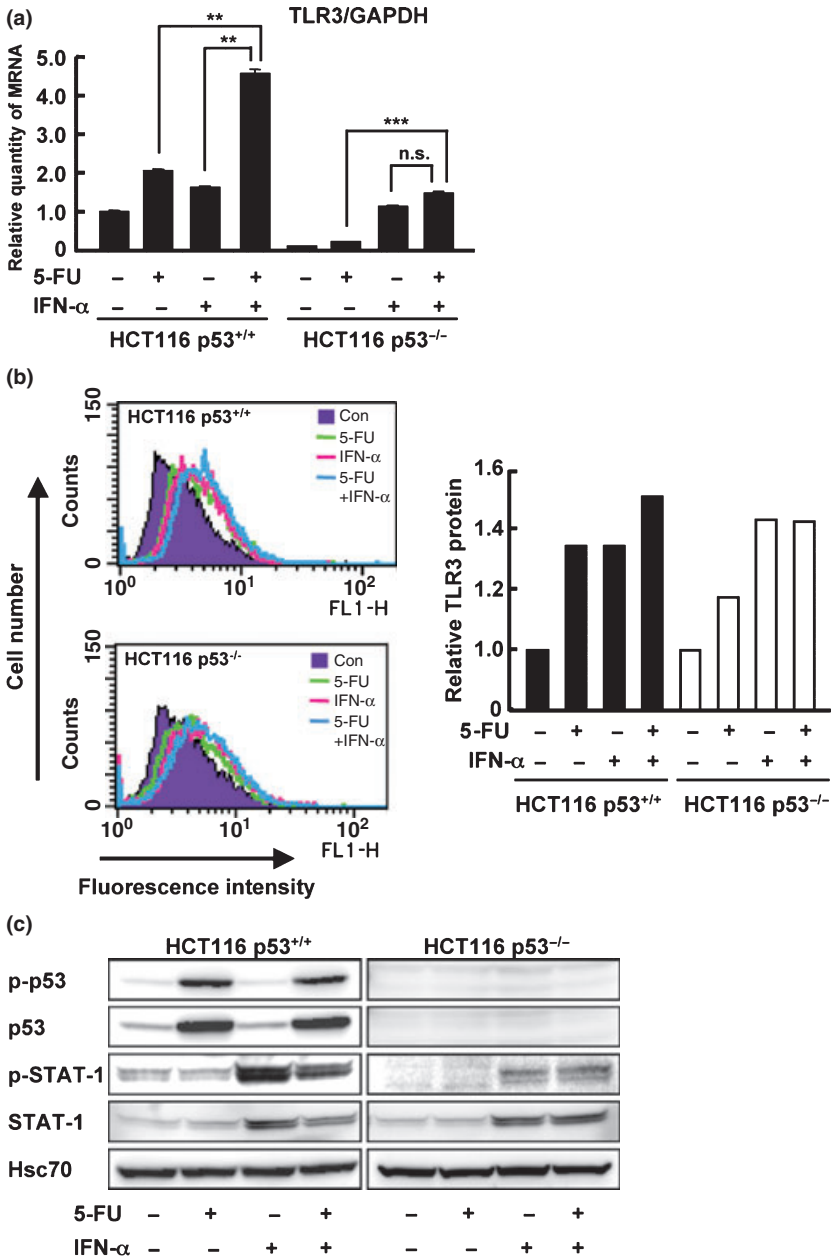

Fig. 5. 5-Fluorouracil (5-FU) + interferon-α (IFN-α) enhanced the up-regulation of toll-like receptor 3 (TLR3) expression in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. (a–c) Cells were treated with the indicated combination of 5-FU and IFN-α. For (a), TLR3 mRNA was determined by real-time PCR. Toll-like receptor 3 (TLR3) mRNA level was normalized to GAPDH (internal control). Data shown are mean ± SD performed in triplicate of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* the indicated group, assessed by ANOVA with Tukey–Kramer. For (b), cells were trypsinized, fixed, permeabilized, and stained with anti-TLR3 and Alexa Fluor 488 antibodies. Fluorescence intensity was measured on a FACSCalibur, and shown as shaded histogram or the indicated colored lines, which represent intracellular TLR3 expression in the respective samples (left panels). Fold induction of TLR3 expression (relative to untreated control) was computed using the geometric mean fluorescence intensity (right panel). For (c), cell lysates were analyzed for the expression of phosphorylated p53 (p-p53), basal p53, phosphorylated STAT-1 (p-STAT-1), and basal STAT-1. Hsc70 was used as internal control.

and addition of caspase inhibitor abrogated the cell death induced by poly I:C and anticancer drugs (Figs 2c,d,4a,b). Whether TLR3 directly activates caspase-3 or needs a molecular mediator to produce cleaved caspase-3 still has to be investigated.

Combination chemotherapy regimens for cancer treatment are frequently favored over single agents in an attempt to produce better tumor response rates. A well-studied combination therapy is that of 5-FU and IFN-α. However, the clinical studies of 5-FU + IFN-α therapy for cancer have shown conflicting outcomes. Wadler *et al.* initially reported a good response rate to IFN-α + 5-FU treatment in colorectal cancer patients.[26,27] Recent investigations by Monden's group and others revealed promising results of 5-FU + IFN-α therapy in hepatocellular carcinoma.[28–31] But phase III trials of this combination in colorectal cancer,[32] and phase II trials in carcinoid and pancreatic cancer,[33,34] revealed no significant benefit from this dual treatment. In our current work, we observed that 5-FU + IFN-α did not significantly induce cell death in HCT116 and A549 cells. But the inclusion of poly I:C in 5-FU + IFN-α combination significantly triggered apoptosis compared with only 5-FU + IFN-α. Although the cytotoxic effects of the triple combination need to be tested on an array of normal cell lines, we found that this triple combination did not induce remarkable apoptosis in HEK293 cells and MEF (Fig. 6d,e). The underlying mechanism of the different response between normal and cancer cells towards these treatments is still unresolved. Khvalevsky *et al.* previously reported that while the up-regulation of TLR3 in HEK293 (normal) cells led to the induction of cytokines, increase of TLR3 in HepG2 (cancer) cells skewed towards the induction of apoptosis.[25] Congruently, we observed that the efficiency of TLR3 induction by anticancer drugs is similar between HEK293 and HepG2 cells (data not shown); thus, even if TLR3 is induced similarly in normal and cancer cells, TLR3 signaling apparently diverges in these cells. The reason for this disparate TLR3 molecular signaling in normal and cancer cells

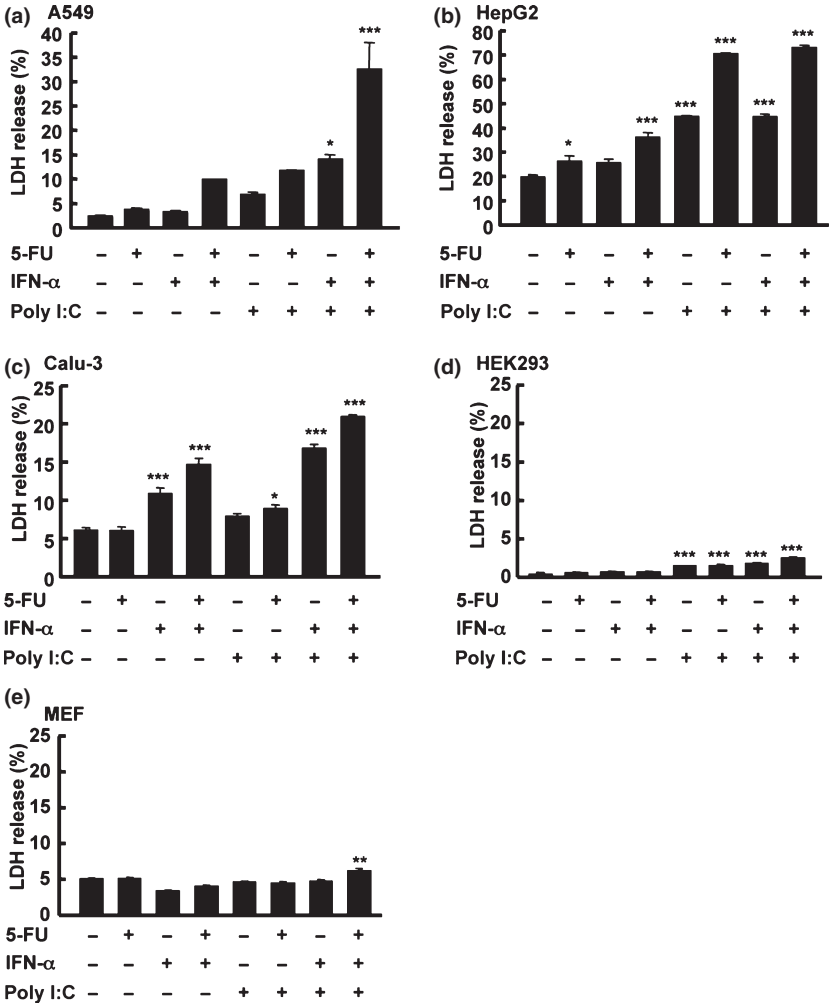

Fig. 6. 5-Fluorouracil (5-FU) + interferon-α (IFN-α) + poly I:C most effectively induced cell death in various cancer cell lines. (a–e) Cancer cell lines harboring wild-type p53 (a,b), mutant p53 (c), and normal cells (d,e) were untreated or treated with the indicated reagents. % LDH release was determined. Values are presented as mean ± SE from triplicate tests. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ *vs* untreated group, determined by ANOVA with Tukey–Kramer. Except for the indicated groups, the difference between other groups and control was not statistically significant.

remains unclear. But this might be one of the factors that makes cancer cells more sensitive to poly I:C treatment. Furthermore, cancer cells exhibit regions with hypoxia and low pH compared with normal cells, and these conditions in tumor cells make them less robust and more sensitive to the cytotoxic effects of anticancer drugs.[35,36] Our present findings showing the ability of anticancer drugs to induce TLR3 expression and augment poly I:C-induced tumor cell death through TLR3 provide an added dimension to therapeutic approaches for cancer treatment.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Toll-like receptor 3 (TLR3) mRNA is up-regulated by anticancer drug treatment.

Fig. S2. Effect of small-interfering toll-like receptor 3 (si-TLR3) oligonucleotide on TLR3 mRNA level.

Fig. S3. Effect of nutlin-3-activated p53 on LDH release and toll-like receptor 3 (TLR3) mRNA expression.

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