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TAK1 inhibitor 5Z-7-oxozeaenol sensitizes neuroblastoma to chemotherapy

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Abstract

Treatment failure in high risk neuroblastoma is largely due to development of chemoresistance. NF- κ B activation is one of the resistance mechanisms for cancer cells to escape from chemotherapy-induced cell-death. TAK1 is an essential component in genotoxic stresses-induced NF- κ B activation; however, the role of TAK1 in the development of chemoresistance in neuroblastoma remains unknown. Using a panel of neuroblastoma cell lines, we found that TAK1 inhibitor 5Z-7-oxozeaenol significantly augmented the cytotoxic effects of doxorubicin (Dox) and etoposide (VP-16) on neuroblastoma cell lines. TAK1 inhibition also enhanced the inhibitory effect of Dox and VP-16 on anchorage-independent growth. Treatment of neuroblastoma cells with 5Z-7-oxozeaenol blocked Dox- and VP16-induced NF- κ B activation and enhanced Dox- and VP16-induced apoptosis. Moreover, 5Z-7-oxozeaenol was able to overcome the established chemoresistance in LA-N-6 neuroblastoma cells. Using an orthotopic neuroblastoma mouse model, we found that 5Z-7-oxozeaenol significantly enhanced chemotherapeutic efficacy in vivo. Together, our results provide a proof-of-concept that TAK1 inhibition significantly increases the sensitivity of neuroblastoma cells to chemotherapy-induced cell-death and can serve as an effective adjunct to current chemotherapeutic regimens for high risk diseases.

Keywords

Neuroblastoma; TAK1 inhibitor; 5Z-7-oxozeaenol; Chemotherapy

Introduction

Neuroblastoma is the most common extracranial solid tumor in children and accounts for 8–10 % of all childhood tumors [1]. The new International Neuroblastoma Risk Group (INRG) classification system divides this disease into four broad categories: very low risk, low risk, intermediate risk and high risk [2]. The clinical hallmark of neuroblastoma is heterogeneity which resulted in a dichotomization in therapeutic strategies [3]. Tumors with favorable biologic features (very low risk, low risk, intermediate risk) are less aggressive and nonmetastatic. These can be treated with surgical resection and shorter courses of chemotherapy. However, the treatment for high risk tumor has shifted over the past two decades towards myeloablative chemoradiotherapy requiring stem-cell transplantation to rescue the immune system [3]. Despite dramatic escalations in the intensity of therapy

provided for the high risk group, the cure rate has shown only modest improvement with 40–50 % of event-free survival (50–60 % of patients with relapse) and 40 % of overall survival at 5 years [4]. Since most high risk neuroblastoma show an initial response to therapy but ultimately relapse, it is reasonable to conclude that acquired drug resistance or selection of therapy-resistant cells prevents current treatment from becoming a cure. Therefore, understanding the molecular mechanism that mediates resistance to chemotherapy is imperative for curing this disease.

The nuclear factor- κ B (NF- κ B) transcription factors are the central mediators of the inflammatory process and key participants in innate and adaptive immune responses [5–7]. Aside from having a critical role in immunity, NF- κ B activation is frequently encountered in tumor cells and is believed to be one of the resistance mechanisms that contribute to cancer development and chemotherapeutic resistance [8–10]. Now, it is recognized that most if not all chemotherapeutic agents and radiation therapies can activate NF- κ B in vitro and in vivo. The DNA-intercalating agent doxorubicin (Dox) and topoisomerase II inhibitor etoposide (VP16) are common chemotherapeutic drugs used in treatment protocols for high risk neuroblastoma [11]. Dox and VP16 enter into cells and cause double-strand breaks (DSBs) that are recognized by the Mre11-Rad50-Nbs1 (MRN) complex which transiently recruits the ataxia telangiectasia mutated kinase (ATM) [12, 13]. These DSBs are also detected by the poly-(ADP)-ribosylating enzyme poly (ADP-ribose) polymerase 1 (PARP-1) resulting in PARP-1 activation [14–16]. Both PARP-1 and ATM are required for NF- κ B activation in response to genotoxic stress [17, 18]. However the signal transduction pathway from nuclear genotoxic stresses to cytosolic I κ B kinases (IKKs) complexes remains largely unknown.

TAK1 is a member of the mitogen-activated protein kinase (MAPK) kinase family and was initially found to function in transforming growth factor- β (TGF- β)- and bone morphogenetic protein (BMP)- mediated MAPK activation [19]. TAK1 is a pivotal intermediate for IKK activation in response to multiple stimuli and plays an essential role in innate and adaptive immune responses [20–23]. Recently, we and several other groups have reported that TAK1 mediates NF- κ B activation in response to genotoxic stresses [24–28]. The balance between pro-apoptotic and pro-survival pathways determines the efficacy of the chemotherapy. Given that NF- κ B activation is one of the major survival signals, we reasoned that inhibition of TAK1 activation could disrupt the balance between cell-death and cell-survival, and sensitize cells to chemotherapy treatments.

Recently, TAK1 inhibition mediated by RNAi-silencing or an orally active TAK1 inhibitor (LYTAK1) has shown to significantly inhibit NF- κ B activity and sensitize pancreatic cancer cells to gemcitabine-induced cell-death both in vitro and in vivo [29]. Additionally, RNAi screening also identified TAK1 as a potential target for enhanced efficacy of topoisomerase inhibitors in breast cancer [30]. Furthermore, TAK1 plays a critical role in non small cell lung carcinoma (NSCLC) growth and KRAS-dependent colon cancer survival [31, 32]. Sustained TAK1 activation in tumor-associated macrophages promotes NSCLC growth and TAK1 inhibition promotes apoptosis in KRAS-dependent colon cancer [31, 32]. By using a panel of neuroblastoma cell lines including a chemoresistant neuroblastoma cell line, we show that TAK1 inhibition significantly enhances the sensitivity of neuroblastoma cells to chemotherapy treatments.

Materials and methods

Cell lines and cell culture

Neuroblastoma cell lines IMR-32, NGP, NB-19, CHLA-255, SK-N-AS, SH-SY5Y cells were grown in RPMI1640 (Cellgro) containing 10 % fetal bovine serum (FBS, SAFC Biosciences), 100 units/mL penicillin, and 100 μ g/mL streptomycin. LA-N-6 cells were

grown in RPMI1640 containing 20 % FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. All cells were grown at 37 °C in 5 % CO₂. NB-19 was kindly provided by Dr. A. Davidoff (St. Jude's Children's Hospital) and LA-N-6 was kindly provided by Dr. R. Seeger (Children's Hospital of Los Angeles).

Antibodies and reagents

Anti-phospho-IKK / (2078S), anti-IKK (2684S), anti-phospho-I B (9246L), anti-I B (9242L), anti-phospho-JNK (9251L), anti-JNK (9252L), anti-phospho-p38 (9211L), anti-p38 (9212L), anti-PARP (9532S), anti-Caspase3 (9662S), anti-Mouse (7076S) and anti-Rabbit (7074S) secondary antibodies were purchased from Cell Signaling Technology. Anti-Actin (A2228) antibody was obtained from Sigma. Doxorubicin (Dox, D1515) and etoposide (VP16, E1383) were obtained from Sigma. 5Z-7-oxozeaenol (499610) was purchased from Calbiochem.

Western blotting assay

Cell lysates were obtained by washing the cells twice with ice cold PBS and then lysing cells with RIPA lysis buffer (25 mM HEPES at pH 7.7, 135 mM NaCl, 1 % Triton X-100, 25 mM D-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM Benzamidine, 20 mM disodium D-nitrophenylphosphate, and phosphatase inhibitor cocktail 2 and 3 (p5726 and p0044, Sigma). After centrifuging at 13,000×g for 15 min at 4 °C, supernatants were collected, resolved by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The membranes were then incubated with corresponding primary antibodies overnight at 4 °C and horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit for 1 h at RT (25 °C). The membranes were then visualized by the ECL-Plus Western detection system (GE Health Care, Buckinghamshire, UK).

CCK-8 cell viability assay

The experiment was performed as previously described [33]. Briefly, cell lines were plated into 96-well plates at a concentration of 1×10^4 cells per well. After incubating the plate for 24 h at 37 °C, the cells were treated with various concentrations of Dox, VP16, 5Z-7-oxozeaenol or their combination for a duration indicated. Relative cell viability was quantified by adding 10 µL of Cell Counting Kit-8 (Dojindo Laboratories) solution, incubating for 1 h at 37 °C, and measuring the absorbance at 450 nm.

Soft agar assay

The experiment was performed as previously described [33]. Briefly, a 5 % solution of agar (214220, Difco Laboratories) was made and autoclaved. This was then allowed to cool to 56 °C in a water bath. A 0.5 % mixture of agar and RPMI1640 containing 10 % FBS was plated into 6-well plates (2 mL per well). After this layer solidified, a 0.3 % of agar solution in RPMI1640 media with 10 % FBS was made and mixed with each cell line at a concentration of 1×10^4 cells per well (2 mL of volume). After letting cells grow at 37 °C in 5 % CO₂ for 2–3 weeks, cells were stained with Thiazolyl Blue Tetrazolium Bromide (MTS, Sigma) per well for 24 h. The wells were then photographed and colonies counted.

Propidium iodide (PI) staining assay

After treating cells with Dox and 5Z-7-oxozeaenol for appropriate period, cells were washed with ice cold PBS twice, harvested and centrifuged at 400 × g for 5 min at 4 °C. The supernatant was aspirated, and the pellets were resuspended at 1×10^6 cells/mL in 1× binding buffer (51-66121E, BD Biosciences). Then 100 µL of cell suspension was transferred into a new tube, 5 µL of propidium iodide (PI) staining solution (51-66211E, BD

Biosciences) was added into each tube, then tubes were covered and incubated for 15 min at RT. After adding 400 μ L of $1 \times$ binding buffer into each tube, the samples were analyzed by flow cytometry within 1 h. Unstained cells were used as a control.

In vivo antitumor efficacy study in orthotopic neuroblastoma mouse model

The orthotopic neuroblastoma mouse model was performed as previously described [34]. Briefly, human luciferase-transduced SH-SY5Y cells were trypsinized and resuspended at 1×10^7 cells per mL in PBS. One hundred microliter of the cell suspension were surgically injected into the left kidney of five week old female nude mice. All mice were housed in a pathogen-free environment and handled in strict accordance with the approved animal protocol. Three weeks after injection, tumor was measured by bioluminescence imaging and a total of 32 mice bearing tumors were randomized into four groups (eight mice in each group): vehicle (distilled water and DMSO), Dox only, 5Z-7-oxozeaenol only, and combination of Dox and 5Z-7-oxozeaenol. Treatments were administered by intraperitoneal (IP) injection as follows: 1 mg/kg Dox and 15 mg/kg 5Z-7-oxozeaenol four times weekly for 2 consecutive weeks. All mice were sacrificed and tumors were weighted at the end point of treatment.

Statistical analysis

Statistical significance in drug-treated versus control groups in vitro was determined by using the Student's t test (two-tailed) and in orthotopic neuroblastoma mouse models was determined by using the Student's t test (two-tailed). All values are expressed as the mean $\pm$ SD. A *P* value of less than 0.05 was considered statistically significant.

Results

TAK1 inhibition significantly enhances the cytotoxic effect of Dox and VP-16 on neuroblastoma cells

Since TAK1 is required for genotoxic stresses-induced NF- κ B activation, we reasoned that pharmacological inhibition of TAK1 activity would block this pathway and cause increased chemosensitivity. In order to test our hypothesis, IMR-32 and SH-SY5Y cells were treated with Dox along with or without TAK1 inhibition. Co-culture of TAK1 inhibitor 5Z-7-oxozeaenol significantly enhanced the cytotoxic effect of Dox on IMR-32 and SH-SY5Y cells (Fig. 1a, c, e). It also significantly enhanced the cytotoxic effect of VP-16 on IMR-32 and SH-SY5Y cells (Fig. 1b, d). This phenomenon is not dependent on MYCN amplification status, because both MYCN amplified (IMR-32) and non-amplified (SH-SY5Y) neuroblastoma cells showed similar results (Fig. 1). To further confirm that our finding is not limited by cell types, we used other two MYCN amplified (NGP and NB-19) and two MYCN non-amplified (CHLA-255 and SK-N-AS) neuroblastoma cell lines and similar results were observed in all cell lines tested (Supplementary Fig. S1). However, TAK1 inhibition alone only had cytotoxic effects on SK-N-AS and IMR-32 cells at a relatively high dose (10 μ M) (Supplementary Fig. S2).

TAK1 inhibition enhances the inhibitory effect of Dox and VP-16 on neuroblastoma colony formation

To examine the effect of TAK1 inhibition on anchorage-independent growth, a soft agar assay was performed with IMR-32 and SH-SY5Y cells. Although TAK1 inhibition alone had no significant effect on cell proliferation in culture, 5Z-7-oxozeaenol significantly inhibited anchorage-independent colony formation of IMR-32 and SH-SY5Y cells in a dose-dependent manner (Supplementary Fig. S3). To further examine the combinatory effect of TAK1 inhibition and chemotherapeutic drugs, we used a low dose of 5Z-7-oxozeaenol (0.5

μM) which could not significantly inhibit anchorage-independent cell growth. Combining a low dose of 5Z-7-oxozeaenol with Dox significantly enhanced the inhibitory effect of Dox on anchorage-independent growth of IMR-32 and SH-SY5Y cells (Fig. 2a, c). A similar effect was seen when combining this low dose of 5Z-7-oxozeaenol with VP-16 (Fig. 2b, d). Together, TAK1 inhibition by 5Z-7-oxozeaenol significantly enhanced the inhibitory effect of Dox and VP-16 on anchorage-independent neuroblastoma cell growth.

TAK1 inhibition inhibits Dox- and VP-16- induced NF-κB, JNK and p38 activation

TAK1 is required for genotoxic stress-induced NF-κB, JNK and p38 activation. Because NF-κB activation is critical for cell survival, we reasoned that the combinatory effect of 5Z-7-oxozeaenol with Dox or VP16 might be caused by inhibition of NF-κB activation. To test this hypothesis, we examined whether TAK1 inhibition could block chemotherapy-induced NF-κB activation in SH-SY5Y and IMR-32 cells. As shown in Fig. 3a, b, TAK1 inhibition by 5Z-7-oxozeaenol completely blocked Dox-induced IKK phosphorylation (Fig. 3a, b). 5Z-7-oxozeaenol treatment also completely blocked Dox-induced JNK and p38 phosphorylation (Fig. 3a, b). Furthermore, VP-16-induced IKK, JNK and p38 phosphorylation were also completely blocked by 5Z-7-oxozeaenol (Fig. 3c, d). We did not observe basal TAK1 activation in seven NB cell lines that we tested, including IMR-32, NGP, NB-19, CHLA-255, SK-N-AS, SH-SY5Y and LA-N-6 (data not shown). These data indicate that TAK1 is not constitutively activated and TAK1 is required for Dox and VP16-induced NF-κB, JNK and p38 activation in neuroblastoma cells.

TAK1 inhibition enhances Dox- and VP-16- induced apoptosis

As NF-κB activation upregulates anti-apoptotic molecules that inhibit cell-death, we reasoned that 5Z-7-oxozeaenol-mediated inhibition of Dox- and VP-16-induced NF-κB activation might promote apoptosis via blocking NF-κB activation. To test this hypothesis, we examined cleavage of Caspase 3 and PARP after treating the cells with Dox. Combinatory treatment of Dox and 5Z-7-oxozeaenol significantly increased the cleavages of Caspase 3 and PARP in the cells compared to Dox treatment alone (Fig. 3e). Furthermore, TAK1 inhibition also increased VP-16-induced Caspase 3 and PARP cleavages in cells (Fig. 3f). Congruent with these results, flow cytometry analysis also showed that the percentage of PI (propidium iodide)-staining positive cells dramatically increased after treatment with Dox and 5Z-7-oxozeaenol compared to Dox treatment alone (Fig. 3g). Together, these results indicate that TAK1 inhibitor 5Z-7-oxozeaenol greatly potentiates efficacy of chemotherapeutic agents via the inhibition of NF-κB activation and subsequent promotion of apoptosis.

TAK1 inhibition overcomes the chemoresistance of LA-N-6 cells

Since we found that TAK1 inhibitor dramatically enhanced efficacy of chemotherapeutic agents in several neuroblastoma cell lines, we reasoned that TAK1 inhibition could overcome the chemoresistance of neuroblastoma cells. To test this hypothesis, we examined the effect of TAK1 inhibition on a chemoresistant neuroblastoma cell line LA-N-6. Congruent with previous reports, LA-N-6 cells are resistant to a much higher dose of Dox compared to other neuroblastoma cells tested (Supplementary Fig. S4). However, co-culture LA-N-6 cells with TAK1 inhibitor 5Z-7-oxozeaenol significantly increased the sensitivity of LA-N-6 cells to Dox and VP-16 (Fig. 4a, b). One μM of Dox or 5 μM of VP-16 caused more than 60 % of LA-N-6 cells to die when co-cultured with TAK1 inhibitor 5Z-7-oxozeaenol (Fig. 4a, b). 5Z-7-oxozeaenol blocked Dox-induced IKK, JNK and p38 phosphorylation in LA-N-6 cells (Fig. 4c). Moreover, TAK1 inhibition significantly increased Dox-induced Caspase 3 and PARP cleavages and the percentage of PI-staining positive cells (Fig. 4d, e). Taken together, these results indicate that TAK1 inhibitor 5Z-7-oxozeaenol can significantly enhance chemotherapeutic efficacy and overcome chemoresistance of neuroblastoma cells.

5Z-7-oxozeaenol enhances the chemotherapeutic efficacy of Dox in vivo

To determine whether TAK1 inhibition enhances chemotherapeutic efficacy in vivo, we established an orthotopic neuroblastoma mouse model by surgical injection of luciferase-transduced SH-SY5Y cells into the left kidney of nude mice. Three weeks after the injection of neuroblastoma cells, tumor establishments were detected by bioluminescence imaging, and mice bearing tumors were randomly divided into four groups and treated as indicated in Fig. 5a. 5Z-7-oxozeaenol administered alone (15 mg/kg) and low dose of Dox administered alone (1 mg/kg) slightly but not significantly inhibited neuroblastoma growth in vivo in our treatment strategy. However, combination of 5Z-7-oxozeaenol with Dox significantly inhibited tumor growth as evidenced by the dramatically decreased tumor weights compared with control and single agent treatment groups by the end point of our treatment strategy (Fig. 5b, c).

Discussion

Neuroblastoma is a highly lethal pediatric solid tumor in which transient chemotherapeutic responses are typically followed by recurrence with poorly-responding chemoresistant disease [3]. Understanding the molecular mechanisms that mediate chemoresistance of recurrent disease and overcoming this chemoresistance are critically important for curing patients who present high risk neuroblastoma. Given the pivotal role of TAK1 in chemotherapy-induced NF- κ B activation, we hypothesized that inhibition of TAK1 activation will block chemotherapy-induced NF- κ B activation and potentiate the efficacy of conventional chemotherapeutic agents (Fig. 5d). Herein, using a panel of neuroblastoma cell lines, we provide convincing evidence that synthetic inhibition of TAK1 activity greatly enhances Dox- and VP-16-induced cytotoxicity in neuroblastoma cells both in vitro and in vivo. Furthermore, using a chemoresistant neuroblastoma cell line LA-N-6, we showed that TAK1 inhibition could overcome established chemoresistance in neuroblastoma. NF- κ B has been shown to be one of master transcriptional factors that promote cell-survival and its activation contributes to chemoresistance [8, 9]. It has been reported that NF- κ B inhibition enhances the cytotoxic effect of topotecan in neuroblastoma in a synthetic lethal screen [35]. In our studies, 5Z-7-oxozeaenol inhibited Dox- and VP-16-induced NF- κ B activation and increased the cytotoxic effect of Dox- and VP-16 in neuroblastoma. Therefore, inhibition of TAK1-mediated NF- κ B activation is very likely to be one of the major underlying mechanisms for the action of 5Z-7-oxozeaenol. However, due to the fact that 5Z-7-oxozeaenol also inhibits Dox- and VP-16-induced JNK and p38 activation, it is likely that inhibition of TAK1-mediated MAPK activation also contributes to the effect of 5Z-7-oxozeaenol on neuroblastoma cells. Taken together, we conclude that pharmacological inhibition of TAK1 activity would block genotoxic stress-induced TAK1-signaling and tilt the balance between cell-death and cell-survival in favor of cell-death in neuroblastoma cells (Fig. 5d). Integration of TAK1 inhibitor with current chemotherapy is ready to be tested in more clinically relevant experiments.

Approximately 50 % of neuroblastoma patients are in the high risk category, with survival rates less than 40 % despite intensive multimodal therapy [4]. Chemotherapy resistance at relapse contributes heavily to treatment failure. Therefore, the ability to overcome established chemoresistance is an urgent need for patients with relapsing high risk neuroblastoma. In this investigation, we found that TAK1 inhibitor 5Z-7-oxozeaenol could overcome the established chemoresistance in a chemoresistant neuroblastoma cell line LA-N-6 through blocking Dox-induced NF- κ B activation. Our results suggest that combination of TAK1 inhibitor 5Z-7-oxozeaenol with chemotherapeutic drugs might be a potential therapeutic option for the treatment of relapsed neuroblastoma patients.

Another interesting observation in this study is that the TAK1 inhibitor 5Z-7-oxozeaenol alone could significantly inhibit colony formation in soft agar assay, although it did not significantly inhibit cell proliferation in cultures, even at high-doses. Anchorage-independent growth is a hallmark of transformed cells and 5Z-7-oxozeaenol is a specific TAK1 catalytic activity inhibitor by irreversibly interacting within the ATP binding site of TAK1 [36, 37]. Our results suggest that TAK1 kinase activity is required for anchorage-independent growth of neuroblastoma cells. It is very likely that a TAK1 kinase activity-dependent signal transduction pathway exists in suspending neuroblastoma cells to maintain its anchorage-independent growth.

Although TAK1 inhibitor 5Z-7-oxozeaenol promotes chemotherapy-induced cell-death in all neuroblastoma cell lines tested, its efficacy in each cell line is quite different. Therefore, systematically understanding the network of TAK1-dependent survival pathways in response to chemotherapy will provide valuable information for potential clinical application of TAK1 inhibitors like 5Z-7-oxozeaenol.

In summary, by using a panel of seven neuroblastoma cell lines and an in vivo model of neuroblastoma, we provide a proof-of-concept that combination of TAK1 inhibitor with chemotherapeutic agents will significantly enhance chemotherapeutic efficacy in neuroblastoma. Because the requirement of TAK1 kinase activity in Dox-induced NF- κ B activation is not limited by cell types, TAK1 inhibition may greatly enhance chemotherapeutic efficacy not only in neuroblastoma but also in other cancer types. Therefore, drug development of TAK1 small molecule inhibitors that can be used in clinics is urgently needed for the treatment of patients with neuroblastoma as well as other cancer types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

5Z-7-oxozeaenol enhances the cytotoxic effect of Dox and VP-16 on neuroblastoma cells. **a**, **b** IMR-32 and **c**, **d** SH-SY5Y cells were seeded into 96-well plates at a concentration of 1×10^4 per well. After 24 h, cells were incubated with drugs for 24 h at indicated concentrations and cell viability was assessed by CCK-8 assay. Results presented as % vehicle $\pm$ SD ($n = 6$). $**P < 0.01$, $***P < 0.001$ (Student's *t* test, two-tailed). **e** IMR-32 and SH-SY5Y cells were seeded in 6-well plates at a concentration of 3×10^5 /well. After 24 h, cells were incubated with drugs for 16 h at indicated concentrations and cell morphology was captured by light microscopy

Fig. 2. 5Z-7-oxozeaenol enhances the inhibitory effect of Dox and VP-16 on anchorage-independent growth of IMR-32 and SH-SY5Y cells. IMR-32 cells (**a, b**) and SH-SY5Y (**c, d**) were plated in 0.3 % agarose/RPMI-1640 media containing the indicated drugs with indicated concentration on top of a 0.5 % agarose/RPMI-1640 layer. After 14 days, colonies were stained with MTT and counted. These experiments were performed in sextuplicate and reported as mean with SDs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test, two-tailed)

Fig. 3.

5Z-7-oxozeaenol inhibits Dox- and VP-16- induced NF- κ B, JNK and p38 activation and promotes apoptosis. **a** SH-SY5Y and **b** IMR-32 cells were treated with Dox at the indicated time points with or without 5Z-7-oxozeaenol, protein extracts were subjected to SDS-PAGE and immunoblotted with antibodies indicated. **c** SH-SY5Y and **d** IMR-32 cells were treated with VP-16 at the indicated time points with or without 5Z-7-oxozeaenol, protein extracts were subjected to SDS-PAGE and immunoblotted with antibodies indicated. **e**, **f** SH-SY5Y cells were treated with Dox (**e**) or VP-16 (**f**) at the indicated time points with or without 5Z-7-oxozeaenol, protein extracts were subjected to SDS-PAGE and immunoblotted with antibodies against cleaved PARP and caspase 3 to detect the apoptotic cells. β -Actin was detected as a loading control for whole cell extracts. **g** SH-SY5Y cells were treated with 5Z-7-oxozeaenol (1 μ M) or Dox (1 μ g/mL) or their combination for 24 h and examined by flow cytometry using propidium iodide (PI) staining to label apoptotic cells

Fig. 4.

TAK1 inhibition overcomes the chemoresistance of LA-N-6 cells. **a, b** LA-N-6 neuroblastoma cells were seeded in 96-well plates at a concentration of 1×10^4 /per well. After 24 h, cells were incubated with drugs for 24 h at indicated concentrations and cell viability was assessed by CCK-8 assay. **c** LA-N-6 cells were treated with Dox at the indicated time points with or without 5Z-7-oxozeaenol, protein extracts were subjected to SDS-PAGE and immunoblotted with antibodies indicated. -Actin was detected as a loading control for whole cell extracts. **d** LA-N-6 cells were treated with Dox at the indicated time points with or without 5Z-7-oxozeaenol, protein extracts were subjected to SDS-PAGE and immunoblotted with antibodies against cleaved PARP and caspase 3 to detect the apoptotic cells. -Actin was detected as a loading control for whole cell extracts. **e** LA-N-6 cells were treated with 5Z-7-oxozeaenol (2 μ M) or Dox (10 μ g/ml) or their combination for 24 h and examined by flow cytometry using PI staining to label apoptotic cells

Fig. 5. 5Z-7-oxozeaenol enhances the inhibitory effect of Dox on neuroblastoma tumor growth in vivo. **a** Treatment strategy used in this study. **b** Representative pictures of three biggest orthotopic tumors in each group. **c** Tumor weights from the last day of treatment. Data was from one experiment with 8 mice per group. ** $P < 0.01$, *** $P < 0.001$, (Student's t test, two-tailed). **d** Working model of pharmacological TAK1 inhibition in neuroblastoma. TAK1 inhibition blocks genotoxic stresses-induced TAK1 signaling in neuroblastoma cells and tilts the balance between cell-death and cell-survival in favor of cell-death in neuroblastoma cells