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ORIGINAL ARTICLE

TAPI-1 Exhibits Anti-tumor Efficacy in Human Esophageal Squamous Cell Carcinoma Cells via Suppression of NF-κB Signaling Pathway

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Abstract

Background TNF-α processing inhibitor-1 (TAPI-1) is a known metalloproteinase inhibitor with potential anti-inflammatory effects. However, its anti-cancer effects on esophageal squamous cell carcinoma (ESCC) have not been uncovered.

Aim In the present study, the effects of TAPI-1 on ESCC cell viability, migration, invasion, and cisplatin resistance and the underlying molecular mechanisms were investigated in TE-1 and Eca109 cells.

Methods To this end, TE-1 and Eca109 cells were exposed to TAPI-1 for indicated time intervals. Cell viability was assessed using cell counting kit-8 assay and apoptosis was evaluated using flow cytometry assay. Migration and invasion were assessed using Transwell assays. Gene expressions were analyzed using quantitative reverse transcription polymerase chain reaction. The activation of NF-κB signaling pathway was elucidated via Western blot and chromatin immunoprecipitation assay.

Results We observed that higher doses (10, 20 μM) of TAPI-1 inhibited ESCC cell viability, while a lower dose (5 μM) of TAPI-1 inhibited ESCC cell migration and invasion and enhanced the chemosensitivity of ESCC cells to cisplatin. Moreover, TAPI-1 suppressed the activation of NF-κB signaling and the target genes expression in the stage of transcription initiation. Furthermore, blocking NF-κB signaling in advance could abolish all the effects of TAPI-1 on ESCC cells.

Conclusion Overall, these results indicated that TAPI-1 impairs ESCC cell viability, migration, and invasion and facilitates cisplatin-induced apoptosis via suppression of NF-κB signaling pathway. TAPI-1 may serve as a potential adjuvant agent with cisplatin for ESCC therapy.

Keywords TAPI-1 · NF-κB signaling pathway · ESCC · Cell viability · Migration and invasion · Cisplatin resistance

Introduction

Esophageal carcinoma is one of the most frequently diagnosed digestive cancers, known as the sixth most common cause of cancer-related death worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is the major histopathological subtype of esophageal carcinoma [2]. Although advances have been made in treatments for ESCC, the poor prognosis remains disappointing due to recurrence, metastasis, and the resistance to chemotherapeutic agents, such as cisplatin [3–5]. Therefore, it is necessary to understand the molecular mechanisms of ESCC pathogenesis and develop efficient adjuvant treatment modalities, such as anti-tumor drugs.

TNF-α processing inhibitor-1 (TAPI-1) is a known broad-spectrum metalloproteinase inhibitor, which inhibits the shedding of TNF-α and other cytokine receptors [6, 7]. In recent years, TAPI-1 has been proposed to ameliorate various inflammatory diseases, such as rheumatoid arthritis,

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kidney injury, and neuroinflammation [8–10]. Additionally, several studies showed that TAPI-1 might display anti-tumor efficacy in pancreatic cancer, breast cancer, and hepatoma [11–15]. However, the effect of TAPI-1 on digestive tract tumors remains to be greatly elucidated. Intriguingly, a previous review has concluded that the matrix metalloproteinase (MMP) family, a potential molecular target of TAPI-1, facilitated ESCC progression [16]. Moreover, our previous study revealed that knockdown of TNF-α-converting enzyme (TACE, also named ADAM17), another molecular target of TAPI-1, significantly inhibited ESCC cell viability [17]. Therefore, we hypothesize that TAPI-1 may exhibit anti-tumor effects on ESCC.

In the present study, our data indicated that TAPI-1 could inhibit ESCC cell viability, migration, and invasion and enhanced the chemosensitivity of ESCC cells to cisplatin, while exhibited low toxicity in normal esophageal epithelial cells. Of note, TAPI-1 remarkably inhibited NF-κB signaling pathway in ESCC cells. Moreover, suppression of NF-κB signaling pathway in advance remarkably abolished the anti-tumor effects of TAPI-1, suggesting that TAPI-1 exhibits anti-tumor efficacy in an anti-NF-κB manner in ESCC cells.

Materials and Methods

Cell Lines and Chemicals

Human esophageal epithelial cell line Het-1A and human ESCC cell lines TE-1 and Eca109 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cell lines were maintained in RPMI medium (Gibco), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100-mg/ml streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

TAPI-1 (HY-16657) and BAY11-7082 (HY-13453) were purchased from MedChem Express and dissolved in dimethyl sulfoxide (DMSO) for storage at – 80 °C. Cisplatin (PHR1624) was obtained from Sigma-Aldrich and dissolved in phosphate-buffered saline (PBS) for storage at – 20 °C.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay was conducted to assess cell viability. In brief, cells were inoculated in 96-well plates with a density of 1×10^3 cells per well. After chemicals treatment, cells were washed twice in PBS. Cells were then subjected to CCK-8 reagents (Dojindo Laboratories, Kumamoto, Japan) with the recommended protocol. The absorbance was measured at 450 nm using a Synergy H1 Microplate Reader (Bio-Tek). The average absorbance of six replicates was calculated.

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

In brief, the total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen). After reverse transcription (TaKaRa, Japan), 2 × QuantiNova SYBR Green PCR Master Mix (QIAGEN) was applied to analyze the relative mRNA levels using a CFX Connect™ Real-Time System (Bio-Rad). The fold change relative to control was calculated using $2^{-\Delta\Delta Ct}$ method. The mRNA levels were normalized to β-actin. Partial primer sequences were listed as follows: 5′-CCACGAAACTACCTTCAACTCCATC-3′ and 5′-ACTCCTGCTTGCTGATCC- ACAT-3′ for β-actin, 5′-TCCCTACAGACAGAGCCACAAG-3′ and 5′-CTTCAC - CTCCGTGATTGCCTTC-3′ for BIM, 5′-GAGCTGAAG CAGATGCAGGACAA-3′ and 5′-TGACGGAGTTGCCAC TTGACTTG-3′ for TRAIL, 5′-TGTGGATGACTG- AGT ACCTGAACC-3′ and 5′-CAGAGACAGCCAGGAGAA ATCAAAC-3′ for BCL2, 5′-AACAGGAACACAGGA GTCATCAGT-3′ and 5′-CGGAGGATTATCG- TTGGTG TCAGT-3′ for CDH1, 5′-TCCTGCTTATCCTTGTGCTGA TGT-3′ and 5′-GTCTTCTTCTCCTCCACCTTCTTCA-3′ for CDH2, 5′-ACTGTGACAAGG- AATATGTGAGCC-3′ and 5′-CAAGGTAATGTGTGGGTCCGAAT-3′ for SLUG, and 5′-CCTACACCAAGAACTTCCGTCTGTC-3′ and 5′-GTGCCAAGGTCAATGTC- AGGAGAG-3′ for MMP2. The remaining primer sequences are listed in Table 1.

Flow Cytometry Assay

Apoptosis was evaluated by the Annexin V-propidium iodide (PI) double staining assay. In brief, ESCC cells were digested into single cells and washed twice with ice-cold PBS. After centrifugation, cells were re-suspended and stained with Annexin V and PI using the FITC Annexin V Apoptosis Detection kit I (BD Pharmingen™) according to the manufacturer's protocols. Apoptosis was analyzed using BD LSRFortessa™.

Migration and Invasion Assays

Cell migration and invasion capacity were evaluated using Transwell inserts (8-μm pores; Corning) in 24-well plates. In brief, 200 μl of serum-free RPMI medium with 1×10^5 cells was added to the upper chamber with Matrigel (BD Biosciences) for invasion assay or without Matrigel for migration assay. After chemicals treatment, 500 μl of RPMI medium with 20% FBS was added to the lower chamber, followed by an incubation for 24 h at 37 °C. Nonmigrating cells remaining at the top of the upper chamber were removed with cotton swabs. The cells that

Table 1 References for cisplatin resistance-related genes and primers for qRT-PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)	References
ABCC10	ACTGACTCTGAACGGCTGCTTA	AAGATGAGACCACCCACGAAGG	[32]
ACTL6A	AATCAGAAGCCAGTCTCCATCCT	CTCTAGTATTCCACGGTGCCTCT	[33]
AKR1C2	TGTAAAGCCAGGTGAGGAAGTGA	AGCAGCCTGTGGTTGAAGTTG	[34]
ATP7A	CTGGTGAAGTCGTGCTGAAGATG	GTGCTAGTACATGAATGGCAGGTC	[35]
CHD1L	ACTGGTATGGTACTGAGCGACTT	TGAGGAGGAAGATGAAGACTGTGA	[36]
CMTM6	ATGGAGAACGGAGCGGTGTA	AGATGAAGGCCAGCAGAGACA	[37]
EIF4E	ACGGAATCTAATCAGGAGGTTGCT	AGAGTGCCCATCTGTTCTGTAGG	[38]
FZD7	CGCCTCTGTTCGTCTACCTCTT	ATGATGGTGCGGATACGGAAGA	[39]
GSTP1	CCTGTACCAGTCCAATACCATCCT	TCCACGCCGTCATTCACCAT	[40]
HMGN5	CCAAGCAAGAAGCAGTTGTTGAAG	TCAGAAGCTGGTGCCTCTGTAAT	[41]
HOXB7	TGCCTCACGGAAAGACAGATCAAG	TCTTCCTCTTCCTCCTCTGCTTCA	[42]
ITGA5	CATCGCTCTCAACTTCTCCTTGG	CGGCTCTTGCTCTGATAATGTAGG	[43]
ITGB1	TGAAGCCAGCAACGGACAGAT	TTACAGACACCACACTCGCAGAT	[44]
LAMP2	GCCATCTCCTACTACAACACCTAC	CTGAAGCAACCTTATCCTGAGTGA	[45]
MAGED1	CTCGGTCTCCTCTTGGTGATTCT	GCACTCGTCTGTAGTCCAGGTATT	[46]
MCL1	AGAGGAGGAGGAGGACGAGTTGTA	AGACCTGCCCATTGGCTTTGTG	[47]
NFE2L2	AATTGCCTGTAAGTCCTGGTCATC	TGCCCTAAGTTCATCTCTTGTGAG	[48]
NGFR	ATCCTGGCTGCTGTGGTTGT	CTGTTGGCTCCTTGCTTGTTCTG	[49]
NSD2	ATCGCCTCCAACAGCATCATC	GCAGGACTCACAGCACAGAA	[50]
RAC1	CATGGCTAAGGAGATTGGTGCTGTA	ACTGCTCGGATCGCTTCGTCAA	[51]
RACK1	ACCACCACGAGGCGATTTGT	GCCACACTCAGCACATCCTT	[52]
SFN	TCTTCCACTACGAGATCGCCAACA	TGATGAGGGTGCTGTCTTTGTAGGA	[53]
SLC2A1	TGTGCTCCTGGTTCTGTTCTTCATC	CTCCTCGGGTGTCTTGTCACTTTG	[54]
SOD2	AAGCTGACGGCTGCATCTGTTG	TAAGCGTGCTCCCACACATCAATC	[55]
SRPX2	AAGCCGTCGTTGGTCTGGAA	GTAGTCACAGCGAGAGTCAAGAAG	[56]
UBQLN4	GAGCCTCGGTCAAGGAGTTCAA	GATTCCGTGCTGGTTCAGTGTG	[57]
VRK1	CCTGGTGTTGAAGATACGGAATGG	TTCTGGTTCTTGAACGGGTCTGTA	[58]
YAP1	CCAGACTACCTTGAAGCCATTCCT	TTCCATCTCCTTCCAGTGTTCCAA	[59]
YBX1	AGGAGAACAAGGTAGACCAGTGAG	CGTCTGCGTCGGTAATTGAAGT	[60]
ZFX	AGTGGCAGGAAAGTGTATCAGTGT	GATGTCGCATTATGTGCTGGTTCT	[61]

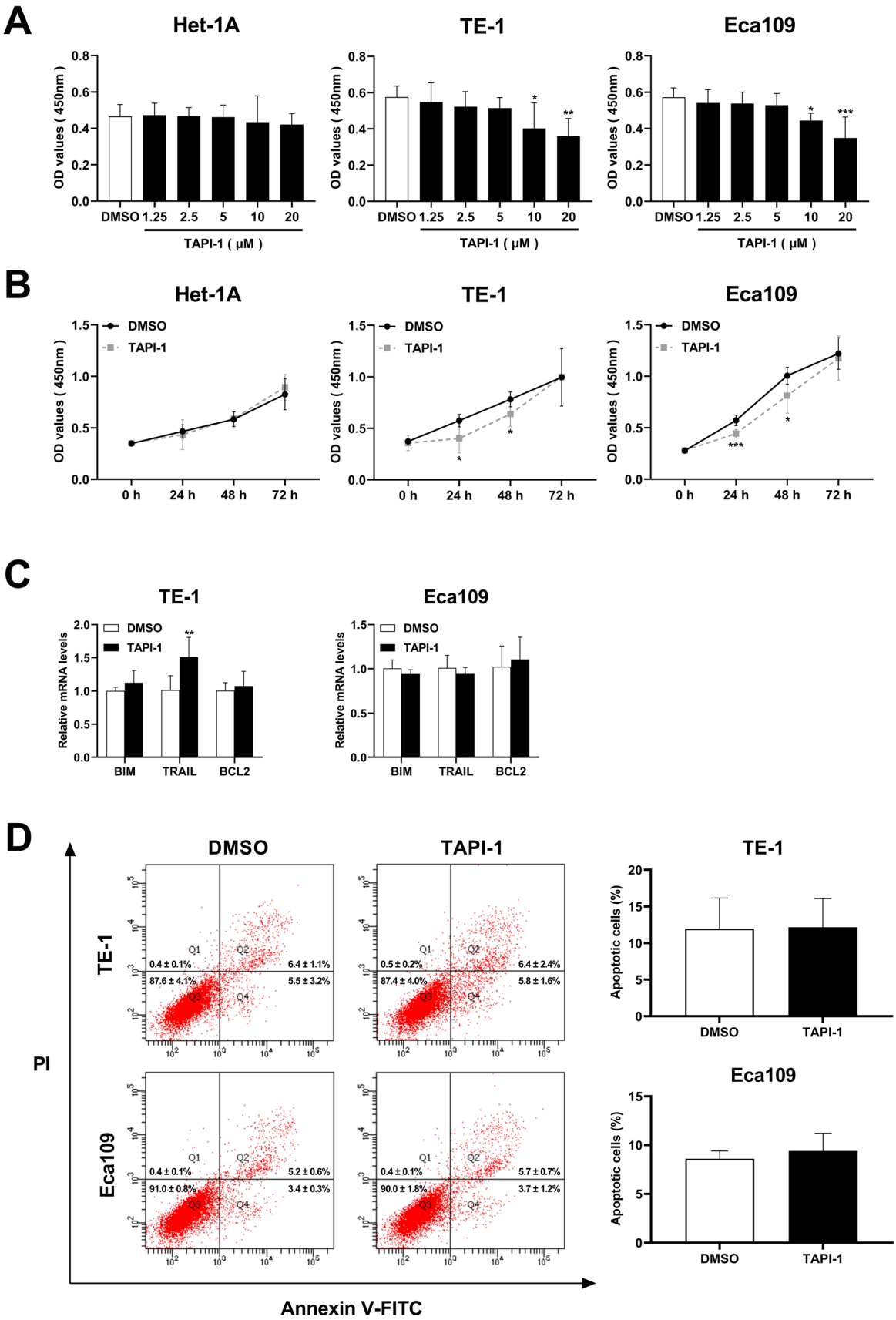
migrated to the bottom of the upper chamber were fixed with 4% formaldehyde and stained with crystal violet. Cells were observed under a microscope at ×200 magnification and then lysed with 500 µl of 0.2% Triton X-100 per well. The absorbance was measured at 550 nm using a Synergy H1 Microplate Reader (Bio-Tek). The average absorbance of three independent experiments was calculated.

Bioinformatics Analysis

The putative transcription factors in the upstream of cisplatin resistance-related genes were speculated via the ChEA3 website (https://maayanlab.cloud/chea3). Ten transcription factors with the highest scores from the Enrichr Queries library are listed in Fig. 4A.

Protein Extraction and Western Blot Analysis

To obtain total protein, cells were lysed in RIPA buffer (Beyotime) containing 0.5-mM PMSF (Sigma) and 1 × phosphatase inhibitor (Roche). Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) was applied for nuclear protein extraction with the recommended protocol. β-actin and H3 were used as internal controls for the total and nuclear proteins, respectively. The following antibodies were used: anti-β-actin antibody (Proteintech, 66009-1-Ig, 1:5000), anti-H3 antibody (Proteintech, 17168-1-AP, 1:2000), anti-NF-κB p65 antibody (Wanleibio, WL01980, 1:500), anti-P-NF-κB p65 (Wanleibio, WL02169, 1:500), HRP Goat Anti-Mouse IgG (Jackson, 115-035-003, 1:10,000), and HRP Goat Anti-Rabbit IgG (Jackson, 111-035-003, 1:10,000). The gray levels were analyzed using ImageJ software.


A
Het-1A
TE-1
Eca109
OD values (450nm)
DMSO 1.25 2.5 5 10 20
TAPI-1 (μM)
B
DMSO
TAPI-1
0 h 24 h 48 h 72 h
C
Relative mRNA levels
BIM TRAIL BCL2
D
DMSO
TAPI-1
PI
Annexin V-FITC
Q1 Q2 Q3 Q4
0.4 ± 0.1% 6.4 ± 1.1% 87.6 ± 4.1% 5.5 ± 3.2%
0.5 ± 0.2% 6.4 ± 2.4% 87.4 ± 4.0% 5.8 ± 1.6%
0.4 ± 0.1% 5.2 ± 0.6% 91.0 ± 0.8% 3.4 ± 0.3%
0.4 ± 0.1% 5.7 ± 0.7% 90.0 ± 1.8% 3.7 ± 1.2%
Apoptotic cells (%)

◂**Fig. 1** The effect of TAPI-1 on ESCC cell viability. **A** CCK-8 analysis of cell viability after exposure of Het-1A, TE-1, and Eca109 cells to DMSO or varying doses of TAPI-1 (1.25, 2.5, 5, 10, and 20 μM). **B** CCK-8 analysis of cell viability after exposure of Het-1A, TE-1, and Eca109 cells to DMSO or TAPI-1 (10 μM) for 0, 24, 48, and 72 h, respectively. **C** TE-1 and Eca109 cells were exposed to DMSO or TAPI-1 (10 μM) for 24 h. qRT-PCR quantitative analysis of BIM, TRAIL, and BCL2 expression at mRNA levels. **D** TE-1 and Eca109 cells were exposed to DMSO or TAPI-1 (10 μM) for 24 h. Apoptosis analysis using flow cytometry and quantitative analysis of apoptotic cells. $*P<0.05$, $**P<0.01$, and $***P<0.001$, compared with the DMSO group

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) was carried out using Pierce™ Agarose ChIP Kit (Thermo, 26156) according to the manufacturer's protocol. The ChIP-grade anti-NF-κB p65 antibody was purchased from CST (#6956, 1:50). Immunoprecipitated DNA was analyzed by quantitative polymerase chain reaction (qPCR) with 2 × QuantiNova SYBR Green PCR Master Mix (QIAGEN). The putative NF-κB-binding sites in c-Myc, CyclinD1 and MMP2 promoters were speculated via the JASPAR website (https://jaspar.genereg.net) and the primers were generated by TsingKe Biological Technology (Beijing, China). The results from three independent experiments were averaged and plotted as percentage of input. The primer sequences were as followed: 5′-CTAAGTGATCAGACACCGTCAG-3′ and 5′-TGTCCC TGGTCAAGTAGGTTT-3′ for c-Myc, 5′-CCAGGCTAG AAGGA- CAAGATGAAGG-3′ and 5′-GCGTCGTTGCAA ATGCCCAAG-3′ for CyclinD1, 5′-GTTAGGCAAGTG ACTTCTCAGT-3′ and 5′-GTAAGCACCTAAGATTAC CT- CTCT-3′ for MMP2.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 8.0 software. All quantifications were performed with at least three independent experiments. Comparison of two groups was performed using an unpaired Student's t test. One-way analysis of variance (ANOVA) was applied when more than two groups were compared. Data were presented as mean ± standard deviation (SD). $P<0.05$, $P<0.01$, and $P<0.001$ represented different degrees of statistical significance.

Results

TAPI-1 Mildly Inhibits ESCC Cell Viability with Low Toxicity

To determine whether exposure to TAPI-1 could influence ESCC cell viability, TE-1 and Eca109 cells were exposed to varying doses of TAPI-1 (0, 1.25, 2.5, 5, 10, and 20 μM) for 24 h, following which, cell viability was determined using CCK-8 assay. Interestingly, following exposure of TAPI-1, TE-1, and Eca109 cell viability was inhibited in a dose-dependent manner. As shown in Fig. 1A, in the presence of 10 and 20 μM of TAPI-1, cell viability decreased significantly in TE-1 and Eca109 cells. Next, we performed the time-course experiments to observe duration of decreased cell viability in TE-1 and Eca109 cells exposed to TAPI-1 (10 μM) for the indicated time points (0, 24, 48, and 72 h). Exposure of ESCC cells to TAPI-1 resulted in remarkably decreased cell viability at 24 and 48 h (Fig. 1B), following which, there was no significant alteration in cell viability at 72 h. Moreover, we carried out the same experiments in human-immortalized esophageal epithelial cell line Het-1A and observed no alteration in cell viability (Fig. 1A and 1B), suggesting that TAPI-1 could inhibit ESCC cell viability without toxicity to normal esophageal epithelial cells.

To further investigate whether exposure to TAPI-1 could influence ESCC cell apoptosis, TE-1 and Eca109 cells were exposed to TAPI-1 (10 μM) or solvent control. Following exposure to TAPI-1 for 24 h, the total RNA was extracted and the relative mRNA levels of apoptosis-related genes were determined using qRT-PCR. As shown in Fig. 1C, we observed no obvious change at the mRNA levels of pro-apoptosis genes BIM and TRAIL, and the anti-apoptosis gene BCL2 after TAPI-1 treatment in Eca109 cells. In TE-1 cells, TRAIL was significantly upregulated in response to TAPI-1 treatment. Moreover, flow cytometry assay showed that neither TE-1 nor Eca109 cell apoptosis was influenced following TAPI-1 treatment for 72 h ($P>0.05$) (Fig. 1D). These data suggested that TAPI-1 markedly inhibited ESCC cell viability and showed no effect on cell apoptosis.

TAPI-1 Inhibits ESCC Cell Migration and Invasion

To investigate whether exposure to TAPI-1 could influence ESCC cell migration and invasion, Transwell chambers with or without Matrigel were applied. To rule out the impact of TAPI-1 on ESCC cell numbers, the concentration of 5-μM TAPI-1 chosen to assess the migration and invasion ability of ESCC cells. TE-1 and Eca109 cells were exposed to TAPI-1 (5 μM) or solvent control for 24 h. In Fig. 2A, qRT-PCR showed that the mRNA levels of SLUG and MMP2 expression were remarkably downregulated in response to TAPI-1 treatment in TE-1 and Eca109 cells. Moreover, we observed that CDH1 (E-cadherin) expression was slightly upregulated after TAPI-1 treatment in TE-1 cells. However, no obvious change in CDH2 (N-cadherin) expression was observed in TE-1 or Eca109 cells. Additionally, Transwell assays indicated that TAPI-1 treatment significantly suppressed the invasion and migration of TE-1 and Eca109 cells, respectively (Fig. 2B, C). These data suggested that

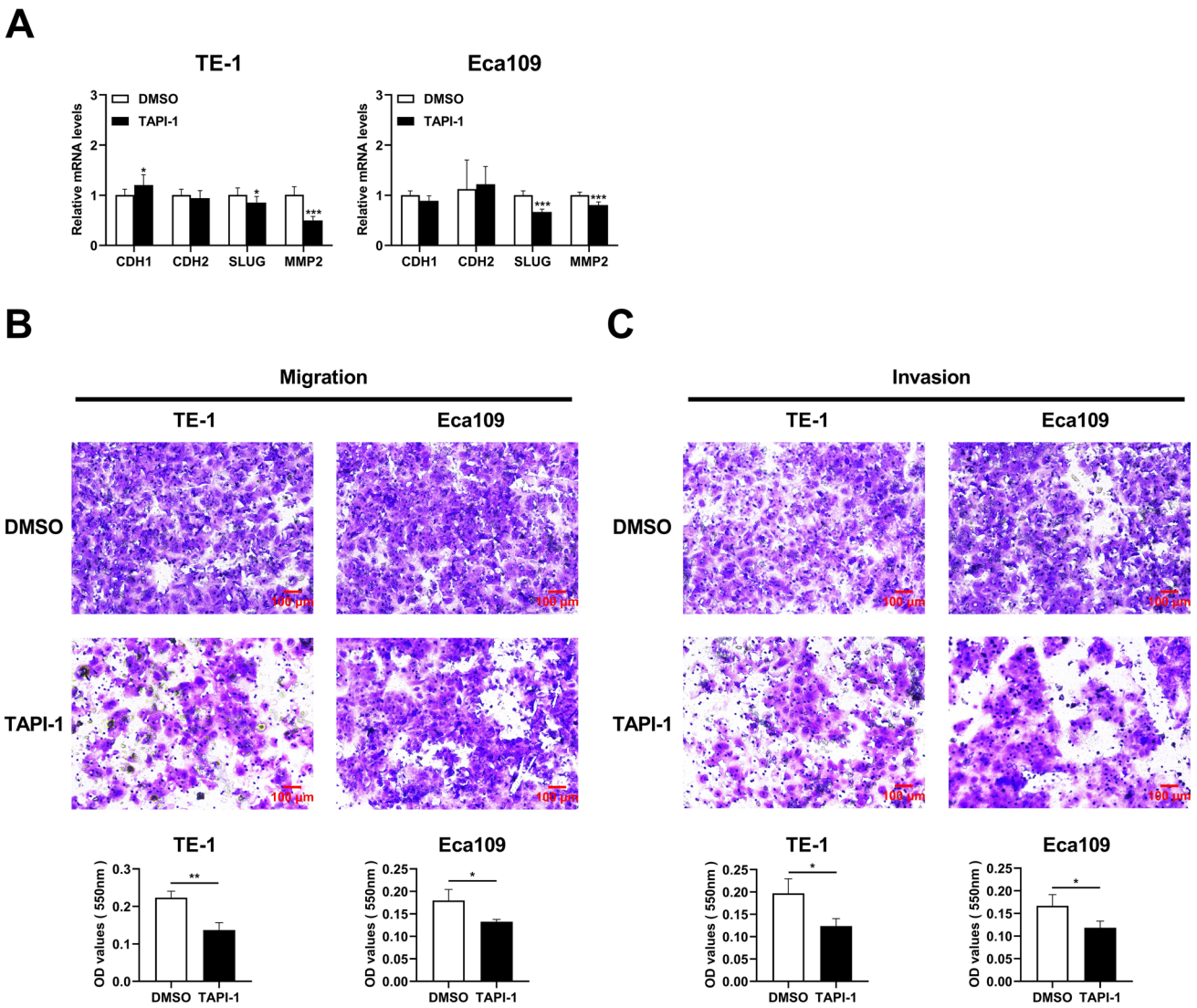


Fig. 2 The effect of TAPI-1 on ESCC cell migration and invasion. TE-1 and Eca109 cells were exposed to DMSO or TAPI-1 (5 μM) for 24 h. **A** qRT-PCR quantitative analysis of CDH1, CDH2, SLUG, and MMP2 expression at mRNA levels. Representative images and absorbance analysis results of Transwell migration assay (**B**) and invasion assay (**C**). $*P<0.05$, $**P<0.01$, and $***P<0.001$, compared with the DMSO group

TAPI-1 could significantly inhibit ESCC cell migration and invasion.

TAPI-1 Promotes the Sensitivity of ESCC Cells to Cisplatin

Chemoresistance remains the leading cause of treatment failure in ESCC. Thus, we then evaluated the impact of TAPI-1 on chemoresistance of ESCC cells using the gold-standard chemotherapeutic drug cisplatin. To rule out the impact of TAPI-1 itself on ESCC cell viability, the concentration of 5-μM TAPI-1 chosen to assess the sensitivity of ESCC cells to cisplatin due to no obvious alteration in ESCC cell viability (Fig. 1A). First, to determine the effect of TAPI-1 on cisplatin resistance-related genes, TE-1 and Eca109 cells were exposed to TAPI-1 (5 μM), following which, the total RNA was extract. Numerous studies have documented a variety of cisplatin resistance-related genes over recent years. From 2016 to 2021, 30 members of the genes that have been recently reported to facilitate cisplatin resistance are listed in Tab. 1. As shown in Fig. 3A, the relative mRNA levels of the 30 cisplatin resistance-related genes were determined using qRT-PCR. A Venn diagram was made to compare the differences in gene expressions between TE-1 and Eca109 cells (Fig. 3B). In Fig. 3B, we observed that 29 genes (ABCC10, ACTL6A, AKR1C2, ATP7A, CHD1L, CMTM6, EIF4E, FZD7, GSTP1, HMGN5, HOXB7, ITGA5, ITGB1, LAMP2, MAGED1,

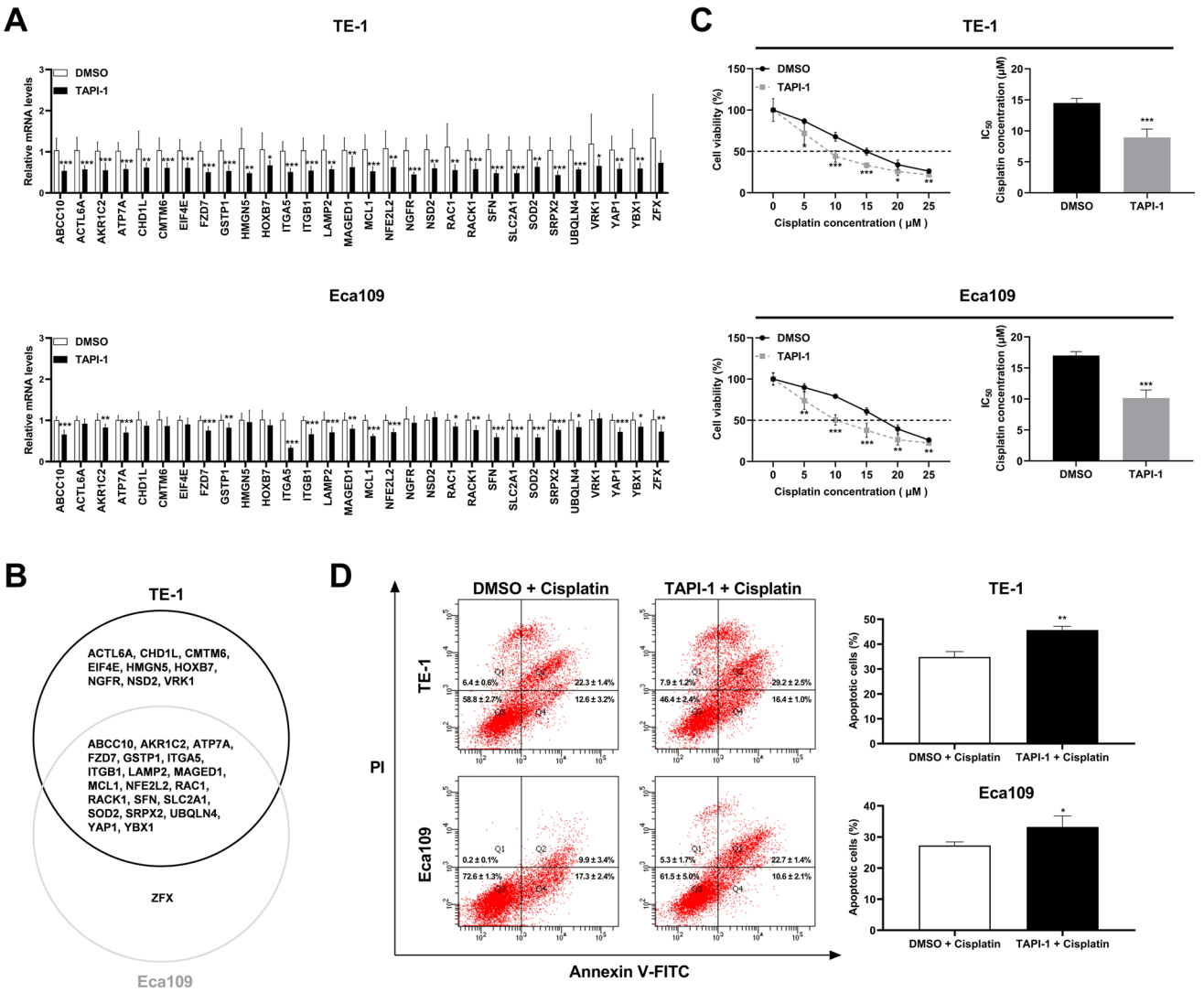
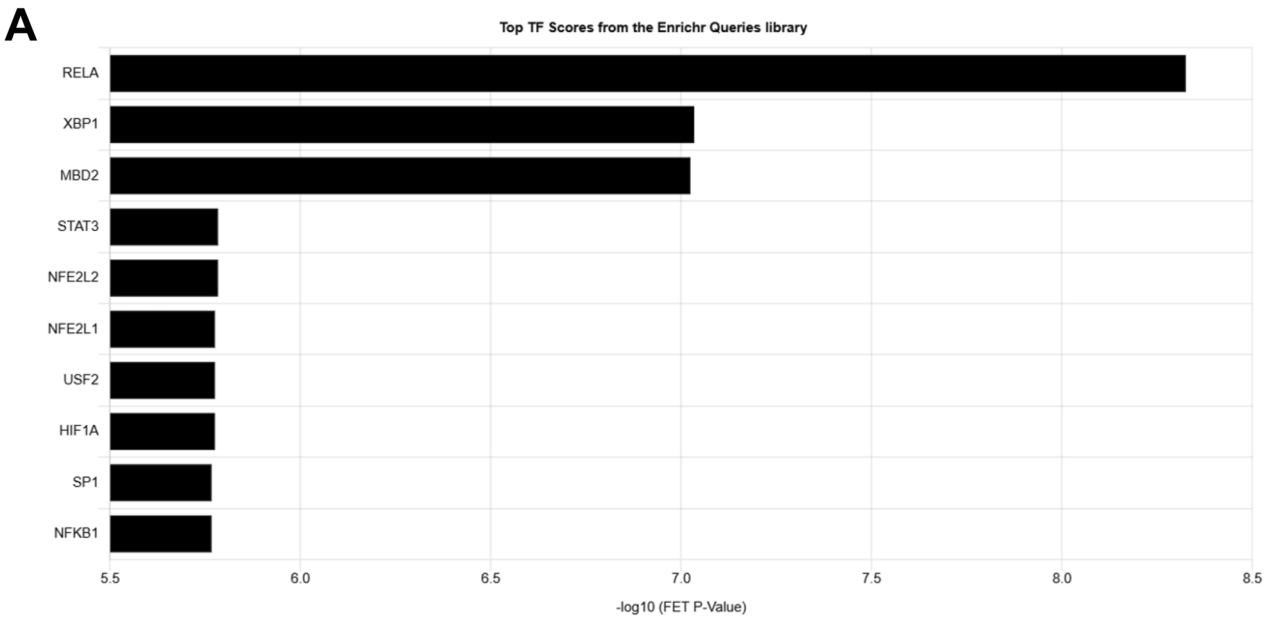


Fig. 3 The effect of TAPI-1 on the sensitivity of ESCC cells to cisplatin. **A** TE-1 and Eca109 cells were exposed to DMSO or TAPI-1 (5 μM) for 12 h. qRT-PCR quantitative analysis of ABCC10, ACTL6A, AKR1C2, ATP7A, CHD1L, CMTM6, EIF4E, FZD7, GSTP1, HMGN5, HOXB7, ITGA5, ITGB1, LAMP2, MAGED1, MCL1, NFE2L2, NGFR, NSD2, RAC1, RACK1, SFN, SLC2A1, SOD2, SRPX2, UBQLN4, VRK1, YAP1, YBX1, and ZFX expression at mRNA levels. **B** Venn diagram analysis of simultaneously downregulated genes in TE-1 and Eca109 cells in response to TAPI-1 treatment. **C** TE-1 and Eca109 cells were exposed to different concentrations of cisplatin (0, 5, 10, 15, 20, 25 μM) with DMSO or TAPI-1 (5 μM) for 48 h. CCK-8 analysis of cell viability and IC_{50} values of cisplatin. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, compared with the DMSO group. **D** TE-1 and Eca109 cells were exposed to cisplatin (10 μM) with DMSO or TAPI-1 (5 μM) for 48 h. Apoptosis analysis using flow cytometry and quantitative analysis of apoptotic cells. $^{*}P<0.05$ and $^{**}P<0.01$, compared with the DMSO + Cisplatin group

MCL1, NFE2L2, NGFR, NSD2, RAC1, RACK1, SFN, SLC2A1, SOD2, SRPX2, UBQLN4, VRK1, YAP1, YBX1) expressions were downregulated in TE-1 cells and 21 genes (ABCC10, AKR1C2, ATP7A, FZD7, GSTP1, ITGA5, ITGB1, LAMP2, MAGED1, MCL1, NFE2L2, RAC1, RACK1, SFN, SLC2A1, SOD2, SRPX2, UBQLN4, YAP1, YBX1, ZFX) expressions were downregulated in Eca109 cells at the mRNA levels in response to TAPI-1 treatment. In summary, 20 genes (ABCC10, AKR1C2, ATP7A, FZD7, GSTP1, ITGA5, ITGB1, LAMP2, MAGED1, MCL1, NFE2L2, RAC1, RACK1, SFN, SLC2A1, SOD2, SRPX2, UBQLN4, YAP1, YBX1) expressions were simultaneously downregulated in TE-1 and Eca109 cells at the mRNA levels after TAPI-1 treatment.

Next, TE-1 and Eca109 cells were exposed to TAPI-1 (5 μM) or solvent control, meanwhile exposed to different concentrations of cisplatin (0, 5, 10, 15, 20, 25 μM) for 48 h. CCK-8 assay revealed that TAPI-1 treatment increased the sensitivity of ESCC cells to cisplatin (Fig. 3C). The IC_{50} of cisplatin in TE-1 and Eca109 cells with TAPI-1 was significantly


A
Top TF Scores from the Enrichr Queries library
RELA
XBP1
MBD2
STAT3
NFE2L2
NFE2L1
USF2
HIF1A
SP1
NFKB1
5.5
6.0
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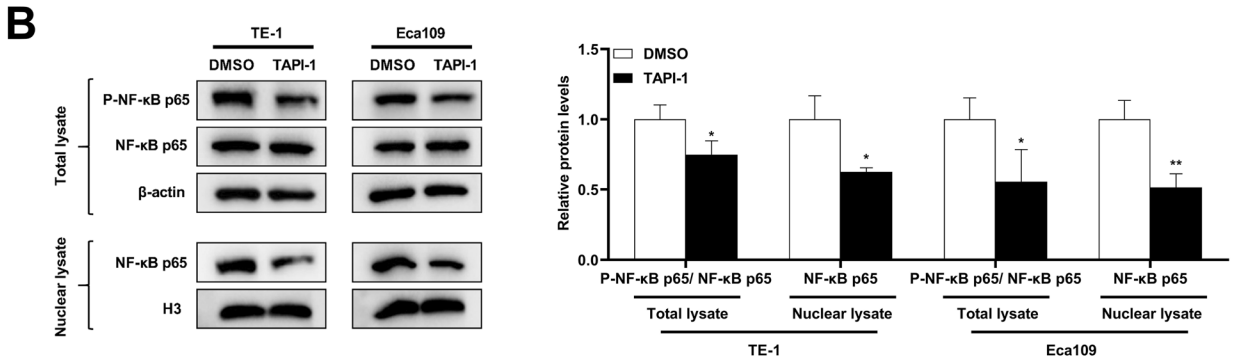
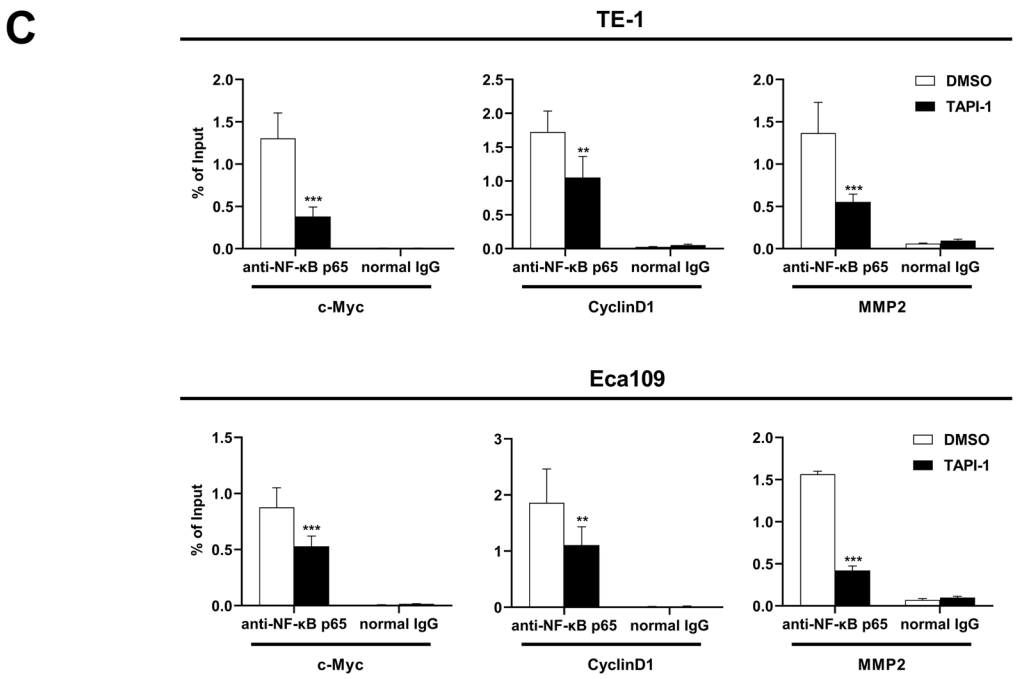
7.0
7.5
8.0
8.5
-log10 (FET P-Value)


B
TE-1
Eca109
DMSO
TAPI-1
DMSO
TAPI-1
Total lysate
P-NF-κB p65
NF-κB p65
β-actin
Nuclear lysate
NF-κB p65
H3
Relative protein levels
DMSO
TAPI-1
P-NF-κB p65/ NF-κB p65
NF-κB p65
P-NF-κB p65/ NF-κB p65
NF-κB p65
Total lysate
Nuclear lysate
Total lysate
Nuclear lysate
TE-1
Eca109


C
TE-1
% of Input
anti-NF-κB p65
normal IgG
c-Myc
CyclinD1
MMP2
DMSO
TAPI-1
Eca109
% of Input
anti-NF-κB p65
normal IgG
c-Myc
CyclinD1
MMP2
DMSO
TAPI-1

◂**Fig. 4** The effect of TAPI-1 on NF-κB signaling pathway in ESCC cells. **A** The predicted transcription factors in the upstream of cisplatin resistance-related genes. TE-1 and Eca109 cells were exposed to DMSO or TAPI-1 (10 μM) for 12 h. **B** Western blot analysis of P-NF-κB p65 and NF-κB p65 protein expression in total lysate and NF-κB p65 protein expression in nuclear lysate. **C** ChIP-qPCR quantitative analysis of the enrichment of NF-κB p65 at the indicated gene promoter regions. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the DMSO group

lower than that of the control cells (Fig. 3C). To further confirm the effect of TAPI-1 on cisplatin resistance in ESCC cells, TE-1 and Eca109 cells were exposed to TAPI-1 (5 μM) or solvent control, meanwhile exposed to cisplatin (10 μM) for 48 h, following which, flow cytometry assay was carried out. As expected, TAPI-1 (5 μM) significantly aggravated TE-1 and Eca109 cell apoptosis in response to cisplatin (10 μM) to varying degrees, respectively (Fig. 3D). These data indicated that TAPI-1 may be a promising adjuvant agent with cisplatin to therapy ESCC.

TAPI-1 Suppresses NF-κB Signaling Pathway in ESCC Cells

To investigate potential molecular mechanisms behind the anti-tumor efficacy of TAPI-1, we speculated the putative transcription factors in the upstream of cisplatin resistance-related genes using the ChEA3 website. Ten transcription factors with the highest scores from the Enrichr Queries library are listed in Fig. 4A. Thus, we hypothesized that RELA (also named NF-κB p65) signaling may be influenced by TAPI-1. To investigate the role of TAPI-1 in the activation of NF-κB signaling pathway in ESCC cells, TE-1 and Eca109 cells were exposed to TAPI-1 (10 μM) for 12 h, following which, Western blot and ChIP assays were carried out, respectively. As shown in Fig. 4B, TAPI-1 treatment remarkably suppressed the activation of NF-κB signaling, as indicated by the decreased phosphorylation and nuclear translocation of NF-κB p65 in TE-1 and Eca109 cells. Furthermore, ChIP was performed to assess the binding intensity of NF-κB p65 to the promoter regions of target genes. Tumor growth and metastasis-related genes c-Myc, CyclinD1, and MMP2 [17] were previously reported as target genes of NF-κB p65 and were chosen for ChIP-qPCR to evaluate the activation of NF-κB signaling in ESCC cells. ChIP-qPCR revealed that NF-κB p65 pulled fewer promoter segments of c-Myc, CyclinD1, and MMP2 in response to TAPI-1(10 μM) treatment in TE-1 and Eca109 cells, respectively (Fig. 4C).

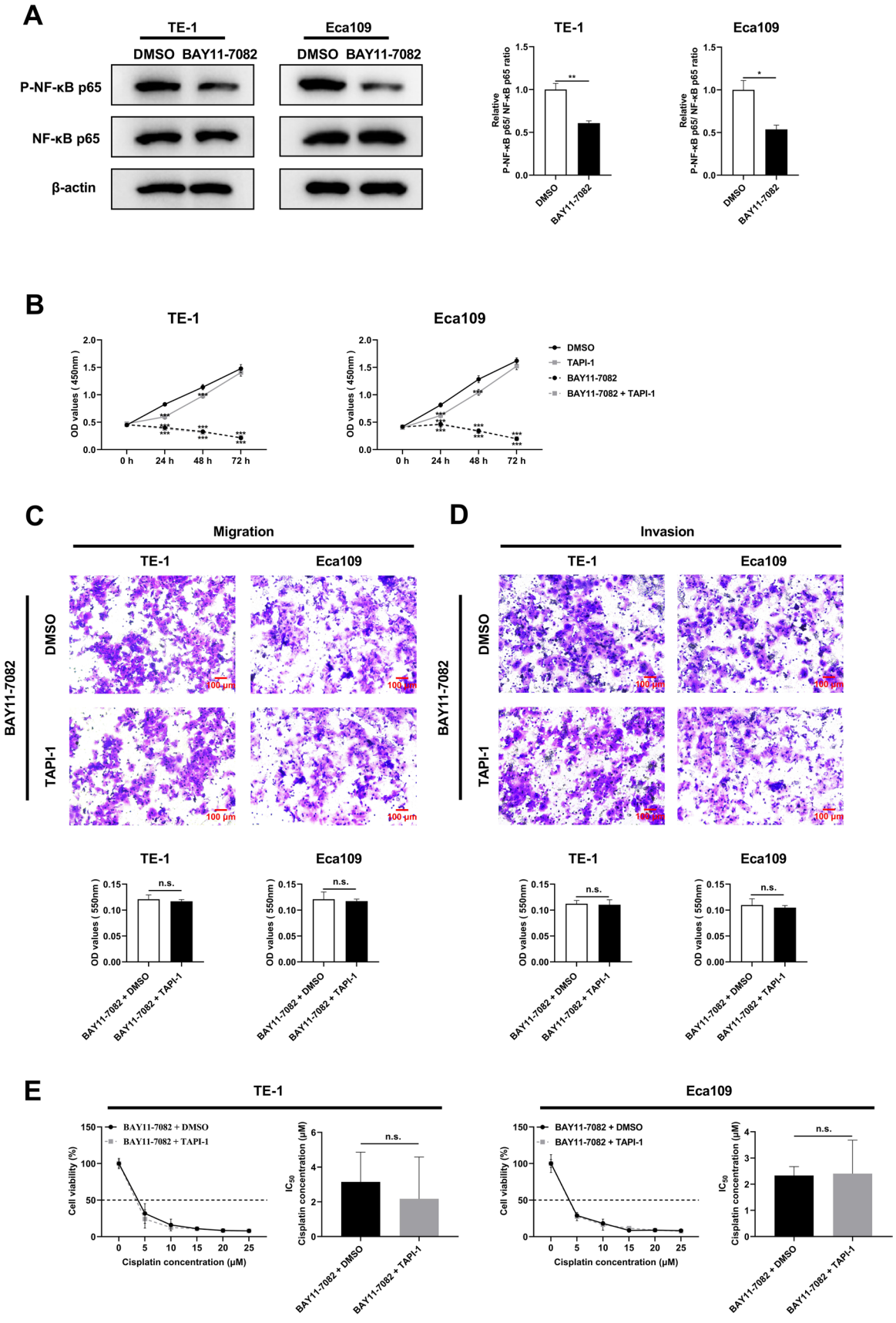
Suppression of NF-κB Signaling Abolishes Anti-tumor Effects of TAPI-1 on ESCC Cells

To further confirm the role of NF-κB signaling pathway in TAPI-1-mediated anti-tumor efficacy in ESCC cells, we blocked NF-κB signaling using BAY11-7082 (10 μM) and observed the decreased phosphorylation of NF-κB p65 in TE-1 and Eca109 cells, respectively (Fig. 5A). CCK-8 assay indicated that the treatment with BAY11-7082 alone or in combination with TAPI-1 could remarkably inhibit ESCC cell viability. However, following the treatment with BAY11-7082, we observed no obvious alteration in ESCC cell viability in the presence or absence of TAPI-1 (Fig. 5B). ESCC cells were pretreated with BAY11-7082 (10 μM) for 6 h, followed by exposure to TAPI-1 (5 μM) for another 24 h. Subsequently, migration and invasion assays were conducted and showed no change of migration and invasion in ESCC cells (Fig. 5C, D). Moreover, CCK-8 assay revealed that a pretreatment with BAY11-7082 (10 μM) could neutralize TAPI-1-enhanced the sensitivity of ESCC cells to different concentrations of cisplatin (0, 5, 10, 15, 20, 25 μM) (Fig. 5E). Taken together, BAY11-7082 abolished anti-tumor effects of TAPI-1 on ESCC cells, which indicated that TAPI-1 exhibited anti-tumor efficacy in ESCC cells in an anti-NF-κB manner.

Discussion

As a common malignant tumor of the digestive tract, ESCC makes about 0.15% population afflicted [18]. In spite of the use of advanced surgery combined with radiotherapy and various chemotherapeutic agents, patients with ESCC typically have a poor five-year survival rate [19], largely due to high recurrence rate, high metastatic rate, and chemotherapeutic drug resistance. Despite proved to display anti-tumor efficacy on several tumor deceases, the effect of TAPI-1 on digestive tract tumors remains largely unclear.

In the present study, we demonstrated that TAPI-1 treatment could significantly inhibit ESCC cell viability, migration, invasion, and cisplatin resistance. Furthermore, RELA was determined as an indirect molecular target of TAPI-1 that conferred cisplatin resistance in ESCC. RELA, also named NF-κB p65, known as a critical transcription factor relevant to inflammation and cancer, has been extensively studied in human cancers over recent years. Several studies revealed that NF-κB signaling is constitutively activated in ESCC cells [20–22], thus facilitating ESCC tumor growth, metastasis and cisplatin resistance [23]. Silencing NF-κB p65 using small interfering RNA effectively alleviated ESCC, implying that NF-κB may be a potential therapeutic target [21, 22]. Several NF-κB inhibitors have been applied for cancer research, such as BAY11-7082. Unfortunately, BAY11-7082 showed toxicity in human normal esophageal epithelial cells [24]. Nevertheless, no significant changes in the cell viability of Het-1A cells were observed in response to varying doses of TAPI-1 (0, 1.25, 2.5, 5, 10, and 20 μM), implying that TAPI-1 may be a desirable anti-cancer drug


A
TE-1
Eca109
DMSO BAY11-7082
DMSO BAY11-7082
P-NF-κB p65
NF-κB p65
β-actin
TE-1
Eca109
Relative P-NF-κB p65/ NF-κB p65 ratio
DMSO
BAY11-7082
B
TE-1
Eca109
OD values (450nm)
0 h
24 h
48 h
72 h
DMSO
TAPI-1
BAY11-7082
BAY11-7082 + TAPI-1
C
Migration
TE-1
Eca109
BAY11-7082
DMSO
TAPI-1
100 μm
OD values (550nm)
n.s.
BAY11-7082 + DMSO
BAY11-7082 + TAPI-1
D
Invasion
E
TE-1
Eca109
BAY11-7082 + DMSO
BAY11-7082 + TAPI-1
Cell viability (%)
Cisplatin concentration (μM)
IC50
Cisplatin concentration (μM)
n.s.

◂**Fig. 5** Pretreatment of BAY11-7082 abolishes the effects of TAPI-1 on ESCC cell viability, migration, invasion, and cisplatin resistance. **A** TE-1 and Eca109 cells were exposed to DMSO or BAY11-7082 (10 μM) for 6 h. Western blot analysis of P-NF-κB p65 and NF-κB p65 protein expression in total lysate. **B** TE-1 and Eca109 cells were treated with DMSO, BAY11-7082 (10 μM), TAPI-1 (10 μM), or BAY11-7082 (10 μM) in combination with TAPI-1 (10 μM) for 0, 24, 48, and 72 h, respectively. CCK-8 analysis of cell viability. TE-1 and Eca109 cells were pretreated with DMSO or BAY11-7082 (10 μM) for 6 h, followed by an exposure to DMSO or TAPI-1 (5 μM) for another 24 h. Representative images and absorbance analysis results of Transwell migration assay (**C**) and invasion assay (**D**). **E** TE-1 and Eca109 cells were pretreated with DMSO or BAY11-7082 (10 μM) for 6 h, followed by an exposure to cisplatin (10 μM) with DMSO or TAPI-1 (5 μM) for another 48 h. $*P<0.05$ and $**P<0.01$, compared with the DMSO group

with low toxicity. Interestingly, similar to our data, several studies showed that TAPI-1 could attenuate Sjogren's syndrome, cardiac hypertrophy, and pancreatic cancer via suppression of NF-κB signaling pathway [11, 25, 26].

Despite these data, the molecular mechanism for signal transduction from TACE or MMPs to NF-κB remains enigmatic in ESCC. Previous studies have documented that EGFR and VEGFR2, two typical substrates of TACE, contributed to ESCC progression [27, 28]. Moreover, EGFR and VEGFR2 were proved to activate NF-κB signaling in other disease models [29, 30]. However, the roles of EGFR and VEGFR2 in TACE-activated NF-κB signaling in ESCC remain to be elucidated. Intriguingly, we observed that the molecular targets of TAPI-1, including TACE and MMPs, were regarded as the target genes of NF-κB p65 in ESCC cells [17, 31]. Additionally, our previous data revealed that TACE and NF-κB signaling formed a feedback loop to exacerbate ESCC progression [17]. The interrelationships between MMPs and NF-κB signaling in ESCC await future investigation.

Conclusion

Taken together, in this study, for the first time, we demonstrated that TAPI-1 significantly inhibited ESCC cell viability, migration, and invasion and efficiently enhanced the sensitivity of ESCC cells to cisplatin via suppression of NF-κB signaling pathway (Fig. 6).

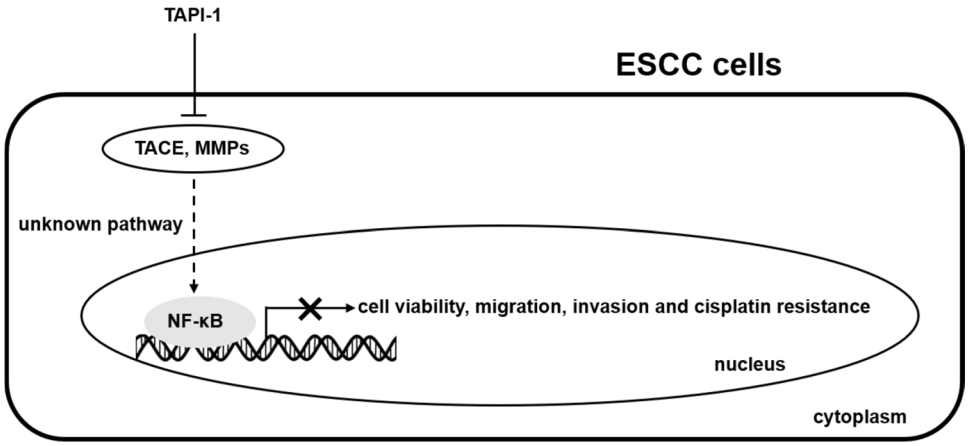


Fig. 6 Schematic diagram of TAPI-1-mediated anti-tumor efficacy in ESCC cells via suppression of NF-κB signaling pathway. The activation of NF-κB signaling is suppressed in response to TAPI-1 treatment via an unknown pathway, leading to the decrease of ESCC cell viability, migration, and invasion and the enhanced sensitivity of ESCC cells to cisplatin

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Author's contribution HL and JQ designed the study and wrote the paper. LG and LL performed the research. JQ analyzed data. DZ revised the paper. All co-authors have reviewed and approved the article before submission.

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Declarations

Conflict of interest There are no relevant financial or non-financial competing interests to report.

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