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Anti-Cancer treatments affecting PI3K/Akt/Mtor and Ras/MAPK pathways in neuroblastoma

Nöroblastomda PI3K/Akt/Mtor ve Ras/MAPK yolaklarını etkileyen anti kanser tedaviler

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Proteins in PI3K/Akt and Ras/MAPK Signaling in Neuroblastoma
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BACKGROUND
In this study, we applied combination therapy of RA and Tac to NB cells with different molecular properties and aimed to evaluate its effects on proliferation, differentiation, and apoptotic pathways in NB.

METHODS
Four cell lines of different characteristics; (KELLY, LAN-5, CHP-134, and SHSY5Y) were cultured and treated with various doses of RA and Tac. The IC50 values were determined by through WST analysis. The IC50 of the RA+Tac combination was applied to the cells. To determine the apoptosis/necrosis rate, the cells were dyed with Annexin V/PI. To examine the protein levels of certain pathways, Western Blot and IHC were performed.

RESULTS
The RA and RA+Tac treatments demonstrated beneficial effects in all the NB cell lines. The combination of RA+Tac treatments is relatively more efficient than RA in promoting apoptosis, inhibiting proliferation, and decreasing the expression levels of signal pathway proteins ($p < 0.05$). Only the Tac treatment did not have a significant effect on the NB cells. In low doses and in combination with RA, Tac was found to be effective on cells.

CONCLUSION
In summary, the NB cells differentiated with the RA treatment were more responsive when RA+Tac was administered. Tac exhibited a synergistic effect combined with RA and affected the crucial signal pathway proteins. Our studies lead to a more comprehensive study of the combination of RA and Tac.

KEYWORDS
Neuroblastoma, retinoic acid, signal pathways, treatment of neuroblastoma, tacrolimus

ÖZ
AMAÇ
Bu çalışmada, farklı moleküler özelliklere sahip Nöroblastom (NB) hücrelerinde Retinoik Asit (RA) ve Tacrolimus (Tac) kombinasyon tedavisi uygulandı ve NB'deki proliferasyon, diferansiyasyon ve apoptoz yolları üzerindeki etkilerin değerlendirilmesi amaçlandı.

YÖNTEM
Farklı özelliklere sahip dört hücre hattı; KELLY, LAN-5, CHP-134 ve SHSY5Y kültürlendi ve çeşitli dozlarda RA ve Tac ile tedavi edildi. IC50 değerleri WST analizi ile belirlendi. RA+Tac kombinasyonunun IC50 değeri hücrelere uygulandı. Apoptoz/nekroz oranını belirlemek için hücreler Annexin V/PI ile boyandı. Belirli sinyal yolaklarının protein seviyelerini incelemek için Western Blot ve IHC uygulandı.

SONUÇ
RA ve RA+Tac tedavisi, tüm NB hücre hatlarında yolak baskılayıcı etkiler gösterdi. RA+Tac tedavisinin kombinasyonu, apoptozu teşvik etme, proliferasyonu inhibe etme ve sinyal yolak proteinlerinin ekspresyon seviyelerini azaltma konusunda RA'dan nispeten daha etkilidir ($p < 0,05$). Sadece Tac tedavisinin NB hücreleri üzerinde anlamlı bir etkisi olmadı. Düşük dozlarda ve RA ile kombinasyon halinde, Tac hücreler üzerinde etkili bulundu. RA tedavisi ile farklılaşan NB hücreleri, RA+Tac uygulandığında tedaviye daha duyarlı hale geldi. Tac, RA ile kombine edildiğinde sinerjik bir etki gösterdi ve sinyal yolak proteinlerini etkiledi. Çalışmalarımız, RA ve Tac kombinasyonunun daha kapsamlı bir incelemesine yol açtı.

ANAHTAR KELİMELER
Nöroblastom, nöroblastom tedavisi, retinoik asit, sinyal yolakları

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Neuroblastoma (NB) is the most common extracranial solid malignancy of childhood (1). NB is responsible for approximately 15% of deaths related to cancer in children. While individuals with low- and intermediate-risk NB have a survival rate close to 100%, those with high-risk NB have a 5-year survival rate of less than 50% (2). The MYCN gene, located in the 2p24 region of the short arm of the second chromosome, plays an important role in the prognosis of NB. MYCN amplification NB is generally associated with the high-risk group, and survival in this group is not good despite intensive multimodal treatment. New chemotherapy regimes and molecular therapies are needed for effective treatment of advanced patients (3). Retinoic acid (RA) is a biologically active compound derived from vitamin A, an essential nutrient required for the proper functioning of the body. Especially during the embryonic period, RA helps cells grow and develop. Retinoids promote cellular differentiation and inhibit proliferation. Thus, it is regarded as a promising candidate for inhibiting the the progression of tumors (4).

Cell fate determination and differentiation is an important process for cells to function in specific tissues. As known, the differentiation process is impaired in NB cells (5). NB cells are derived from neural crest cells, and the differentiation ability of neural crest cells is impaired, so the cells are unable to become a mature cells. Neural crest cells give rise to stromal Schwann cells and neuroblastic cells, and these types of cells have varying degrees of differentiation in NB (6). As is well known, the degree of differentiation and tumor grade of Schwann cells is a predictive biomarker for NB. The PI3K/Akt/mTOR signal pathway plays an important role in the proliferation of NB. Pre-clinical and clinical studies have demonstrated that mTOR inhibitors, such as rapamycin and its variants, show long-term objective tumor response. Tacrolimus (Tac), a derivative of rapamycin, is being investigated as a drug that can modulate the PI3K/Akt/mTOR pathway, which is crucial for cellular proliferation (7). Tac has a negative effect on tumor growth by inhibiting the mTOR pathway (8). Simultaneously, Ras/MAPK pathway, involved in cellular death, differentiation, survival, and proliferation, is also a significant signaling pathway in pediatric solid tumors (9). It is necessary to interrogate new agents which may be effective against NB. There are no studies in the literature where RA and Tac are combined and applied to NB cells. In our research, we aimed to assess the effects of RA in combination with Tac on different types of NB cells. Apart from their use after organ transplantation, rapamycin and mTOR inhibitors are being commonly for treatment of cancer.

Materials and Methods

The following were used in our research: RPMI 1640 Media (Cegrogen), MTT (AppliChem), S-100 antibody (Santa Cruz, sc-53438), p44 antibody (Cell Signaling Technology, 9102), Akt antibody (Cell Signaling Technology, 9272), Ras antibody (Cell Signaling Technology, 3965), GAPDH antibody (Cell Signaling Technology, 97166), Ki67 antibody (Santa Cruz, sc-101861), Bcl-2 antibody (Santa Cruz, sc-65392), Caspase 3 antibody (Santa Cruz, sc-7272), BCA protein assay kit (Thermo Scientific), Annexin V/PI kit (BD Bioscience), Retinoic Acid (Sigma, R2625), Tacrolimus (MedChemExpress, HY-13756), and Cisplatin (MedChemExpress, HY-17394).

Cell culture and treatment

Different types of NB cell lines (KELLY, SHYS-5Y, LAN-5 and CHP-134) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% L-Glutamin+ antibiotic solution and maintained in a 5% CO2 incubator. The cells at 80% confluency were treated with increasing concentrations of RA (0.1 μM, 0.2 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, and 10 μM), Tac (10 nm, 20 nm, 50 nm, 100 nm, 500 nm, 1000 nm, and 2000 nm), and combination doses of RA + Tac for 24, 48, and 72 hours.

Apoptosis assay

2 x 105 cells/well were seeded in six-well plates and treated with RA, Tac, RA+Tac, and Cisplatin (CDDP) for 48 hours. Then, cells were mechanically detached using cell scraper and harvested. The cell pellet dissociated with Binding Buffer (1X) and stained with 5 μL Annexin V and 10 μL PI dye for 15 minutes. Analyses were performed by using flow cytometry.

Immunocytochemistry

IHC was performed after the cells were treated with IC50 doses of drugs. Briefly, the cell pellet was obtained and dissociated with cell culture medium. 10 μL of cell suspension was dropped to the slide, and methanol was used to fixed the cells into the slide's surface. Then, cells were fix by incubating in 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature. Slides were treated with the Antigen Retrieval Buffer at 95°C for 15 minutes. Cells were rinsed in PBS three times. 0.1% Triton X-100 in PBS was used to permeabilize the cells for 10 minutes at room temperature. Cells were rinsed in PBS three times. Then, blocking was performed by using 10% fetal bovine serum in PBS for 1 hour at room temperature. Cells were treated with the primary antibodies (S-100, Ki-67, Bcl-2, and Caspase-3) at a dilution of 1:200 overnight. Cells were

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rinsed in 1% goat serum in PBS 3 times for 10 minutes. Then, the secondary antibody was dropped to the slide's surface, covered with a coverslip and treated with the cells for an hour. Cells were rinsed in PBS three times. 1 drop of DAB chromogen solution and 1 drop of DAB enhancer were mixed and applied to the slide's surface and incubated for 15 minutes. Cells were rinsed in wash buffer three times. Slides were treated with hematoxylene for 5 minutes. Cells were rinsed in wash buffer three times. Slides were then treated for 5 minutes in increasing alcohol series (20%, 40, 60, 80%, and 100%). Then, slides were kept in xylol for an hour. Finally, slides were covered with a coverslip of an appropriate size. Slides were visualized under a light microscope.

Protein isolation from cells

NB cell lines were collected by centrifugation and washed with ice-cold PBS with 2 times. 1 mL of ice-cold RIPA lysis buffer for 1x107 cells was added to the cells and centrifugated at 13,000 x g for 20 minutes at 4°C. Then, the pellet was discarded, and the supernatant containing the protein was carefully collected in a new tube and kept on the ice for further analysis. BCA assay was performed to determine the protein concentration. Briefly, BSA standarts included in an assay was dissolved and prepared a series of standart solutions with known concentrations ranging from 0 to 2 mg/mL. 10 µL of each BSA standard solution and protein sample was added into separate wells of a 96-well microplate. 200 µL of the BCA working reagent was added to each well. Then, plate was incubated at 37°C for 30 minutes. After incubation, the absorbance was measured at 562 nm in a microplate reader.

Western blot

25 µg of isolated proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes. In the subsequent step, the membranes were blocked with 5% fat-free dry milk for 1 h at room temperature. The blots were incubated with Akt (1:1000), Ras (1:1000), p44 (1:1000) and GAPDH (1:1000) antibodies overnight at 4 °C. At room temperature, the membranes were incubated for 1 h with goat antirabbit IgG secondary antibodies conjugated to horseradish peroxidase (CST, 1:3000). The relative expression of each target protein was measured using GAPDH as an endogenous reference. The images were analyzed using the ImageJ 1.54 g program.

Statistical analysis

The data will be presented as mean ± standard deviation. Statistical analyses were performed using the SPSS 29.0 (IBM) software package at a significance level of $p < 0.05$. The conformity of the data to a normal distribution was assessed using the Kolmogorov-Smirnov test. The intergroup data were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney U test.

Results

Cell viability assay

Various doses of RA and Tac were administered to the cells. The viability assays of chemotherapeutics on KELLY, LAN-5, CHP-134, and SHSY5Y cells were evaluated using the MTT method at 24, 48, and 72-hour time points. RA showed a reduction effect on cell viability at a dose of 0.5 µm on all cells. At low doses, Tac does not affect viability. The Tac at a dose of 500 nm provided the IC50 value for all cell lines. RA treatment showed 38.7% ±1.8% cell viability after 48 hours (Figure 1a). Tac treatment demonstrated an average value of 33.7% ±1.2% cell viability during a period of 48 hours (Figure 1b). The levels of Tac and RA dosages were assessed using various combinations. The combination of RA and Tac (0.5 µm+500 nm) resulted in an IC50 value of 25.9% ± 1.7 after 48 hours of measurement (Figure 1c).

Apoptosis assay

During the early stages of apoptosis, the phosphatidylserine molecules on the cell surface are translocated to the outer surface of the membrane by a "flip-flop" movement in the membrane of an apoptotic cell. Annexin V is a protein that binds to phosphatidylserine molecules that are translocated outside the cell. Necrotic cells are marked with PI. Based on the results of the MTT results of drugs, the cells were treated for 48 hours with the IC50 concentration of RA, Tac and RA+Tac combination. Subsequently, cells were stained with Annexin V and PI to assess the percentages of apoptotic and necrotic cells. The BD Accuri C6 software was used to calculate and analyze all flow cytometry data. According to our findings, the rates of early+late apoptosis had similar rates among the cell lines treated with RA and RA+Tac. RA treatment was induced cell apoptosis on all cell lines. The rate of necrosis was low. The percentage of live cells in Tac treatment was higher than to other drug treatments. The synergistic effect of RA+Tac induced significant apoptosis. As a represented Figure 2a, apoptosis rate was relatively higher as a result of RA treatment, rather than RA+Tac combination on CHP-134 and SHSY-5Y cells. On the other hand, RA+Tac combination showed a slightly necrotic effect on cells. As shown in Figure 2b, RA+Tac combination had a comparatively higher apoptotic effect on LAN-5 and KELLY cells compared to RA treatment alone. (Figure 2).

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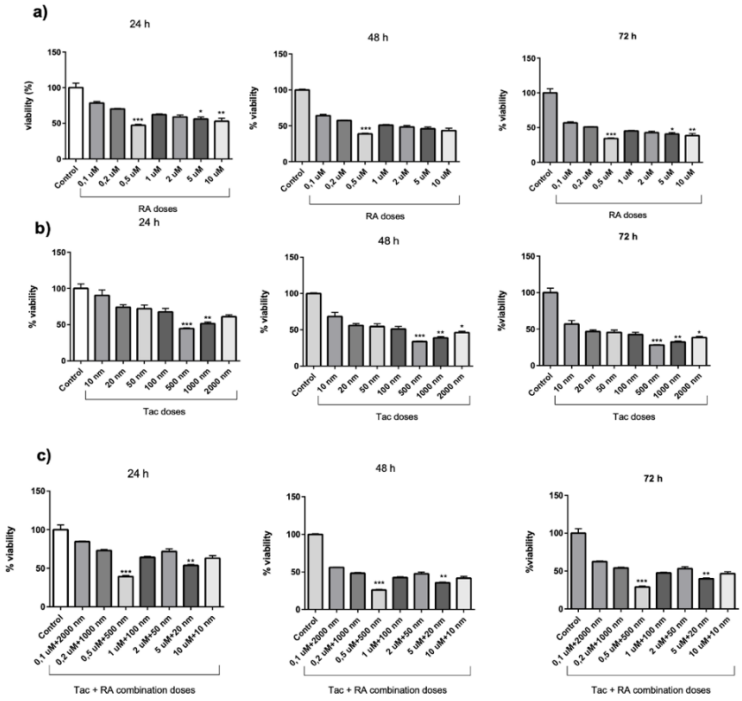

Figure 1. The cell viability of all chemotherapeutics: RA, Tac, and the combination of both agents. A various doses of all agent have administrated to all cell lines. As a result, a significant reduction in the number of viable cells after the treatment with RA, Tac, and their combination doses was found after 48 hours.

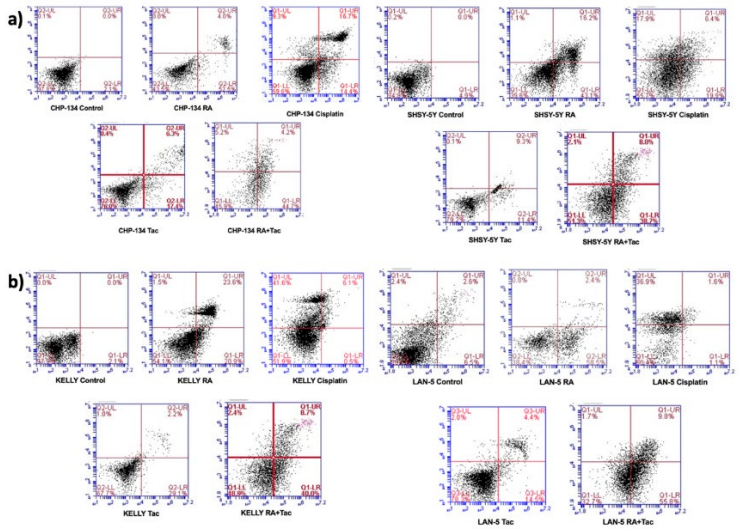

Figure 2. The apoptotic and necrotic effects of RA, Tac and combination. 2a. The CHP-134 and SHSY-5Y cell lines showed similar exposure to therapeutic agents in terms of apoptotic and necrotic cell death. 2b. The apoptotic effects of RA, Tac, and RA+Tac on KELLY and LAN-5 cell lines. RA exhibited an induction of proliferation on both cell lines. The apoptosis rate was relatively higher on LAN-5 with the treatment of both RA alone and RA+Tac combination compared to KELLY cell.

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Protein expressions

The expression of Ras, Akt, and p44 proteins was detected by Western Blot. RAS protein is widely involved in various physiological processes, such as proliferation, apoptosis, and cell survival. As known, RA prevents downregulation of RAS (10). In our study, after the drug treatment, the expression of the RAS protein in all the cell lines was analyzed. Compared to the control group, the expression of RAS protein in all cell lines was significantly reduced in both the RA and RA+Tac treatment groups, in all cell lines. The Tac treatment did not affect to lower the RAS levels. Compared to the control, RA+ Tac significantly decreased the Ras levels in all cell lines ($p < 0.001$) (Figure 3).

P44 is associated with MAPK pathway and is involved in many different physiological processes, such as inflammatory response, oxidative stress, and apoptosis (11). In our study, blotting analysis showed that RA treatment reduced the p44 levels . On SHSY-5Y and LAN-5 cells, RA decreased the p44 levels ($p < 0.05$). RA+Tac treatment is found relatively more effective on both cell lines ($p<0.01$). On KELLY and CHP-134 cells, RA inhibited the expression significantly ($p < 0.01$). RA+Tac treatment was relatively less effective than RA alone to reduce the expression ($p < 0.05$) (Figure 4a).

One more crucial protein investigated in the study was Akt. The activity of the protein p-Akt exhibited a notable reduction in all cell lines treated with RA and RA+Tac, compared to the control group. The resulting decrease was statistically significant ($p < 0.01$). A relative decrease was observed between the treatment groups of RA alone and RA+Tac ($p < 0.05$) (Figure 4b).

Immunocytochemistry

IC50 values of RA, Tac and RA+Tac were administered to NB cells. After the cells were collected, samples were fixated into the slide's surface. As shown in Figure 5a, S-100 differentiation marker exhibited a weak positivity in control group. The differentiating agents, RA and RA+Tac, showed more than 50% positive effect on cells. Ki67, a proliferation marker, was evaluated in NB cells (Figure 5b).

As a result of RA treatment, the proliferation rate was lower than the control group. The group treated with RA and RA+Tac had a significant reduction compared to the control ($p < 0.01$). There was no significant difference between the RA and RA+Tac groups ($p > 0.05$). The cellular expression of Caspase-3, a well-established indicator of programmed cell death, was evaluated (Figure 5c).

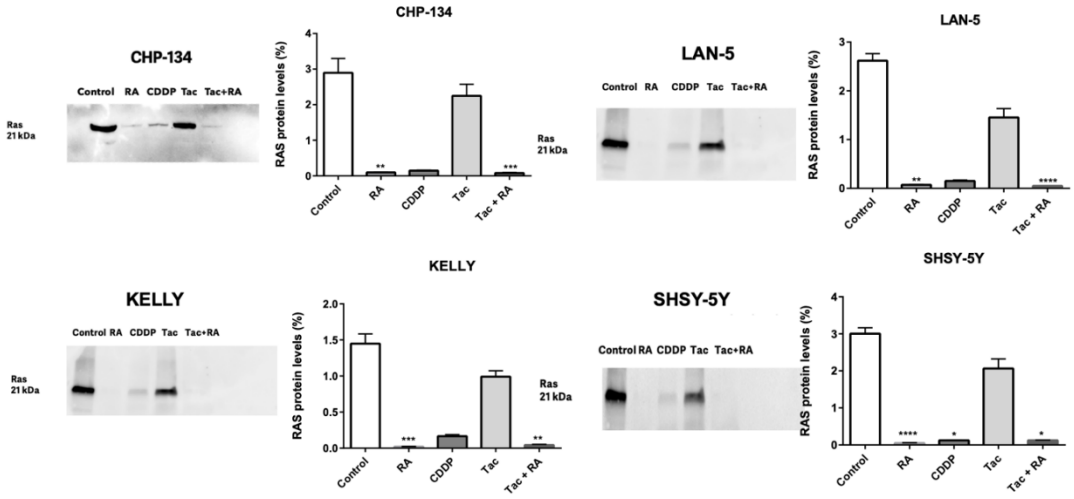

Figure 3. Ras protein levels determined by Western Blot. On all NB cell lines, RA and RA+Tac treatment significantly decreased the Ras levels.

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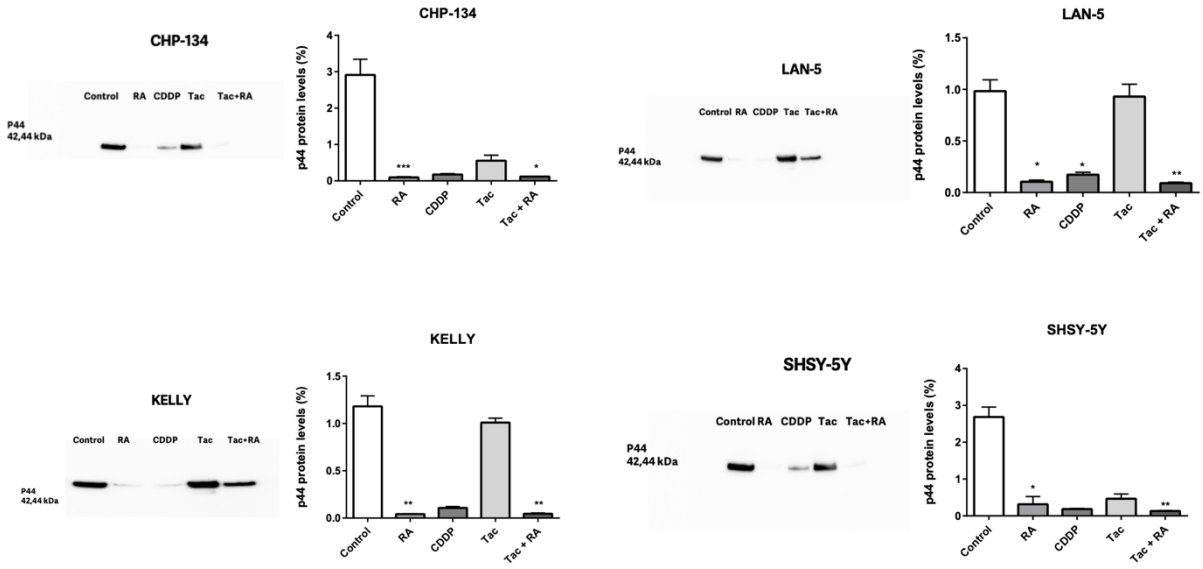

Figure 4. Determining the p44 levels by Western Blot. As shown, Tac was insufficient to suppress the protein expression. RA and RA+Tac treatment suppressed the p44.

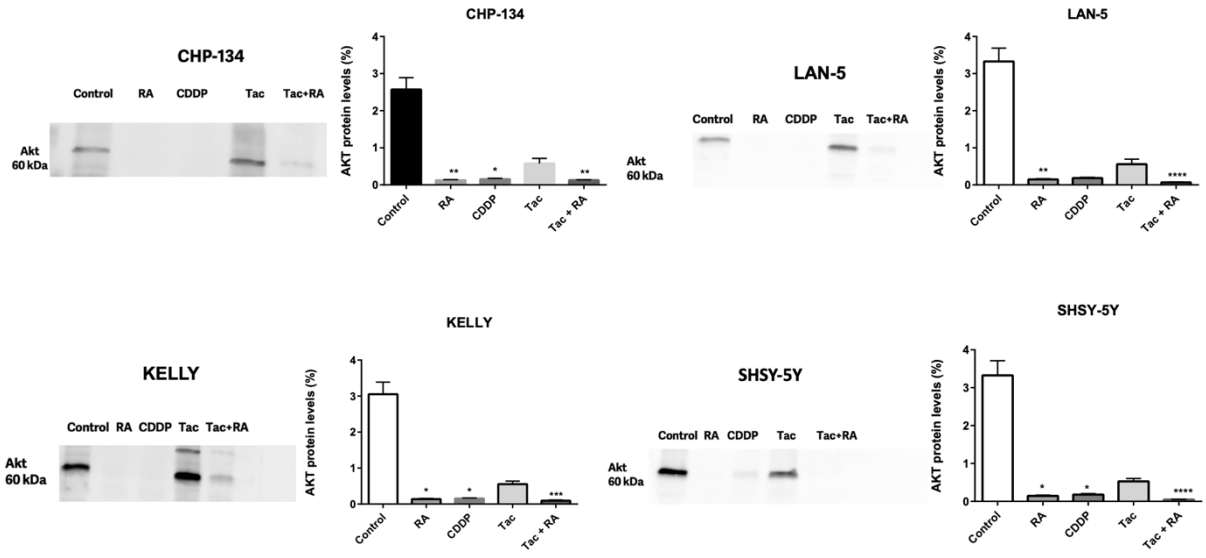

Figure 5. p-Akt levels explained by blotting. RA and RA+Tac treatment suppressed the protein expression.

The expression of Caspase-3 was significantly elevated in the groups that treated RA alone and RA+Tac. The two groups exhibited a statistically significant difference, compared to each other ($p < 0.05$). Tac alone did not induce apoptosis in NB cells. Bcl-2, a further mark of programmed cell death, is a biomarker that inhibits apoptosis. Immunocytochemical staining with Bcl-2 was performed (Figure 6d). As a result, the control group exhibited a high level of Bcl-2 expression. The expression level in Tac treatment group did not exhibit a significant decrease compared to the

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control group ($p > 0.05$). Bcl-2 showed very low positivity in RA treatment group. In RA+Tac group, Bcl-2 showed weak positivity.

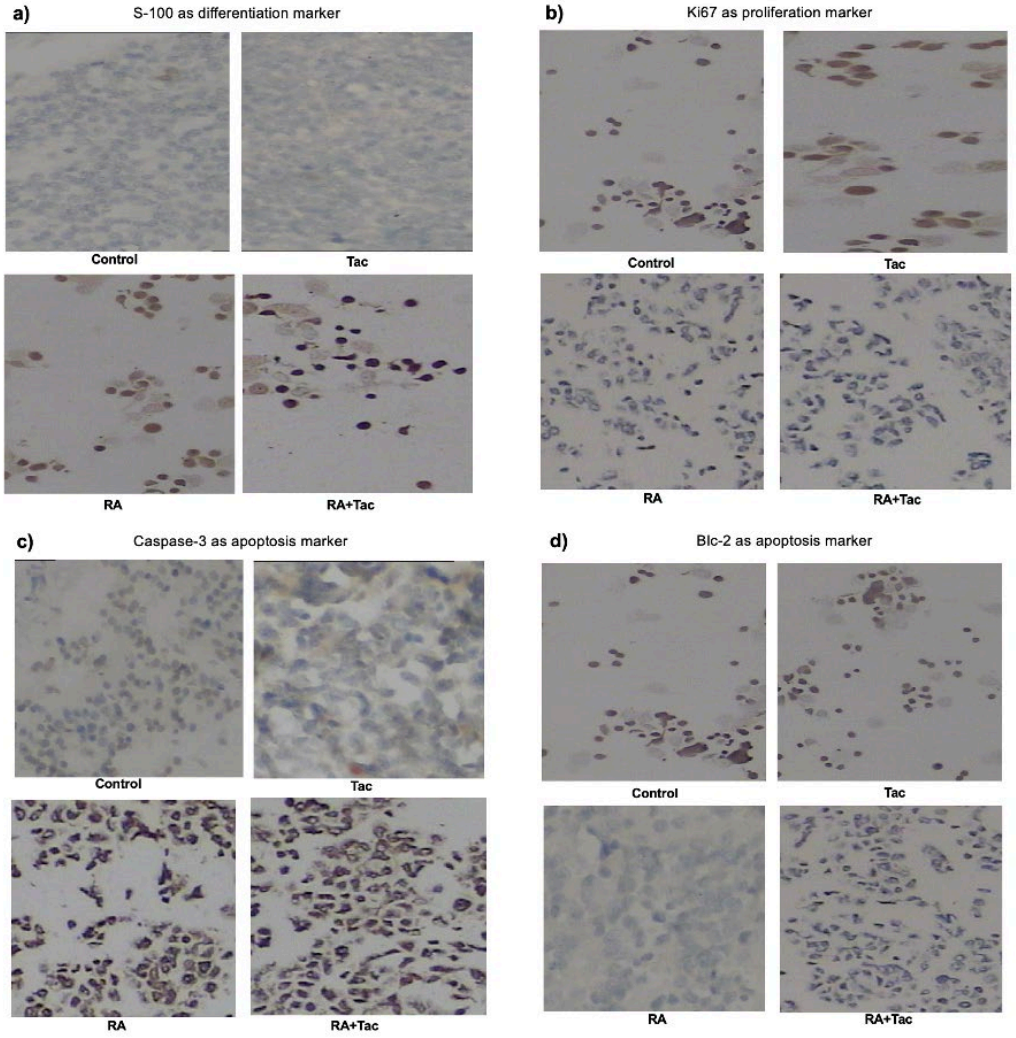

Figure 6. Differentiation, proliferation, and apoptosis biomarkers evaluated by immunocytochemistry. a) Weak positivity in control and Tac treatment. RA and RA+Tac treatment showed intense positivity in more than 50%. b) Ki67, a proliferation marker showed weak positivity resulting decreased expression levels in RA and RA+Tac treatment group. c) Caspase-3 exhibited strong positivity in RA and RA+Tac treatment, compared to control and Tac group. d) Bcl-2 is described as the anti-apoptotic protein. RA and RA+Tac showed weak positivity with Bcl-2. Tac showed a moderate positivity, compared to control. Immunocytochemical stainings, original magnification 100X.

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Discussion

As known, NB is a unique type of cancer characterized by newborns who may initially have either localized or metastatic disease (12). Interestingly, in some cases, NB might regress without any medical intervention.

However, older children can succumb to the disease after months to years of arduous therapy (13). Several biological factors contribute to the understanding of the clinical behavior in NB, such as histologic abnormalities, cytogenetic features, and molecular changes, specifically the amplification of the MYCN oncogene (14). Studies showed that treating the cells with retinoids can trigger the cells to differantiate, reprogram the enhancer landscape, and resulting in down-regulation of MYCN expression (15). Furthermore, some studies explain that the combination of MYCN inhibitors with RA suppresses the mTOR pathway and triggers apoptosis (16). In our study, we aimed to investigate the differentiation and apoptotic effect of RA on NB cells. To evaluate this, we used MYCN amplified cell lines; CHP-134, LAN-5, and KELLY and non-amplified cell; SHSY-5Y. Studies showed that treating MYCN amplified cell lines with RA induces differentiation in human NB cell lines (17,18). Similarly, we found a higher rate of Caspase-3 and S-100 expression after RA treatment, compared to the control group. In addition, the levels of Bcl-2, an anti-apoptotic biomarker, were reduced in the RA and RA+Tac treatments.

Tac is the best known mTOR inhibitor of rapamycin and a calcineurin inhibitor, it modulates mTOR in the absence of rapamycin with antiproliferative efficacy shown for many cancers (19). Tacrolimus with low doses has been found to trigger apoptosis and necrosis combined with mTOR inhibitors (20). In addition, Tac is currently used as the first-line immunosuppressant by organ transplant recipients in the clinical setting (21). A study demonstrated that Min6 cells treated to low concentrations of Tac exhibited an increased rate of apoptosis (22). Our findings showed that Tac treatment in NB cell lines had a significant impact on apoptotic indicators. However, by combining Tac with RA, the apoptotic effect was further enhanced. Therefore, we suggest that Tac and RA exhibit a synergistic impact on each other, depending on the dosage.

Ras/ERK and PI3K/Akt/mTOR signaling pathways are the main mechanisms of the cell to control cellular survival, differentiation, proliferation, metabolism, and motility. The PI3K/Akt/mTOR pathway is responsible to cell division, metabolism, and survival. Akt protein is an important molecule in cellular phenomena, many different intra-cellular processes including survival and proliferation in NB (23). We also performed Western blot to evaluate the p-Akt, Ras, and p44 levels. We found that the level of Akt expression in Tac treatment was lower compared to the control group. However, the combination of Tac and RA exhibited the most significant effect. A significant decrease was noted in the cells that administered only- RA in comparison to the control. Our findings indicated that Tac had a moderate level of success in reducing p-Akt levels. Tac has a similar effect on p-Akt levels, according to a study in the literature (22). Our investigation correlates with the current research in this field.

Presently, several genetic characteristics, such as RAS mutations, are being utilized as focusing points for the discovery of novel treatments for NB patients (9). Mutations affecting the RAS–MAPK pathway frequently occur in relapsed NB tumors, which suggests that activation of this pathway is associated with a more aggressive phenotype (24). p44/42 mitogen-activated protein kinase (MAPK), also named extracellular signal-regulated protein kinases (ERK1/2) is over expressed in various cancers including NB. p44 is associated with Ras protein in a downstream signal cascade (25). Studies have shown that the expression of RAS protein decreases in cells induced by RA (26). Similarly, our data demonstrated a significant decrease in expression of Ras in RA and RA+Tac. A similar study noted that RA affects the PI3K, ERK1/2, and p44/42 proteins (27). Tac had no effect in establishing an impact on the Ras mechanism. However, the combination group exhibited notable effect on Ras levels. Although Tac treatment exhibited high levels of p44, an important decreasing was found in RA and RA+Tac groups. It is evident that Tac is unable to independently affect the Ras-MAPK-ERK pathway.

Conclusion

To sum up, RA treatment regulates the differentiation, proliferation, and programmed cell death of NB cells. Tac is a calcineurin inhibitor that has relatively little effect on the signal pathways in cancer cells, as reported in the literature. This study demonstrated that Tac by itself was not effective in treating NB. However, combined with RA, Tac had a synergistic impact and altered crucial signal pathways in NB. Additional investigation is needed to be conducted to explore the pharmacology and impact of Tac in cancer. Since this study is done in vitro, additional analysis is necessary.

Author contributions

O.G, S.A, Z.A, and N.O designed the research. O.G and A.E performed the cell culture experiments. O.G, and A.E performed the Western Blot experiments. O.G performed the flow cytometry. O.G and A.E performed the immunocytochemistry staining. O.G and S.A performed the

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statistics. All the authors have equal contributions to the paper. All the authors contributed to the data interpretation and writing.

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Data availability

The data sets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors have no relevant financial or non- financial interests to disclose.

Ethical standarts

The study was approved by the Ethics Committee of the Dokuz Eylül University, Türkiye with the license number 2018/04-14. All aspects of this study, were performed in accordance with the principles of the Declaration of Helsinki (64th, 2013).

Informed consent

All participants gave their informed consent prior to their inclusion in the study.

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