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The antitumor effect of tlr4 inhibition in head and neck cancer cell lines

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Head and neck cancers (HNC) are a group of heterogeneous tumors frequently associated with high-risk HPV or TP53 mutations. HPV-positive cancers are associated with a better prognosis, whereas TP53-mutated tumors are frequently more aggressive. Effective treatment for advanced-stage and metastatic disease is still unavailable. The role of innate immunity in cancer has not been fully explored, and Toll-like receptor 4 (TLR4) has been demonstrated to be an interesting molecular therapeutic target for several types of cancer. Here, we used four HNC cell lines with different molecular profiles, including mutations in TP53 and HPV infection, and evaluated the effect of the TLR4 pathway on tumorigenesis by modulating its activity via the agonist lipopolysaccharide (LPS) and the inhibitor TAK242. We showed that the induction of the TLR4 pathway increased proliferation in the SCC78 and SCC143 cell lines, whereas the inhibition of TLR4 decreased proliferation, colony formation capacity, and migration in all the cell lines studied. TLR4 inhibition reduced IL-6 mRNA expression in SCC78 and SCC143, and MKI67 in SCC78. Also, TAK242 downregulated p53 pathway in SCC78, a TP53 GOF mutation cell line. In combination with cisplatin, TAK242 demonstrated an additive effect, increased cisplatin sensitivity in SCC154, and altered the death mechanism induced by cisplatin in SCC78 cells. In conclusion, TLR4 inhibition led to antitumor effects independent of HPV infection status or TP53 status, suggesting that TLR4 may be a broad-spectrum target for HNC therapy.

Keywords Head and neck cancer, Toll-like receptors, TAK242, TLR4, HPV, TP53

Head and neck squamous cell carcinomas (HNSCC) are a group of tumors that comprise mucosal epithelial tissue in the oropharynx, nasopharynx, larynx, and oral cavity[1,2]. In addition to differences between anatomical sites, many other characteristics contribute to disease heterogeneity[3,4]. Genetic background, sexual habits, tobacco use, alcohol consumption, oncogenic viral infections, and other factors are important features that influence the development and outcome of this disease[4–6].

Alcohol consumption, tobacco smoking, and high-risk human papillomavirus (HPV) infection are major risk factors for the development of head and neck cancer (HNC)[4]. High-risk HPV infection promotes carcinogenesis through E6 and E7 oncogenic protein expression, which deregulates the normal function of the cells, mainly via pRb and p53 degradation, respectively[7]. HPV-positive tumors often have a better prognosis, possibly because of greater antitumor immunity or fewer genetic mutations, contributing to a better treatment response[6,8,9]. Indeed, the incidence of HPV-associated tumors, mainly at the oropharynx site, has increased in recent years, mostly in Europe and America[10]. However, in Asia, oral tumors are more common and are associated with tobacco[4].

Another important feature of HNC is TP53 mutation, the most frequently mutated gene in this type of cancer[11–13]. p53 is a transcription factor that induces cell cycle arrest, DNA repair, and cell death in response to DNA damage and cellular stress signals[14,15]. Its mutations are found in 75–85% of HPV-negative HNC[11]. In

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addition, TP53 mutations are often associated with shorter survival, radiotherapy, and chemotherapy resistance and a worse prognosis[11,16].

In 2020, there were more than 800,000 estimated cases of HNC worldwide, which almost half of it resulted in death[17], mostly due to the high prevalence of recurrence and metastatic disease[18,19]. Cisplatin is a first-line chemotherapy used for HNC treatment[20]. This drug is activated in the cell cytoplasm after hydrolization, becoming a potent electrophile capable of causing DNA damage, leading to cell cycle blockage and apoptosis[21]. It is frequently combined with radiotherapy, but some different combinations also occur in the clinics, such as with cetuximab, pembrolizumab, nivolumab and docetaxel[20,22]. Even so, the current treatments are followed by adverse side effects, high toxicity and many patients are ineligible to cisplatin, due to comorbidities or intolerance and resistance development[23,24]. Therefore, combining cisplatin with other drugs could be an interesting approach to reduce the dose of cisplatin administration, the side effects, and consequently increasing treatment efficacy. It also highlights the urgent need for new targeted therapies that lead to better outcomes with low side effects and toxicity, demonstrating that new molecular targets with potential antitumor activity are worth investigating.

Toll-like receptors (TLRs) are a family of innate immune receptors expressed in different types of cancer[25–27]. They are associated with protumor or antitumor effects depending on the tumor site and type of TLR[27–36]. The role of TLR4 has been extensively investigated in different types of cancer, and its activation via lipopolysaccharide (LPS) in breast cancer has been associated with a protumor phenotype and has shown differential effects depending on p53 status[37]. In cervical cancer, a type of HPV-positive cancer, TLR4 is highly expressed and related to protumor effects[38]. In HNC, TLR4 activation stimulates cell migration[39] and induces resistance to anti-EGFR therapy[40]. However, both analyses were performed only on p53-mutated cell lines, and the role of the TLR4 pathway in HNC cell lines with different p53 statuses or HPV infections remains unclear.

Here, we aimed to investigate the effects of TLR4 activation with LPS and inhibition with TAK242 in HNC cell lines with different p53 statuses (wild type (WT) or mutated) and the presence or absence of HPV. To study the effect of TLR4 inhibition, TAK242, a small molecule that interacts with the intracellular portion of TLR4 and prevents the ligation of adaptor molecules, was used. We also analyzed the effect of TLR4 on the effects of cisplatin. We found that TLR4 inhibition by TAK242 induced antitumor effects, inhibiting cell migration, proliferation, colony formation in all HNC cell lines studied. Interestingly, a gain-of-funtion (GOF) TP53 was downregulated by TAK242. Such findings demonstrate that TLR4 inhibition leads to antitumor effects independent of HPV infection or TP53 pathway, indicating that TLR4 could be a potential target for this type of tumor.

Results

TLR4 Inhibition affects the proliferation and colony formation of HNC cell lines

To study the effects of TLR4 activation and inactivation in HNC cell lines with different TP53 and HPV statuses, four cell lines were used in this study: SCC78 and FaDu, both with TP53 mutations; SCC154, containing the HPV16 genome and WT TP53; and SCC143, with WT TP53 and no HPV. A comparison of p16 expression in SCC154 and HeLa cells (HPV18 positive control) confirmed that both cell lines expressed comparable levels of p16, as expected in HPV-infected cells (Fig. 1A-B, Supplementary Fig. 1). All the cell lines expressed the TLR4 protein (Fig. 1C, Supplementary Fig. 2A-C), and Fig. 1D shows the basal activity of TLR4 pathway by NFκB expression in all cell lines (Supplementary Fig. 2D).

Next, the IC50 values of TAK242 for the different cell lines were found to be 47.42 μM for FaDu cells, 40.37 μM for SCC78 cells, 72.97 μM for SCC154 cells, and 61.79 μM for SCC143 cells (Fig. 2).

To evaluate the role of TLR4 in HNC tumor cells, proliferation and colony formation were assessed after treatment with TAK242 or LPS. LPS increased proliferation only in SCC143 and SCC78 cells after 72 h of treatment (96 h) (Fig. 3A) and did not affect colony formation in any of the cell lines (Fig. 3B). In contrast, TLR4 inhibition by TAK242 decreased proliferation in all the cell lines studied (Fig. 3A). In SCC78 cells, this effect was observed as soon as 24 h of treatment (48 h), and in SCC154, SCC143, and FaDu cells, TAK242 reduced cell proliferation after 72 h of treatment (96 h). TLR4 inhibition also reduced the colony formation capacity of all the cell lines, reducing both the size and quantity of the colonies (Fig. 3B-C). These data showed that inhibiting TLR4 decreases cell proliferation and colony formation independent of p53 or HPV status in HNC cell lines.

TLR4 Inhibition abrogated cell migration in HNC cell lines

We then evaluated the impact of the TLR4 pathway on cell migration. The SCC78 cell line could not be maintained at high confluence, so it was not used in this assay. The FaDu, SCC143, and SCC154 cell lines were first treated with mitomycin-C to inhibit cell proliferation and induce cell cycle arrest, as shown in Supplementary Fig. 3A-D. Next, the cells were subjected to a wound healing assay after LPS or TAK242 treatment. TLR4 activation by LPS did not impact the migration of any cell line. However, TLR4 inhibition by TAK242 prevented the migration of all three cell lines (Fig. 4A-C) after 12 h of treatment in FaDu and SCC143 cells and after 24 h in SCC154 cells. These data showed that inhibiting TLR4 with TAK242 led to cell migration inhibition in all HNC cell lines studied, independent of p53 or HPV status.

TAK242 down regulates TP53 pathway on HNC TP53 mutated cell line

To investigate the modulation of genes involved in proliferation, the TLR4 and TP53 pathways, we selected a TP53 mutated and a TP53 WT cell line – SCC78 and SCC143, respectively. TAK242 reduced interleukin 6 (IL-6) mRNA expression in both SCC78 and SCC143 (Fig. 5A and E). TLR4 inhibition significantly decreased MKI67 mRNA expression only in SCC78 (Fig. 5B), while in SCC143, it demonstrated only a tendency of decreasing (Fig. 5F).

Interestingly, while analyzing TP53 pathway, TAK242 reduced TP53 and p21 mRNA expression in the TP53 mutated cell line SCC78 (Fig. 5C-D), but the same effect was not observed in the TP53 WT cell line, SCC143. In SCC143, TP53 was not detected and TAK242 did not alter p21 expression (Fig. 5G).

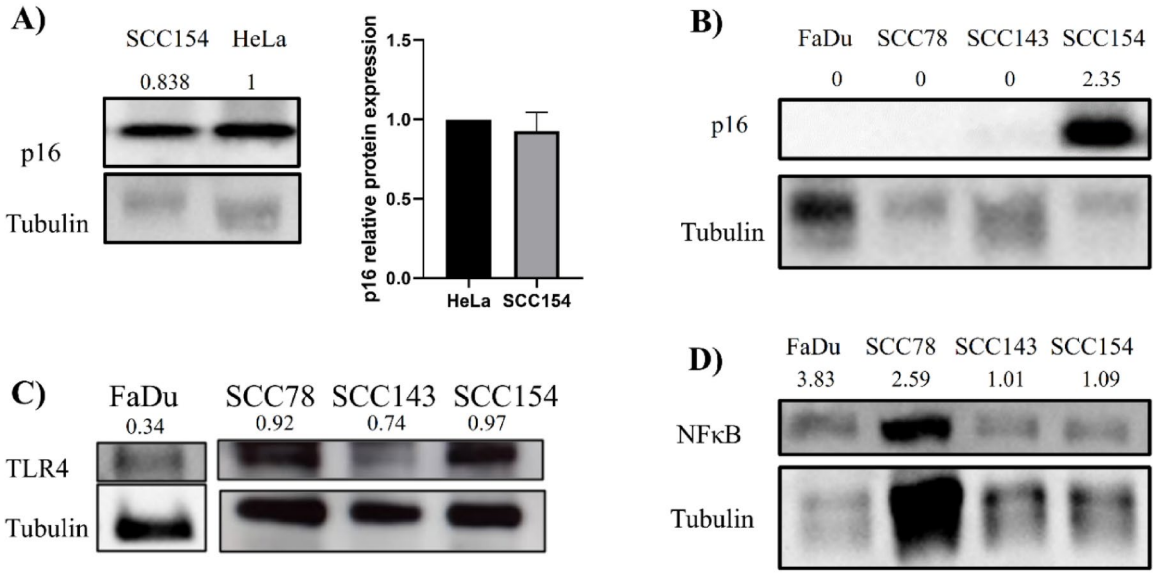


Fig. 1. Detection of p16, TLR4, and NFκB expressions in the cell lines studied. (**A**) p16 protein expression in the HeLa and SCC154 cell lines. (**B**) p16 expression in the FaDu, SCC78, SCC143, and SCC154 cell lines. (**C**) TLR4 and tubulin expression in the FaDu, SCC78, SCC143, and SCC154 cell lines. FaDu western blot was made in a different gel from the other cell lines SCC78, SCC143, and SCC154. (**D**) NFκB and tubulin expression in FaDu, SCC78, SCC143, and SCC154 cell lines.

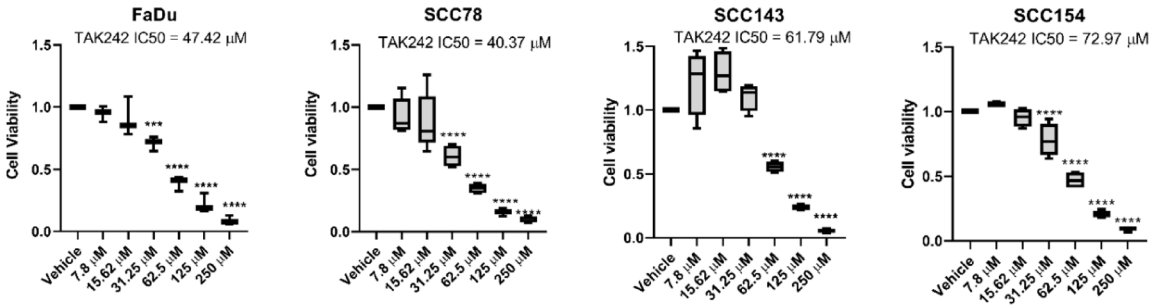
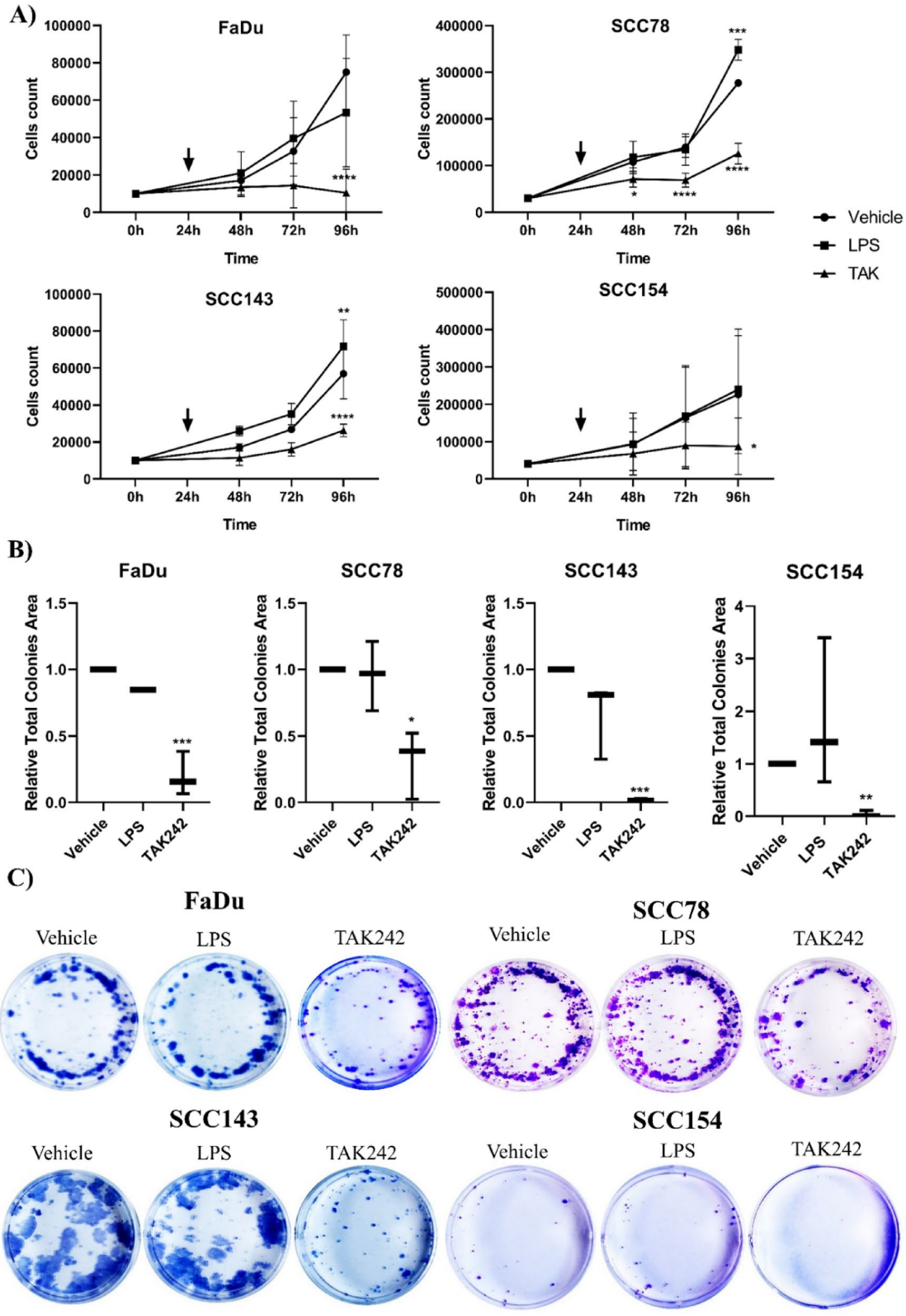


Fig. 2. Determination of the TAK242 IC50 in HNC cell lines. From left to right: viability of FaDu, SCC78, SCC143, and SCC154 cells treated with DMSO (vehicle) and different TAK242 concentrations (0 μM-250 μM). Cell viability was measured after 72 h of treatment with a TLR4 inhibitor. The results are from at least four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Combining TAK242 and cisplatin treatments in HNC cell lines

Cisplatin is a drug frequently used to treat HNC in the clinic. Therefore, we investigated whether TLR4 influences the response to cisplatin. The experiments were performed only with SCC78, SCC143, and SCC154; SCC78 was chosen as the representative TP53-mutated cell line. A cell viability assay revealed that the cisplatin IC50 values were 8.422 μM, 5.190 μM, and 2.851 μM in SCC78, SCC143, and SCC154, respectively (Fig. 6A). The SCC154 (HPV+) cell line demonstrated greater sensitivity to cisplatin than did SCC143 (TP53MUT, HPV-) and SCC78 (TP53WT and HPV-negative) cells, whereas SCC78 cells presented the lowest sensitivity and SCC143 medium sensitivity to cisplatin (Fig. 6B).

LPS alone increased SCC78 and SCC154 cell viability (Fig. 7A and C). However, activating more TLR4 did not significantly affect cisplatin sensitivity in any of the cell lines (Fig. 7A-C). However, inhibiting constitutively activated TLR4 through TAK242 could affect the cisplatin sensitivity of SCC154. Compared with the addition of cisplatin alone, the addition of TAK242 to cisplatin significantly increased cisplatin sensitivity in this cell line (Fig. 7C). Next, we analyzed the dose-effect curve and the combination index (CI) of cisplatin and TAK242



combination in SCC78, SCC143 and SCC154 (Fig. 7D-F). The value of CI obtained for all three cell lines was equal to 1.0, indicating that the TAK242 + Cisplatin combination has an additive effect on the HNC cell lines.

Cell death analysis revealed that cisplatin alone could induce apoptosis (7AAD+/Annexin V-FITC+/7AAD-/Annexin V-FITC + and caspase 3/7 activation) and necrosis (7AAD+/Annexin V-FITC-) in all three cell lines (Fig. 8A-C). Neither LPS nor TAK242 alone induced cell death in SCC78, SCC143 or SCC154 cells (Fig. 8A-C).

Interestingly, the combination of TAK242 and cisplatin shifted apoptotic cell death (7AAD+/Annexin V-FITC+/7AAD-/Annexin V-FITC+) to necrotic cell death (7AAD+/Annexin V-FITC-) in SCC78 cells, although it did not significantly affect caspase activation after TAK242 + cisplatin treatment (Fig. 8A; Supplementary

◀**Fig. 3**. Reduction in the proliferation and colony formation of HNC cell lines after TLR4 inhibition. (**A**) Proliferation assay. At 0 h, 1×10^4, 3×10^4, 1×10^4, or 4×10^4 FaDu, SCC78, SCC143, SCC154, or FaDu cells were seeded in a 24-well plate. After 24 h (arrow), the cells were treated with 10 µg/mL LPS or the corresponding IC50 concentration of TAK242 and counted at 48 h, 72 h, and 96 h. The results are from at least four independent replicates. (**B**) Colony formation assay. The cells were plated at low confluence and treated with LPS (10 µg/mL) or TAK242 (IC50) for 72 h. After 10–15 days, the total area of the colonies in the plate was measured, and the results are presented relative to those of the control. The results are from at least three independent replicates. (**C**) Representative images of cell well plates stained with crystal violet for the colony formation assay. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 4A and 5 A). On the other hand, in the SCC143 cell line, there was a reduction in necrotic cell death after the combination of cisplatin and TAK242 (Fig. 8B; Supplementary Fig. 4B and 5B), whereas in SCC154, there was no difference in cell death after the combination of cisplatin and TAK242 (Fig. 8C; Supplementary Fig. 4C and 5). LPS did not affect cell death in combination with cisplatin in any cell line. Indeed, combining TAK242 with cisplatin could influence the cisplatin response, decreasing cell viability or altering the death outcome, although this effect depends on the type of cell line.

Discussion

The current standard treatment approach for initial and advanced HNC includes chemotherapy and radiotherapy, mainly cisplatin[23,41,42]. Such therapy can lead to numerous cytotoxic effects, and there are few other options for alternative treatment in cases of ineligibility or resistance to cisplatin[23,43]. New drugs and molecular targets are necessary to improve or replace cisplatin, especially for advanced HNC treatment[23]. Several studies have demonstrated that the TLR4 pathway is functionally important for this type of cancer but also highlight that investigations into the effects of inhibiting this pathway, especially in combination with chemotherapeutic drugs, are lacking[44]. This study investigated the impact of TLR4 activation and inhibition on HNC cell lines with different TP53 and HPV statuses and its implications for cisplatin treatment.

TLR4 activation via LPS is frequently associated with protumor effects in HNC. LPS increases proliferation, invasion, migration, cytokine production, and apoptosis resistance induced by TNF-α[45] and induces resistance to anti-EGFR therapy in SCC-4 and CAL27 cells[40], both of which are TP53-mutated squamous cell carcinomas. LPS also led to resistance to cisplatin-induced apoptosis in CAL-27 cells[44] and increased proliferation and resistance to apoptosis induced by cisplatin in PCI-1, PCI-13, and PCI-30 cells[46], with only PC-13 presenting TP53 mutations[47]. Similarly, in this study, LPS increased proliferation in SCC-78 and SCC-143 cells but did not affect colony formation, migration, or cisplatin sensitivity in any of the cell lines studied.

On the other hand, pathway inhibition with TAK242 induced antitumor effects in all cell lines, indicating that the TLR4 pathway must be constitutively activated in these cell lines and highlights the importance of inhibiting the TLR4 pathway in these cases. Furthermore, the antitumor potential of TLR4 inhibition has been demonstrated in different types of cancer. TAK242 reduced cell proliferation and colony formation capacity and induced apoptosis and cell cycle arrest in ovarian cancer cell lines[48]. In glioblastoma cells, TAK242 reduced cell viability, migration, and invasion[49]. Additionally, TAK242 decreased the clonal growth and proliferation of anoikis-resistant breast cancer cell lines[50] and inhibited the migration and invasion of lung cancer cells[51]. Here, we demonstrated that TAK242 decreased proliferation, colony formation capacity, and migration in all human HNC cell lines studied, increase cisplatin sensitivity in SCC154 cells in a viability assay, and alter the effect of cisplatin on SCC78 cells.

TAK242 did not influence caspase activation or Annexin-V or 7AAD staining, demonstrating that it does not directly affect cell death. However, TAK242 successfully arrests proliferation and reduces the colony formation capacity of cells, which can be an important strategy for controlling tumor growth[52]. Blocking proliferation could lead to senescence, halting tumor progression depending on the context[53]. In some cases, senescent cells can activate immune surveillance and contribute to an inflammatory tumor microenvironment (TME)[52,53]. Despite having a context-dependent role in cancer, inducing cell arrest can be attractive in cases where conventional therapy is not possible or is no longer effective, making it an interesting strategy for managing chronic diseases and unresponsive tumors.

We also showed that TAK242 inhibited cell migration in all the cell lines studied. Inhibition of cell migration prevents metastasis, which is responsible for the worst outcomes of HNC[54]. The frequency of distant metastasis varies from 3 to 52%[55], and once the disease is diagnosed, the time to death is approximately 4 months[56]. The options for treatment are conventional chemotherapy and radiotherapy[57], and the causes of death are frequently treatment failure or resistance to treatment[58], indicating the necessity of new therapies to prevent the progression of local disease and metastasis dissemination.

Another important issue in HNC is the heterogeneity of the disease. The presence of HPV or TP53 mutations, for example, is an important feature that influences prognosis[4,6,59]. Therefore, treatments that are effective in different types of tumors will have broader effects. In this study, we demonstrated that TLR4 inhibition reduces tumor progression in HNC cell lines independently of HPV or TP53 status. This is the first study in which an HPV-positive cell line and a wild-type-TP53-HPV-negative cell line were evaluated after TAK242 treatment.

The TLR4 pathway modulation was evaluated in a TP53 mutated and a TP53 WT cell line. TAK242 decreased IL6 mRNA expression in both SCC78 and SCC143, confirming the TLR4 pathway inhibition by TAK242. TAK242 also decreased MKI67 expression in SCC78, a marker of cell proliferation, confirming our previous results. In SCC143, on the other hand, this effect was not significant, but demonstrated a tendency of down regulation.

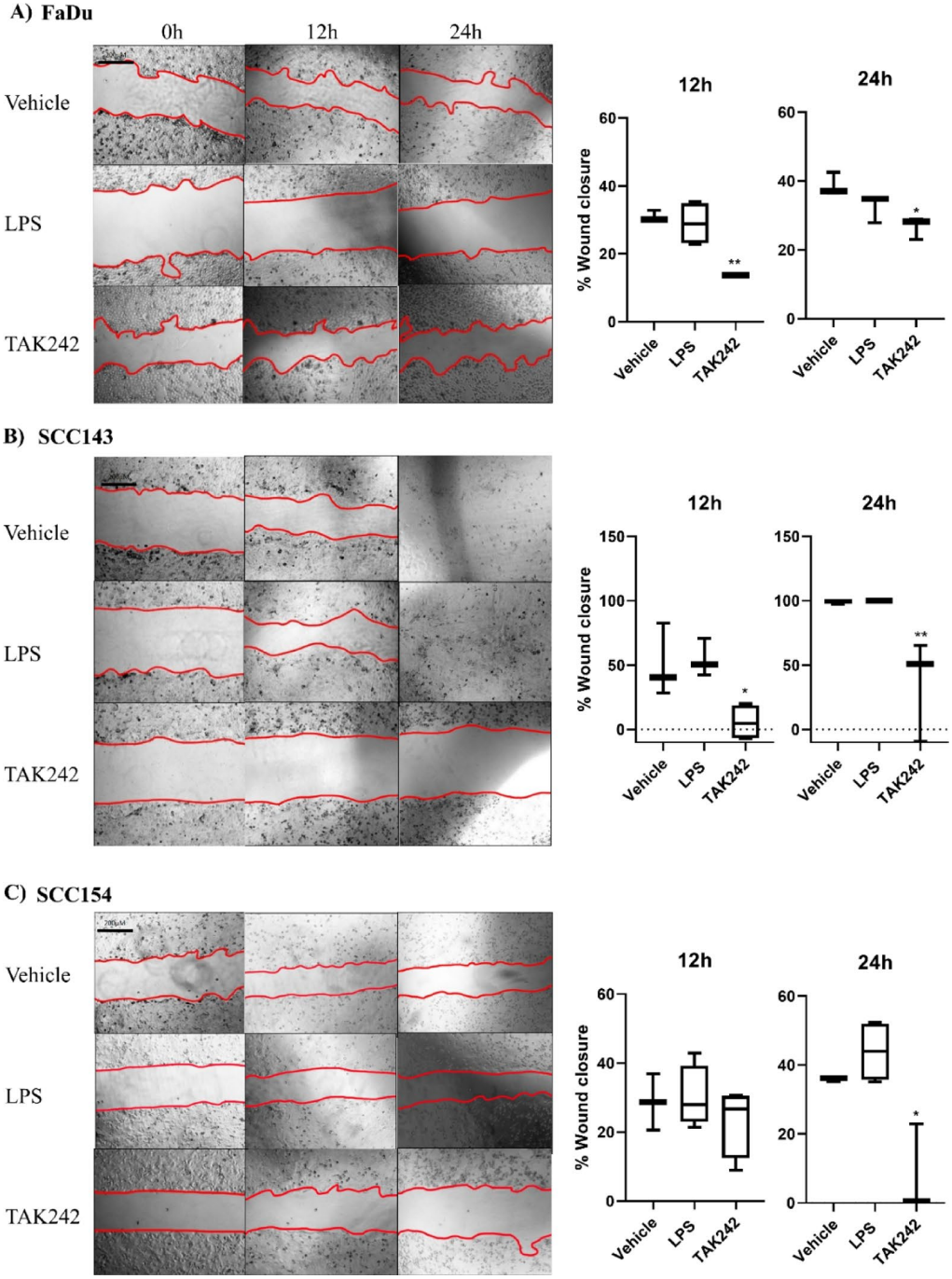


Fig. 4. Reduction in the migration of HNC cells after TLR4 inhibition. (**A**) FaDu, (**B**) SCC143, and (**C**) SCC154. The cells were seeded at high confluence in a 24-well plate. After mitomycin C treatment for 2 h, a wound was made in each well. The cells were treated with 10 µg/mL LPS or 47.42 µM TAK242 in FaDu cells, 61.79 µM in SCC143 cells, or 72.97 µM in SCC154 cells. One representative experimental image at 0 h, 12 h, and 24 h is presented on the left, and the empty gap area between the red lines is indicated. The graphics show the percentage of filled gap area after 12 h and 24 h relative to the 0 h time point. The results are from at least five independent replicates. *, $p < 0.05$; **, $p < 0.01$.

The relation between TLRs and TP53 may vary depending on TP53 status. TP53 WT can regulate the expression of several members of TLR family, including TLR4, which have at least one canonical or non canonical p53 response element[60,61]. Also, drugs that induce p53 consequently increase TLRs expression, and such interactions are lost in some TP53 mutations[62,63]. Here we observed that in SCC78 – the TP53 mutated cell line, TAK242 downregulated the TP53 pathway by decreasing the mutant TP53 mRNA expression and as a consequence its downstream target p21, while in SCC143 – the WTP53 cell line, we could not detect TP53 expression, indicating that this cell line may have developed a mechanism to suppress TP53 expression. Therefore, the use of TAK242

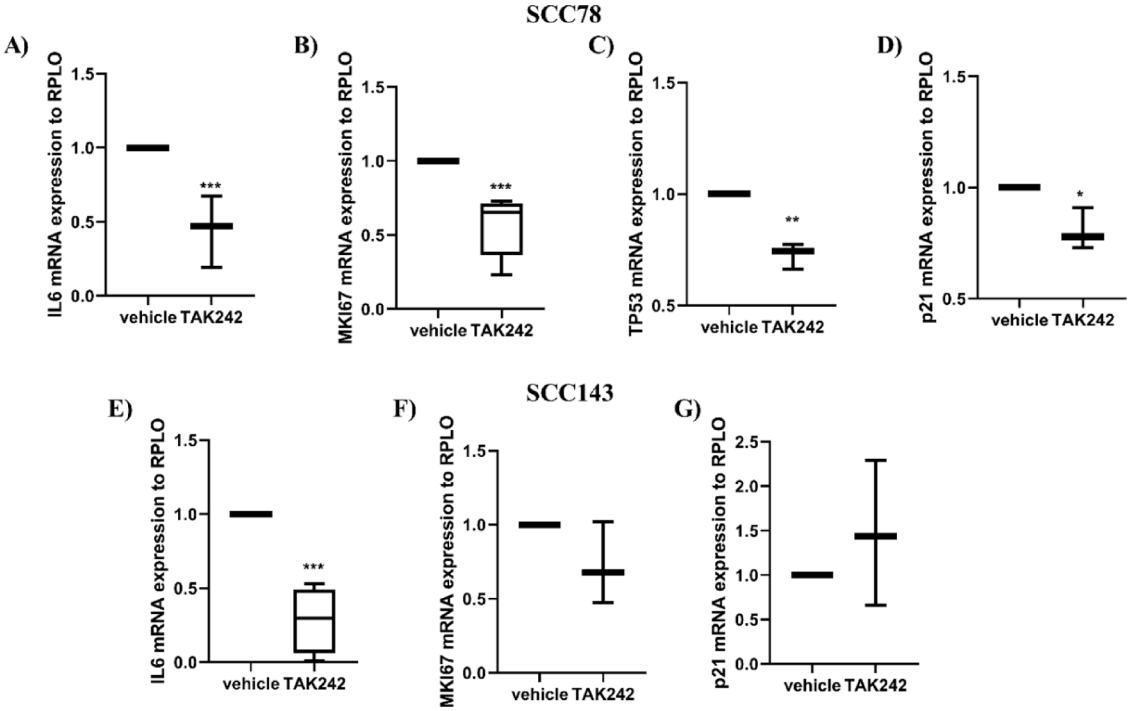


Fig. 5. TAK242 differentially modulates mRNA expression in TP53 WT and TP53 mutated cell lines. Gene expression was assayed in SCC78 (**A**–**D**) and SCC143 (**E**–**G**) after 48 h of TAK242 treatment, and analyzed by quantitative RT-PCR of three biological replicates for IL6, MKI67, TP53 and p21. The graphics show the relative mRNA expression to RPLO. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

did not alter p21 mRNA levels, as TP53 expression was absent. Particularly, SCC78 has a R158G TP53 variant, which has been documented as a GOF mutation in non-small cell lung cancer[64]. GOF mutations may activate tumorigenic genes and even modulate drug sensitivity[14]. In this case, the downregulation of the TP53 pathway with GOF mutation could be beneficial to the treatment. However, the relation between TP53 and TLRs appears to depend on the status of TP53, including the types of mutation, which is not yet completely clear and requires further investigations.

Additionally, the modulation of TLR4 may impact other cancer therapies. Ju et al.[40] demonstrated that TLR4 activation with LPS increased resistance to cetuximab, an anti-EGFR therapy, in HNC cell lines (SCC-4, SCC-9, and CAL-27). Additionally, the inhibition of TLR4 with TAK242 increased the sensitivity of ovarian cancer cells to doxorubicin[48], and the combination of cisplatin and TAK242 decreased the viability of breast cancer cell lines[50]. Here, we demonstrated that the combination of TAK242 with cisplatin had an additive effect in all cell lines and resulted in a decrease in the viability of SCC154 cells.

In addition, the influence of TAK242 on the cisplatin response differed among SCC78 cells, which were the most resistant to cisplatin treatment in our study. Interestingly, the TAK242 + cisplatin combination resulted in a shift in cell death, reducing apoptosis and increasing necrosis in SCC78 cells. Induction of necrosis in the TME can be an interesting inducer of antitumor immunity mediated by damage-associated molecular pattern (DAMP) release and activation of the proinflammatory response in different types of cancer[65]. These findings may contribute to future investigations in which TAK242 could complement cisplatin treatment.

Overall, we confirmed that the antitumor potential of TLR4 inhibition also occurs in HNC, affecting cell proliferation and migration despite molecular and etiological differences between the cell lines analyzed. We also demonstrated that the combination of TLR4 inhibition with cisplatin could improve current treatments. This study contributed to the investigation of TAK242 use in HNC and revealed that TLR4 could be a potential molecular target for this type of cancer, regardless of HPV or TP53 status. These findings mainly provide alternative strategies for advanced and metastatic diseases, supporting further studies on TLR4 inhibition therapy – either as a standalone treatment or in combinations with other therapies.

Materials and methods

Cell culture

Four HNC cell lines were used in this study: SCC78 (UPCI: SCC078) (RRID: CVCL_C044), a TP53-mutated and HPV-negative, floor of mouth squamous cell carcinoma cell line from a male patient with a history of alcohol and nonsmoker; SCC143 (UPCI: SCC-143) (RRID: CVCL_C057), a TP53 WT and HPV-negative, floor of mouth squamous cell carcinoma cell line of a female patient with no alcohol history and smoker; SCC154 (UPCI: SCC-154) (RRID: CVCL_2230), a TP53 WT and HPV16-positive cell line base of tongue squamous cell carcinoma cell line of a male patient; and FaDu (RRID: CVCL_1218), a TP53-mutated and HPV-negative,

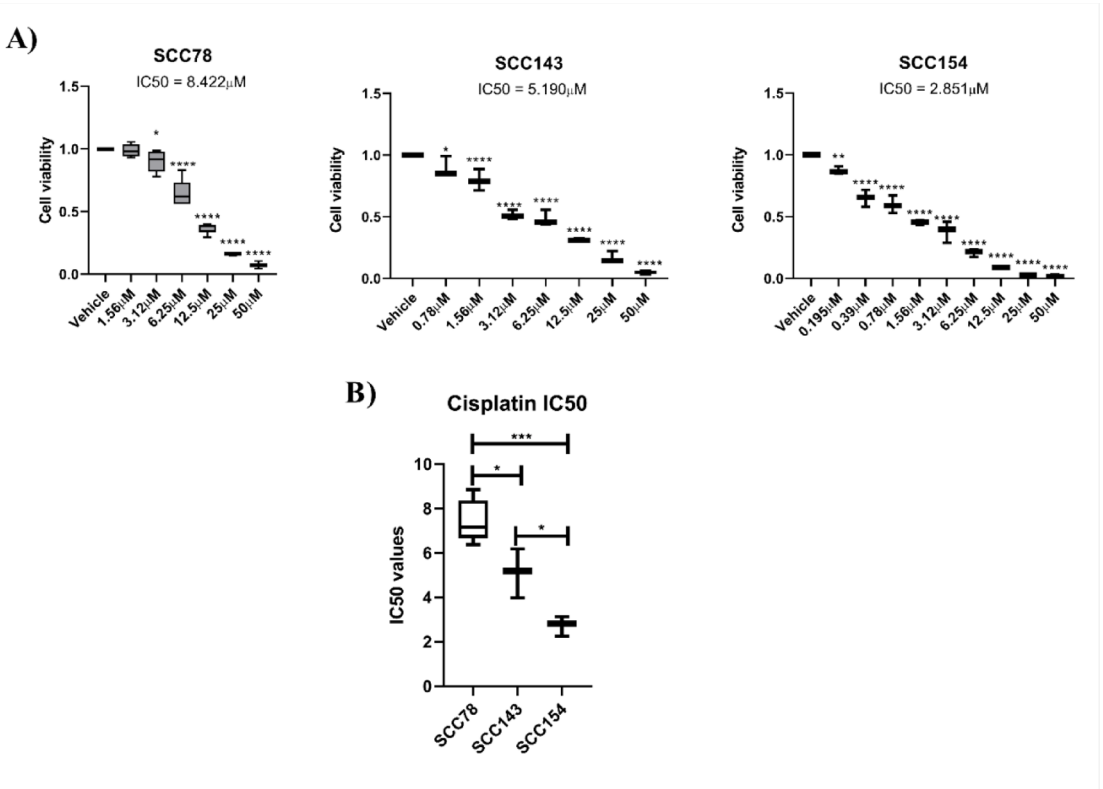


Fig. 6. Determination of the IC50 of cisplatin in HNC cell lines. (**A**) From left to right: Cell viability assay of SCC78, SCC143 and SCC154 cells treated with different concentrations of cisplatin (0 μM-50 μM). Cell viability was measured after 72 h of treatment. (**B**) Comparison of the IC50 values of cisplatin in SCC78, SCC143, and SCC154 cells. The results are from at least four independent replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

hypopharynx squamous cell carcinoma cell line from a male patient with a history of alcohol and smoker. The HeLa cell line (CCL-2 ATCC) (RRID: CVCL_0030), an HPV18-positive cervical adenocarcinoma cell line from a female patient, was used as an HPV-positive control. The TP53-mutated cell lines SCC78 and FaDu presented the following mutations: R150G c.884 C > G(p. Arg282Gly) (18, 19), and c.676-1G > A (p. Stop at 244)[66]. SCC78, SCC143 and SCC154 cells were acquired from Pittsburgh University (UPCI: SCCs)[67], and FaDu and HeLa cells were purchased from the American Type Culture Collection (ATCC).

HNC cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic solution and 1% GlutaMAX, and HeLa cells were grown in minimum essential medium (MEM) at 37 °C with 5% CO_2. The cells were cultured for eight passages, and aliquots from initial passages were used.

Cell viability and proliferation assays

To determine the IC50s of cisplatin (CAS 15663-27-1) (Merck kGaA, Darmstadt, Germany) (232120) and TAK242 (CAS 243984-11-4) (Merck kGaA, Darmstadt, Germany) (614316), 2,500 cells were seeded per well in a 96-well plate, and after 24 h, they were treated with serial dilutions ranging from 0 to 50 μM cisplatin or 0-250 μM TAK242, the vehicle (DMSO) of TAK242 was used as control of the experiments. To analyze cell viability, 2500 cells were seeded per well in a 96-well plate. After 24 h, the cells were treated with 10 μg/ml LPS (manufacturer's recommended dose) (lipopolysaccharide obtained from *E. coli* 055:B5) (L6529) (Merck kGaA, Darmstadt, Germany), the corresponding TAK242 or cisplatin IC50, LPS (10 μg/ml) + cisplatin IC50 combination or the TAK242 IC50 + cisplatin IC50 combination. After 72 h of treatment, 10 μL of PrestoBlue Cell Viability Reagents (Thermo Fisher Scientific, Massachusetts, USA) (A13261) were added. The plates were incubated for 1 h 30 min, and fluorescence was measured at 570 (excitation) nm and 600 nm (emision). The IC50 values determination and ordinary one-way ANOVA statistical analysis with Tukey's and Dunnett's multiple comparisons test were performed via GraphPad Prism 8 software (RRID: SCR_000306) (GraphPad Software, Inc., California, USA), and the combination index of TAK242 and Cisplatin was perfomed via Calcusyn software (RRID: SCR_020251) (Calcusyn v2.1, Biosoft Bioinformatics Software Platform) with at least four replicates.

For the proliferation assays, the following cells were seeded in 24-well plates: 3×10^4, 1×10^4, 4×10^4, and 1×10^4 of SCC78, SCC143, SCC154, and FaDu cells, respectively. The next day, cells were treated with 10 μg/ml LPS, or the corresponding IC50 of TAK242. After 24 h, 48 h, and 72 h of treatment, viable cells were counted via a Neubauer chamber and Trypan blue reagent (Merck kGaA, Darmstadt, Germany) (V900876), and two-

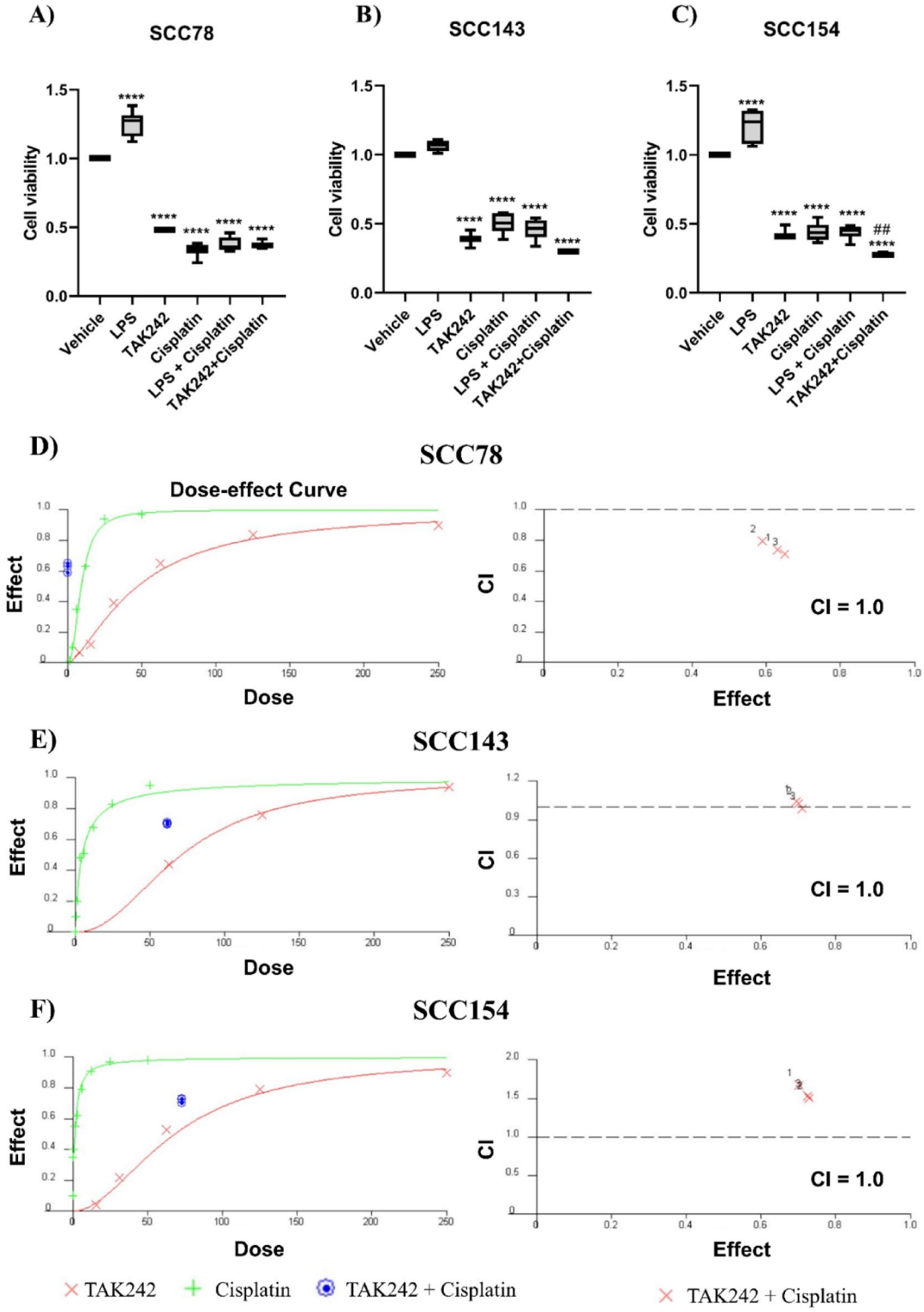


Fig. 7. Evaluation of the effect of TLR4 modulation on cisplatin sensitivity. The cells were treated for 72 h with different combinations of LPS (10 µg/mL), TAK242 (corresponding to the IC50 value), cisplatin (corresponding to the IC50 value), and DMSO (vehicle), and the viability of (**A**) SCC78, (**B**) SCC143, and (**C**) SCC154 cells was measured. Comparisons were made between each treatment group and the control group (*) or between the combined treatments and both individual treatments (#). At least four replicates of the experiments were performed independently. In (**D**–**F**) the TAK242 + cisplatin combination was analyzed by Calcusyn software (RRID: SCR_020251) (Calcusyn v2.1, Biosoft Bioinformatics Software Platform) to determine the combination index (CI) in SCC78, SCC143 and SCC154, respectively. In the left graph, the Dose-effect curves of TAK242 (red curve), Cisplatin (green curve), and TAK242 + cisplatin (blue circles). In the right graphs, the combination index plot of TAK242 + Cisplatin indicating the CI value. ****, $p < 0.0001$; ##, $p < 0.01$.

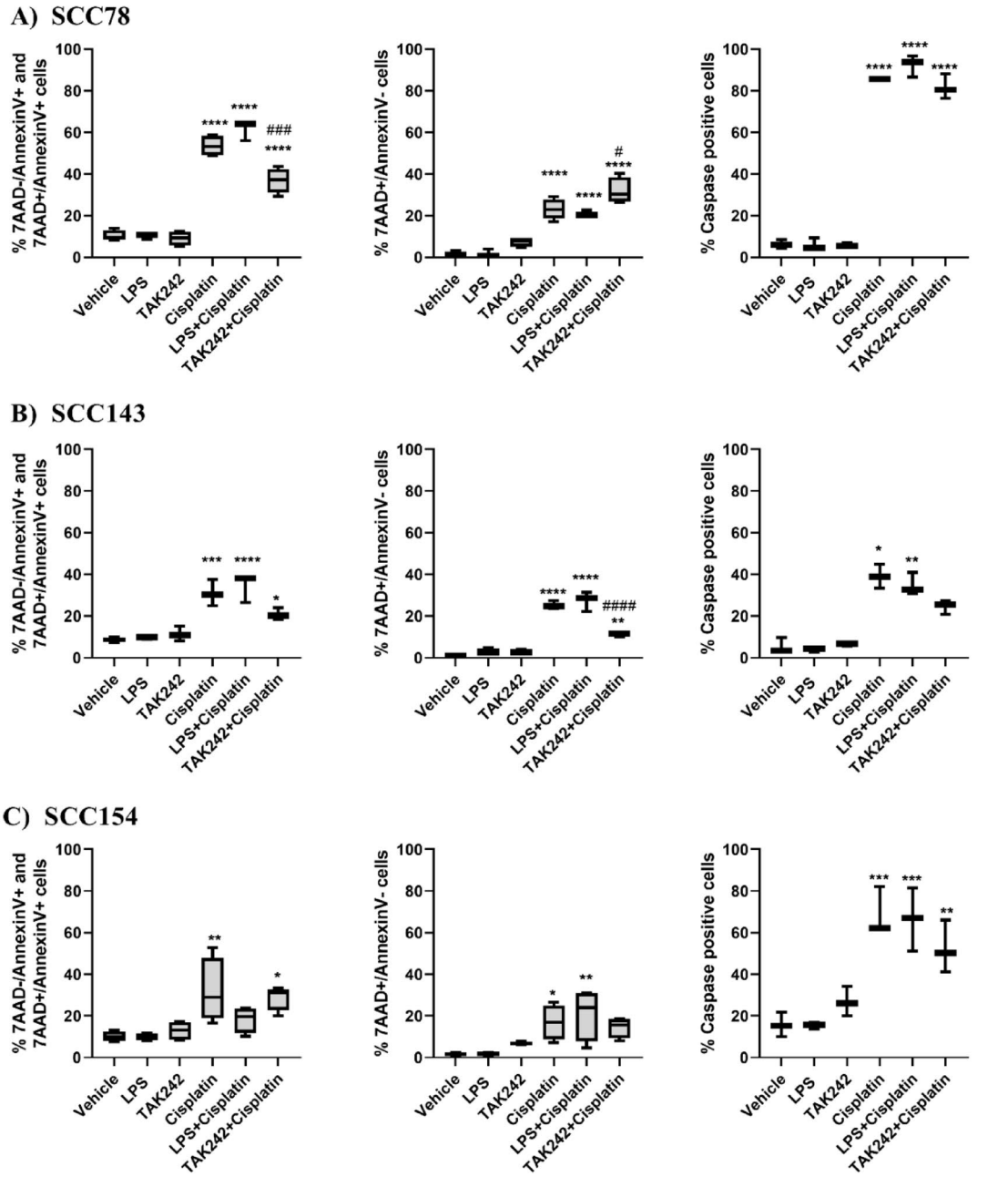


Fig. 8. Cell death mediated by LPS, TAK242, and cisplatin. The cells were treated for 72 h with different combinations of LPS (10 µg/mL), TAK242 (corresponding to the IC50 value), cisplatin (corresponding to the IC50 value), and DMSO (vehicle). Then, cells were stained with Annexin-V-FITC and 7AAD or with CellEvent Caspase-3/7 Green Detection Reagent and analyzed by flow cytometry. In (**A**) SCC78, (**B**)143, and (**C**)154, apoptotic cells were considered those with Annexin-V+/7AAD- and Annexin-V+/7AAD + staining, and necrotic cells were considered those with Annexin-V-/7AAD + staining. Comparisons were made between each treatment group and the control group (*), and the combined treatments were combined with each individual treatment (#). The experiments were independently performed in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; #, $p < 0.05$; ####, $p < 0.0001$.

way ANOVA statistical analysis with Dunnett's multiple comparisons test was performed via GraphPad Prism 8 software with at least four replicates.

Colony formation assay

Cells were seeded at low confluence, with 500 cells per well in a 6-well plate. The next day, the cells were treated with 10 μg/ml LPS or the corresponding IC50 of TAK242. After 72 h, the culture medium was removed. After 10–15 days of treatment, the cells were fixed with methanol and stained with 0.5% crystal violet in 20% methanol. The percentage of the plate area covered with cells was measured via ImageJ 1.53k (RRID: SCR_003070) (National Institutes of Health, Maryland, USA) software, and ordinary one-way ANOVA statistical analysis with Tukey's multiple comparisons test was performed via GraphPad Prism 8 software with three biological replicates.

Western blot

The cells were lysed via protein extraction buffer containing 150 mM NaCl, 50 mM Tris – HCl (pH 7.5), 0.5% NP40, 0.1 mM EDTA, and complete Mini-EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) (11836170001). After extraction, proteins were quantified via Quick Start Bradford Dye reagent (Bio-Rad Laboratories, Inc., California, USA) (#5000205), and 100 μg of protein sample was analyzed via SDS–PAGE (10–12% acrylamide gel) via western blotting.

The following primary antibodies were used: anti-TLR4 (1:100) (ab13556) (RRID: AB_300457) (Abcam, Cambridge, UK), anti-p16 (1:1000) (sc-56330) (RRID: AB_785018) (Santa Cruz Biotechnology, Texas, USA), and anti-alpha/beta-tubulin (1:5000) (2148 S) (Cell Signaling Technology, Massachusetts, USA), anti-alpha tubulin (1:200) (PAS-16891) (Thermo Fisher Scientific, Massachusetts, USA), anti-NFκB (1:200) (ab7972) (Abcam, Cambridge, UK) antibodies. The following secondary antibodies were used: anti-rabbit (1:5000) (7074 S) (Cell Signaling Technology, Massachusetts, USA) and anti-mouse (1:5000) (7076 S) (Cell Signaling Technology, Massachusetts, USA) antibodies.

Cell death analysis

Cell death analysis was performed via flow cytometry by measuring Annexin-V, 7AAD, and activated Caspase-3/7-stained cells. For this experiment, 2×10^5cells (SCC143, SCC78, and FaDu) or 1×10^5cells (SCC154) were seeded in a 6-well plate. After 24 h, the cells were treated with 10 μg/mL LPS, the respective TAK242 or cisplatin at the IC50, or a combination of both. After 72 h of treatment, apoptotic and necrotic cells were detected via the eBioscience Annexin V-FITC Apop Kit (Thermo Fisher Scientific, Massachusetts, USA) (88-8005-72), and active caspase-3/7 -positive cells were stained via the CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific, Massachusetts, USA) (C10423), both following the manufacturer's protocol. The cells were analyzed via flow cytometry via a BD FACSCalibur (BD Life Sciences, New Jersey, USA), and the populations were identified via FlowJo v.10 software (RRID: SCR_008520) (https://www.flowjo.com/) (FlowJo LLC, Oregon, USA). Apoptotic cells were considered those with Annexin-V+/7AAD- and Annexin-V+/7AAD + staining, and necrotic cells were considered those with Annexin-V-/7AAD + staining. Ordinary one-way ANOVA statistical analysis with Tukey's multiple comparisons test was performed via GraphPad Prism 8 software with at least five biological replicates.

Wound healing

First, the following cells were treated with different doses of mitomycin C (CAS 50-07-7) (Merck kGaA, Darmstadt, Germany) (10107409001): 0.25 μg/mL, 0.5 μg/mL, and 1 μg/mL in FaDu; 0.75 μg/mL and 1 μg/mL in SCC143; and 0.125 μg/mL and 0.25 μg/mL in SCC154. A cell proliferation assay was performed to evaluate which dose of mitomycin was able to inhibit proliferation (Supplementary Fig. 3A). The cell cycle arrest was subsequently evaluated after 2 h of mitomycin C treatment at the corresponding doses (0.25 μg/mL in FaDu; 1 μg/mL in SCC143; and 0.125 μg/mL in SCC154) (Supplementary Fig. 3A-C).

For the wound healing assay, cells were seeded at high confluence (1×10^5cells of SCC143; 4×10^5cells of SCC154 and FaDu cells) in a 24-well plate. After 24 h, the plates were treated with mitomycin C for 2 h at the following concentrations: 0.25 μg/mL in FaDu; 1 μg/mL in SCC143; and 0.125 μg/mL in SCC154. The medium containing mitomycin C was discarded, and a wound was made in the middle of each well of the plate. Then, the cells were treated with 10 μg/mL LPS or the TAK242 IC50. The plates were monitored with a ZEISS Axio Observer Z1 (Carls Zeiss AG, Jena, Germany) microscope, which captured images every two hours for 24 h. The area of the gap in pixels was measured with ImageJ 1.53k software (National Institutes of Health, Maryland, USA), and ordinary one-way ANOVA statistical analysis with Dunnett's multiple comparisons test was performed via GraphPad Prism 8 software with at least five biological replicates.

Quantitative polymerase chain reaction (qPCR)

For RNA extraction, 2×10^5cells of SCC78 and SCC143 were plated in a 6-well plate. After 24 h, cells were treated with DMSO (vehicle) or the correspondent TAK242 IC50. After 48 h, RNA was extracted with TRIzol Reagent (Thermo-Fisher Scientific, Massachusets, USA) as described by the manufacturer. After, 1 μg of RNA was treated with DNase (RQ1 RNase-Free DNase) (Promega, Winsconsin, USA), followed by cDNA synthesis with SuperScript III Reverse Transcriptase (Thermo-Fisher Scientific, Massachusets, USA), and qPCR with Power SYBR Green PCR Master Mix (Thermo-Fisher Scientific, Massachusets, USA), also following the manufacturer's protocol. The primers used were: RPLO F 5'-CCTCATATCCGGGGGAATGTG-3'; RPLO R 5'-GCAGCAGCT GGCACCTTATTG-3'; p21 R 5'-tggagtggtagaaatctgtcatgc-3'; p21 F 5'-ggacagcagaggaagaccatgt-3'; TP53 R 5'-T GGTTTCTTCTTTGGCTGGG-3'; TP53 F 5'-GCGTGTTTGTGCCTGTCCTG-3'; MKI67 R 5'-TGAAATAGC GATGTGACATGTGCT-3'; MKI67 F 5'-TGTGCCTGCTCGACCCTACA-3'; IL6 F 5'-CCACACAGACAGCCA CTCAC-3'; IL6 R 5'-AGGTTGTTTTCTGCCAGTGC-3'. The data was obtained by calculating ΔCt, ΔΔCt, and Fold Change relative to RPLO expression and Unpaired T test statistical analysis was performed via GraphPad Prism 8 software with three biological replicates.

Data availability

All the data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

NMA: Conceptualization, formal analysis, funding acquisition, investigation, methodology, writing – original

draft; GBC and KASC: investigation; IGSR, RET, LLV and MGM: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing – review and editing.

Declarations

Competing interests
The other authors declare that they have no potential conflicts of interest.

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