Toll-like receptors (TLRs) play a crucial role in the innate immune response and subsequent induction of adaptive immune responses. Recently, it has been noted that TLRs on tumor cells are involved in tumor development, and several TLR agonists, such as the TLR3 agonist poly(I:C) and the TLR9 agonist CpG ODN, are being developed as vaccine adjuvants and cancer immunotherapeutics. In this study, we investigated whether combining poly(I:C) with a TLR9 agonist CpG ODN would result in a stronger anti-tumor effect on hepatocellular carcinoma cells (HCCs). Surprisingly, we found that simultaneous transfection of poly(I:C) and ODN M362 exhibited a lower pro-apoptotic effect on HCCs than transfection with poly(I:C) alone. Simultaneous co-transfection was accompanied by down-regulation of poly(I:C)-related innate receptors, pro-inflammatory cytokines and apoptotic genes induced by poly(I:C), indicating that ODN M362 blocked the activation of poly(I:C)-triggered innate immune responses and cellular apoptosis. Further studies indicated that these effects were partly due to the phosphorothioate-modification of CpG ODN, which blocked the entry of poly(I:C) into tumor cells. This entry blockade was avoided by administering poly(I:C) after CpG ODN. Moreover, poly(I:C)-mediated pro-apoptotic effects were enhanced in vitro and in vivo by pre-treating HCC cells with CpG ODN. Our findings thus suggest that when combining poly(I:C) and CpG ODN for cancer therapy, these agents should be used in an alternating rather than simultaneous manner to avoid the blocking effect of phosphorothioate-modified TLR9 ligands.

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example, TLR4 activation suppresses TLR3-induced anti-tumor immunity by promoting IL-10 [15]. Therefore, it is important to better define the cross-talk between different TLRs so that appropriate combinations of TLR ligands may be designed for use in anti-tumor therapies.

Hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide and is ranked as the third most common cause of cancer mortality in China. HCC has become a serious threat to the health and lives of individuals worldwide, and an urgent need exists to find a more effective strategy for treating this condition. HCC cells express a broad repertoire of TLRs, including TLR3 and TLR9 [16,17]. Studies have reported that polyinosinic-polycytidylic acid (poly(I:C))-containing liposomes promote HCC cell apoptosis and result in cell death [18]; furthermore, activating TLR9 in HCC cells also inhibits cell growth and induces apoptosis [17]. However, the anti-tumor effect of poly(I:C) in combination with a TLR9 agonist has been less well studied. The aim of this work was to investigate the anti-HCC effects of poly(I:C) in combination with a TLR9 agonist, CpG oligodeoxynucleotide (ODN).

Materials and methods

**Cell lines and culture conditions**

The human hepatoma cell lines HepG2, H7402 and PLC/PRF/5 were cultured and maintained in our laboratory. All cell lines were grown in RPMI Medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37 °C in 5% CO2.

**Transfection of CpG oligodeoxynucleotide (ODN) and poly(I:C)**

Transient transfection was carried out with LipofectamineTM 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. One day before transfection, 1.5 × 106 cells were seeded. After being washed with phosphate-buffered saline (PBS), these cells were transfected with Lipofectamine 2000/CpG ODN, Lipofectamine 2000/poly(I:C) or Lipofectamine 2000/poly(I:C) + CpG ODN. ODN M362 control (ODN M362 Ctrl), ODN M362, ODN 2006-G5 and poly(I:C)-fluorescein were purchased from InvivoGen (San Diego, CA, USA). Poly(I:C) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Proliferation analysis**

Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at 0.3 × 104 cells/well in 96-well plates. After 48 h of culture with or without TLR ligands, cells were incubated with 20 μL MTT (10 mg/mL; Sigma) for another 4 h. After centrifugation at 2500 rpm for 20 min, 100 μL supernatant was discarded, and 100 μL 20% sodium dodecyl sulfate (SDS) was added; the plates were then incubated for at least 12 h at 37 °C. After incubation, absorbance was read by a scanning multwell spectrophotometer (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

**Detection of apoptosis**

Cells in the early and late stages of apoptosis were detected with an annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (BestBio, Shanghai, China). In brief, cells were transfected with poly(I:C) (1 μg/mL), CpG ODN (0.5 μg/mL) or a combination of both, and incubated for 6–24 h before analysis. Media and cells were collected and washed with PBS. Cells that were positive for annexin V–FITC (early apoptosis) or for both annexin V–FITC and PI (late apoptosis) were counted, and the apoptotic index was calculated. A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was also carried out to detect cell apoptosis using the One-Step TUNEL Apoptosis Assay Kit (Beijing, Jiangsu, China). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Staining was evaluated using fluorescence microscopy.

**Quantitative real-time PCR analysis**

Total RNA was extracted with Trizol reagent (Invitrogen) and used to create cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV; Invitrogen) according to the manufacturer’s protocol. Expression of cytokines, receptors and apoptosis-related genes was detected by the real-time polymerase chain reaction (qRT-PCR) method, which was performed using the SYBR Green Master Mix (Toyobo, Osaka, Japan) on an iCycler iQ real-time PCR system (Bio-Rad). Primers are listed in Table 1. The GAPDH gene was used for RNA normalization.

**Western blot analysis**

Cells were solubilized in lysis buffer (BestBio) that included a cocktail of protease inhibitors (BestBio). The whole cell extracts were run on 10% SDS–polyacrylamide gels electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes and immunoblotted with the indicated antibodies, as previously described [19]. The bands were examined using densitometry with AlphaEaseFC software (Alpha Innotech Corp., San Leandro, CA, USA). Antibodies against retinoic acid-inducible gene-I (RIG-I), NF-κB and IκBα were obtained from Cell Signaling Technology (New England BioLabs, San Leandro, CA, USA). Antibodies against β-actin and p–NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Fluorescence microscopy**

HepG2 and H7402 cells were transfected with fluorescently labeled poly(I:C) in the absence or presence of ODN M362, ODN M362 Ctrl or ODN 2006-G5 (0.5 μg/mL), either simultaneously or in turn. Cells were then examined by fluorescence microscopy to evaluate the efficiency of poly(I:C) entry into cells.

**In vivo studies**

Female athymic (nude-nude) mice (5 weeks old) were purchased from HFK BioScience Co., Ltd. (Beijing, China) and maintained at an animal facility under specific pathogen-free conditions. Mice were subcutaneously (s.c.) inoculated with 1 × 107 H7402 cells. After 2 weeks, these mice received an intra-tumoral injection of ODN M362 (5.0 μg in 100 μL), poly(I:C) (2.5 μg in 100 μL) complexed with liposomes or a combination of both; alternating injections of ODN M362 and poly(I:C) were performed every 4 days. The mice were killed on day 40, and tumor volume was calculated as length × width2/2. All experimental procedures were performed in accordance with the guidelines for ethics and regulations of animal experiments defined by Shandong University, China.

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**Table 1**

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**References**

1. Shandong University, China.
2. Female athymic (nude-nude) mice (5 weeks old) were purchased from HFK BioScience Co., Ltd. (Beijing, China) and maintained at an animal facility under specific pathogen-free conditions. Mice were subcutaneously (s.c.) inoculated with 1 × 107 H7402 cells. After 2 weeks, these mice received an intra-tumoral injection of ODN M362 (5.0 μg in 100 μL), poly(I:C) (2.5 μg in 100 μL) complexed with liposomes or a combination of both; alternating injections of ODN M362 and poly(I:C) were performed every 4 days. The mice were killed on day 40, and tumor volume was calculated as length × width2/2. All experimental procedures were performed in accordance with the guidelines for ethics and regulations of animal experiments defined by Shandong University, China.
Statistical analyses

Statistical analysis was performed using paired Student’s t-test. A value of \( P < 0.05 \) was considered statistically significant.

Results

The TLR9 agonist ODN M362 induces apoptosis of HCC cells, but suppresses poly(I:C)-induced apoptosis when co-transfection occurs simultaneously

Based on a recent report [17], the HCC cell lines HepG2 and H7402 were transfected with the TLR9 agonist ODN M362 (0.25–4 \( \mu \)g/mL) for 24 or 48 h, and cell proliferation was determined by an MTT assay. As shown in Fig. 1A, the inhibition rate was approximately 50% in H7402 cells treated with 4 \( \mu \)g/mL ODN M362 for 48 h. To further analyze the effect of ODN M362 on HCC cell apoptosis, annexin V/PI staining assay was carried out after H7402 cells were treated with ODN M362. Fig. 1B shows that the number of annexin V-positive apoptotic cells increased by 1.4-fold in H7402 treated with 0.5 \( \mu \)g/mL ODN M362 for 24 h. These results indicate that ODN M362 can induce apoptosis and suppress the proliferation of HCC cells.

It has been reported that poly(I:C)-containing liposomes could induce apoptosis of human hepatoma cells [18,20]. We wanted to determine whether combining poly(I:C) with a TLR9 agonist CpG ODN would result in a stronger pro-apoptotic effect on HCC cells than using CpG ODN or poly(I:C) alone. Therefore, we compared the pro-apoptotic effects of ODN M362, poly(I:C) or a combination of both on HCC cells. Fig. 1C illustrates that the apoptotic rates in H7402 cells transfected with either poly(I:C) or ODN M362 alone were 54.20% and 28.39%, respectively. Surprisingly, the apoptotic rate in cells co-transfected with both poly(I:C) and ODN M362 decreased to 29.98%. Using an immunofluorescence assay, we directly observed the same phenomenon (Fig. 1D). Similar results were observed in HepG2 cells (Supplementary Fig. S1). These findings suggest that poly(I:C)-induced apoptosis of HCCs is attenuated when the TLR9 agonist, ODN M362, is simultaneously co-transfected with poly(I:C).

ODN M362 suppresses the expression of poly(I:C)-induced genes

To further identify the mechanism underlying the antagonistic effect of ODN M362, we analyzed the expression of the receptors that recognize poly(I:C), including RIG-I, melanoma differentiation-
associated protein-5 (MDA5), laboratory of genetics and physiology-2 (LGP2) and TLR3. As shown in Fig. 2A, when compared with control cells, the expression levels of RIG-I, MDA5, LGP2 and TLR3 increased by between 15- and 27-fold in poly(I:C)-treated HCC cells, but did not significantly change in ODN M362-treated cells. Furthermore, the expression levels of these receptors were dramatically reduced in cells co-transfected with poly(I:C) and ODN M362 compared to cells transfected with poly(I:C) alone. Activation of the NFκB signaling pathway, which could be activated by both TLRs and RLRs, was also reduced (Fig. 2B).

We then measured the expression levels of apoptosis-related genes in cells treated as above. Poly(I:C) up-regulated the expression of apoptosis-related genes, especially Noxa, which increased by approximately 9-fold; however, this up-regulated expression was reduced to only 3-fold when poly(I:C) was combined with ODN M362 (Fig. 2C). Finally, the expression levels of pro-inflammatory cytokine genes associated with poly(I:C) stimulation, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and type I interferons (IFNs), were also measured by qRT-PCR. As shown in Fig. 2D, when poly(I:C) was combined with ODN M362, the pro-inflammatory cytokine response was significantly attenuated compared with that for poly(I:C) alone. For example, IFN-β transcription was elevated by more than 300-fold when poly(I:C) alone was transfected into HCC cell lines, but this was reduced to a 9-fold increase when ODN M362 was used in combination with poly(I:C). These data shed further light on the inhibitory action of ODN M362 on poly(I:C)-induced effects when co-transfection is carried out simultaneously.

**ODN M362 also antagonizes poly(I:C)-induced apoptosis in a colon cancer cell line**

To understand whether these antagonistic effects of ODN M362 also occurred in other types of tumor cells, we carried out experiments on the colon cancer cell line, HCT116. We found that ODN M362 also suppressed the poly(I:C)-induced apoptosis of HCT116 cells (Fig. 3). These data suggest that CpG ODNs have a protective effect against poly(I:C)-induced apoptosis, both in HCC cells and in cells derived from other tumors, such as colon cancer.

**CpG ODN blockade of poly(I:C)-induced apoptosis depends on phosphorothioate modification**

Some CpG ODN molecules contain a phosphodiester backbone similar to native DNA; however, since phosphodiester bonds are highly sensitive to degradation by endonucleases, most synthetic CpG ODNs are now designed with a nuclease-resistant phosphorothioate backbone to increase their half-life. Both of these forms of CpG ODN exert immunostimulatory activities via the TLR9 signaling pathway. However, it has been previously reported that the effects of TLR9 ligands to protect cancer cells against TRAIL-induced apoptosis depend on phosphorothioate modification [21].
Supplementary was similar to that of liposome-treated control cells, with only a modified ODN M362 or ODN M362 Ctrl, the cellular morphology when poly(I:C) was co-transfected with phosphorothioate-phosphodiester CpG ODN 2006-G5 was similar to that of cells treated with poly(I:C) alone, and that most of the cells were apoptotic. Interestingly, these findings showed that ODN M362 Ctrl displayed a similar effect to that of ODN M362, indicating that the inhibitory effects of CpG ODN on poly(I:C)-induced apoptosis depended on the phosphorothioate modification.

Phosphorothioate-modified CpG ODN suppresses poly(I:C)-induced apoptosis by blocking poly(I:C) entry into tumor cells

Luganini et al. have reported that phosphorothioate-modified ODNs inhibit human cytomegalovirus replication by blocking virus entry into host cells [22]. Therefore, we speculated that phosphorothioate-modified ODN M362 blocked poly(I:C) entry into tumor cells. In order to test this idea and directly observe the entry process, fluorescently labeled poly(I:C) was transfected into HCC cells in the presence or absence of ODN M362 Ctrl, ODN M362 or ODN 2006-G5. As predicted, both the phosphorothioate-modified ODN M362 and ODN M362 Ctrl prevented poly(I:C) entry into cells, whereas phosphodiester ODN 2006-G5 did not (Fig. 5). These findings indicate that phosphorothioate-modified CpG ODN can block the entry of poly(I:C), suppressing poly(I:C)-induced apoptosis in HCC cells.

The pro-apoptotic effect of poly(I:C) may be improved by pre-treating HCCs with CpG ODN

Based on the above observations, we further tested whether the CpG ODN-mediated inhibitory action could be avoided by using CpG ODN and poly(I:C) in a sequential rather than simultaneous manner. Since the pro-apoptotic activity of CpG ODN alone was observed to be lower than that of poly(I:C) alone (Fig. 1 and Supplementary Fig. S1), HCC cells were first pre-transfected with ODN M362 and then transfected with fluorescently labeled poly(I:C). Four hours later, we found that poly(I:C) was able to enter HCC cells efficiently using this strategy (Fig. 6A). Interestingly, we observed that poly(I:C)-induced apoptosis was significantly augmented in HCC cells pre-transfected with ODN M362: the apoptotic rate was approximately 83%, much higher than that in cells treated with ODN M362 alone, poly(I:C) alone or both simultaneously (Fig. 6B). Furthermore, our in vivo study showed that injection with ODN M362 12 h before poly(I:C) treatment exerted the optimal therapeutic effect (Fig. 7A and B): the tumor-growth inhibition rate was 87.94 ± 0.08% compared to 61.59 ± 0.14%, 40.03 ± 0.32% and 49.27 ± 0.24% for poly(I:C) alone, ODN M362 alone or both simultaneously, respectively. These results demonstrate that combining poly(I:C) and the TLR9 agonist CpG ODN may lead to a strong anti-tumor effect on HCCs if these agents are administered in an alternating rather than simultaneous manner.

Discussion

TLRs are expressed not only on immune cells but also on tumor cells, where they play an important role in tumor development. In certain cases, triggering TLRs expressed on tumor cells initiates apoptosis and inhibits proliferation [18,23]. For instance, previous reports have indicated that triggering TLR9 inhibits cell growth and induces apoptosis in neuroblastoma cells [24], and it can also sensitize lung cancer cells to apoptosis, leading to growth arrest [25]. However, in other cases, triggering TLRs can induce chronic inflammation and promote tumor growth [2,5]. It is therefore of great importance to better define the function of each TLR expressed on a wide range of tumor cells.
Fig. 4. The phosphorothioate modification was responsible for CpG ODN-mediated inhibitory effects on poly(I:C)-induced apoptosis. A. Microphotographs representative of morphologic changes induced in H7402 cells by treatment with 1 μg/mL poly(I:C) in the presence of 0.5 μg/mL CpG ODN M362, CpG ODN 2006-G5 or ODN M362 Ctrl for 12 h. B. Apoptotic rates of H7402 cells treated as above were determined by annexin V/PI staining. Data are representative of at least 3 independent experiments. Statistical analysis of apoptosis rates (right panel) shows the fold changes increased over the control cells (Lipo group); *P < 0.05.

Fig. 5. Phosphorothioate-modified CpG ODN inhibited poly(I:C) entry into tumor cells. H7402 cells were transfected with 0.5 μg/mL fluorescently labeled poly(I:C) (poly[I:C]-flu) in the absence or presence of 0.5 μg/mL ODN M362, ODN 2006-G5 or ODN M362 Ctrl. The resulting green fluorescence intensity in transfected cells was examined by microscopy (left) and FACS (right). The experiment was repeated 3 times. Representative images are presented.
Many reports have described that HCC tumor cells from patients express TLR3 and TLR9, which are both associated with the prognosis of HCC [26]; moreover, HCC-derived cell lines are positive for both TLR3 and TLR9 [16,17,27]. Both cell-surface and cytoplasmic TLR3 expressions have been observed in HCC cells [20]; TLR9 is mainly expressed on endosomes, but cell-surface expression of TLR9 has also been observed on HCCs [28]. As previously reported, TLR3 expressed in HCC cells plays an important role in terms of cell survival and pro-apoptotic activity. In other studies, stimulation of cell-surface TLR3 with poly(I:C) did not affect cell viability, whereas cytoplasmic stimulation with transfected poly(I:C) did induce apoptosis, which correlated with up-regulation of RIG-I-like receptors [18,20]. Additionally, transfection with the TLR9 agonist ODN M362 induced HCC apoptosis and inhibited HCC growth both in vitro and in vivo [17]. Therefore, we speculated that combining poly(I:C) with the TLR9 agonist CpG ODN might enhance cell apoptosis more than using either agent alone. However, when poly(I:C) and ODN M362 were co-transfected simultaneously into HCC cells, the apoptotic rate significantly decreased compared to transfection of poly(I:C) alone (Fig. 1 and Supplementary Fig. S1). This decreased apoptotic rate was accompanied by a down-regulation of the receptors that recognize poly(I:C), including RLRs and TLR3; the pro-inflammatory cytokines induced by both RLRs and TLRs; and the apoptosis-related genes induced by poly(I:C) (Fig. 2).

Chiron et al. have reported that phosphorothioate-modified TLR9 ligands protect cancer cells against TRAIL-induced apoptosis and that this is dependent on phosphorothioate modification of CpG ODN rather than on TLR9 stimulation [21]. We therefore analyzed whether phosphorothioate modification was also involved in CpG ODN-induced suppression of poly(I:C). We found that while both the phosphorothioate-modified ODN M362 and ODN M362 Ctrl inhibited poly(I:C)-induced apoptosis (Fig. 4), the synthetic phosphodiester CpG ODN (ODN 2006-G5) did not. This result indicates that attenuation of the poly(I:C) pro-apoptotic function by TLR9 ligands is associated with the phosphorothioate modification of CpG ODN.

One possibility consistent with these results is that the phosphorothioate-modified CpG ODN obstructed the entry of poly(I:C) into tumor cells. Indeed, we directly observed that phosphorothioate-modified CpG ODN blocked poly(I:C) entry when these agents were transfected simultaneously (Figs. 5 and 6). In contrast, if HCC cells were first pre-transfected with CpG ODN, subsequent poly(I:C) transfection resulted in efficient entry of poly(I:C) into cells. Moreover, poly(I:C) transfection after CpG ODN pre-treatment significantly augmented the effects of poly(I:C) on HCC cell apoptosis in vitro (Fig. 6B) and enhanced the anti-tumor effect of poly(I:C) in nude mice xenografted with human HCC tumors in vivo. These findings illustrate that the inhibitory effects of phosphorothioate-modified CpG ODN are due to blocking poly(I:C) entry into cells and that using poly(I:C) after CpG ODN can avoid this inhibition.

Recently, Hasan et al. reported that antimicrobial peptides inhibited poly(I:C)-induced immune responses [29], and this correlated with the formation of a strong complex between the antimicrobial peptide and poly(I:C), partially inhibiting the ability of poly(I:C) to bind to TLR3. Likewise, phosphorothioate-modified ODNs were shown to bind to several molecules, such as TRAIL [21] and the V3 loop of HIV gp120 [30], and interfere with the binding of these molecules to their receptors. Based on these previous findings, we
hypothesized that the phosphorothioate-modified backbone of CpG ODN could form a complex with or cause conformational changes in poly(I:C) that consequently prevented entry of poly(I:C) into tumor cells. The precise molecular interactions that likely occur between CpG ODNs and poly(I:C) to block poly(I:C) entry, however, remain to be established. Therefore, we speculate that poly(I:C) may be influenced by many molecules in the microenvironment, including peptides, phosphorothioate-modified ODN and other molecules. This suggests that possible interactions among TLR agonists should be considered carefully when used in combination with each other for clinical use. For example, when TLR agonists are combined for augmenting anti-tumor immunotherapy, poly(I:C) should be used after CpG ODN—particularly when using a phosphorothioate-modified CpG ODN—to avoid disruption of poly(I:C)-induced apoptosis by phosphorothioate-modified TLR9 ligands. These findings provide novel evidence that favors the establishment of anti-tumor strategies that effectively utilize the combined application of TLR agonists.

Grant support

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Authors’ contributions

Conceived and designed the experiments: Zhang J, Zhang Y, Tian Z. Performed the experiments: Zhang Y, Lin A, Sui Q. Analyzed the data: Zhang Y and Zhang J. Contributed reagents/materials/analysis tools: Zhang Y, Lin A, Zhang C. Wrote the paper: Zhang Y, Zhang J.

Conflict of interest

There are no potential conflicts of interest to disclose.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.09.013.

References


