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Combination of 13 *cis*-retinoic acid and tolfenamic acid induces apoptosis and effectively inhibits high-risk neuroblastoma cell proliferation

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ABSTRACT

Chemotherapeutic regimens used for the treatment of Neuroblastoma (NB) cause long-term side effects in pediatric patients. NB arises in immature sympathetic nerve cells and primarily affects infants and children. A high rate of relapse in high-risk neuroblastoma (HRNB) necessitates the development of alternative strategies for effective treatment. This study investigated the efficacy of a small molecule, tolfenamic acid (TA), for enhancing the anti-proliferative effect of 13 *cis*-retinoic acid (RA) in HRNB cell lines. LA1-55n and SH-SY5Y cells were treated with TA (30 μM) or RA (20 μM) or both (optimized doses, derived from dose curves) for 48 h and tested the effect on cell viability, apoptosis and selected molecular markers (Sp1, survivin, AKT and ERK1/2). Cell viability and caspase activity were measured using the CellTiter-Glo and Caspase-Glo kits. The apoptotic cell population was determined by flow cytometry with Annexin-V staining. The expression of Sp1, survivin, AKT, ERK1/2 and c-PARP was evaluated by Western blots. The combination therapy of TA and RA resulted in significant inhibition of cell viability ($p < 0.0001$) when compared to individual agents. The anti-proliferative effect is accompanied by a decrease in Sp1 and survivin expression and an increase in apoptotic markers, Annexin-V positive cells, caspase 3/7 activity and c-PARP levels. Notably, TA + RA combination also caused down regulation of AKT and ERK1/2 suggesting a distinct impact on survival and proliferation pathways via signaling cascades. This study demonstrates that the TA mediated inhibition of Sp1 in combination with RA provides a novel therapeutic strategy for the effective treatment of HRNB in children.

1. Introduction

Neuroblastoma (NB) is an extra-cranial solid tumor that originates from the sympatho-adrenal lineage of neural crest during embryologic development and is found in infants and children, most commonly under the age of 5 years. NB arises in the sympathetic nervous system and is frequently seen in the adrenal gland or nerve tissue in the abdomen and chest. It is the most common pediatric solid tumor and accounts for approximately 15% of childhood cancer deaths (Hoehner et al., 1996). The complexity and aggressiveness of NB is determined by a number of factors such as disease stage, age at diagnosis, and cellular/genetic make-up of the tumor and is categorized into low-, intermediate- and high-risk based on these factors (van Ginkel et al., 2007; Cheung, 2013; Maris, 2010; Carosio et al., 2007). The standard-of-care treatment for high-risk neuroblastoma (HRNB) includes surgery, intensive chemotherapy, irradiation, autologous bone marrow transplant and biologic therapy with isotretinoin (13-*cis*-retinoic acid or RA) and CH14.18 antibody. (Matthay et al., 2009). Despite recent improvements

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in therapeutic regimens, prognosis of high-risk neuroblastoma (HRNB) is still poor with approximately 50% mortality rate (van Ginkel et al., 2007; Maris, 2010; Carosio et al., 2007; Ebb et al., 2001; Preis et al., 1988).

Retinoic acid (RA) is currently used as adjuvant therapy. It is typically given during the remission maintenance phase of NB; however, the data from the children's cancer group study (CCG-3891) indicated certain limitations of RA in improving event-free survival rate (Matthay et al., 2009). RA induces the differentiation of neuroblasts and skeletal myoblasts (Preis et al., 1988; Xun et al., 2012). RA inhibits cell growth and induces differentiation in NB cells including the cell lines established from refractory tumors. It has been suggested that RA effectively targets residual tumor cells that are resistant to chemotherapy or radiation therapy. RA improves mitochondrial respiration and increases the metabolic rate in differentiated cells, while reducing the availability of nutrients to undifferentiated cells and suppresses cell growth (Xun et al., 2012). Even though RA has clinical advantages in NB, it also has several limitations. Inter-and intra-patient variations in the RA plasma concentration and pharmacokinetics have been reported (Veal et al., 2013; Veal et al., 2007). In addition, RA can cause acute and long term toxicity and disrupts various biological functions (van Ginkel et al., 2007). In order to improve cellular response to RA in NB treatment, drug combination therapies leading to reduced toxicity and increased event free survival are currently being explored.

Specificity protein1 (Sp1) is the first mammalian transcription factor to be cloned (Black et al., 2001). Over the past decade, molecular studies conducted on Sp1 demonstrated the crucial role of Sp1 in the regulation of cell growth, differentiation and apoptosis. Elevated levels of Sp1 have been implicated in the development of various cancers and several lines of evidence suggest that there is a correlation between Sp1 expression and poor prognosis in cancer patients (Abdelrahim et al., 2006; Basha et al., 2011a; Chang and Chen, 2005; Lu and Archer, 2009). In addition, previous studies have shown that Sp1 transcription factor regulates the expression of survivin, a key mediator of apoptosis (Doolittle Helen, 2010; Mita et al., 2008). Recently, we have shown that Sp1 is expressed in NB clinical specimens and targeting Sp1 with the small molecule/non-steroidal anti-inflammatory drug (NSAID) tolfenamic acid (TA), inhibits NB cell proliferation (Eslin et al., 2013).

In this study, we have investigated the individual and combined effects of TA and RA for inhibiting HRNB cell growth. HRNB cells, LA1-55n and SH-SY5Y, were treated with optimized concentrations of TA, RA, TA + RA and cell viability was measured at 24 and 48 h post-treatment. In order to understand the effect on apoptosis, critical markers (caspase 3/7 activity, expression of cleaved PARP and percentage of apoptotic cell population) were monitored. The expression of Sp1, survivin, AKT and ERK1/2 was also determined.

2. Material and methods

2.1. Cell lines

LA1-55n (*MYCN* amplified), SH-SY5Y (*MYCN* non-amplified) and SMS-KCNR (*MYCN* amplified) cell lines were derived from bone marrow metastases of childhood cancer patients with NB. Cells were procured from Sigma–Aldrich (St. Louis, MO), ATCC (Manassas, VA) and Children's Oncology Group (Lubbock, TX). Cells were grown in RPMI 1640 media (ATCC) with fetal bovine serum and supplemented with 100 U/ml penicillin and 100 U/ml streptomycin. All cultures were maintained at 37 °C and 5% CO_2 as previously described (Saulnier Sholler et al., 2009; Suresh et al., 2012; Chetty et al., 2005).

2.1.1. Chemicals and reagents

Antibodies for Sp1 and survivin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). c-PARP, ERK1/2, pERK1/2, AKT and pAKT antibodies were acquired from Cell Signaling Technology (Danvers, MA) while β-actin was obtained from Sigma Chemical Co. (St. Louis, MO). Annexin-V/7-AAD kit for apoptosis was purchased from BD Biosciences (San Jose, CA). Tolfenamic acid (TA), dimethyl sulfoxide (DMSO), 13-*cis*-retinoic acid (RA) and protease inhibitor were purchased from Sigma. Dulbecco's phosphate-buffered saline (PBS) was purchased from Hyclone Laboratories (Logan, Utah), and cell lysis buffer was obtained from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) protein assay kit and Supersignal West Dura were purchased from Pierce (Rockford, IL) and Caspase-Glo 3/7CellTiter-Glo kits were obtained from Promega (Madison, WI).

2.1.2. Cell viability

The NB cells were grown in RPMI1640 media and treated with DMSO (control), TA, RA, or TA + RA and cell viability was measured using Cell Titer-Glo kit (Promega, Madison, WI). Briefly, 4000 cells/well were plated in 96-well white walled clear bottom plates (Lonza, Basel, Switzerland) and incubated for 48 h, unless otherwise noted, at 37 °C. LA1-55n, SH-SY5Y and SMS-KCNR cells were treated with increasing concentrations (0, 10, 20, 30, 40 and 50 μM) of TA or RA for 24 h and 48 h. At the end of the incubation period, 100 μl of assay reagent was added, mixed, and the plate was incubated in the dark for 20 min. Luminescence values were obtained from each well using a SYNERGY HT microplate reader. All treatments were performed in triplicate and the data was normalized for control (DMSO-treated) cells and plotted as percent cell viability versus drug concentration.

2.1.3. Combination index analysis

Logarithmically growing cells (4000 cells/well) were plated in 96-well white walled clear bottom plates (Lonza, Basel, Switzerland) and grown at 37 °C in a cell culture incubator. Cells were treated with fixed ratio TA/RA (2:3) for 48 h and cell viability was measured using Cell Titer-Glo kit. The dose-effect relationship analysis for TA and RA was calculated to determine the combination index (CI) by CalcuSync software (BIOSOFT, Cambridge, UK), where CI values <1, =1 and >1 represents synergistic effects, additive effects, antagonistic effects respectively.

2.1.4. Cell apoptosis using flow cytometry

Apoptotic cells were measured using Annexin-V/7-AAD apoptosis detection kit (BD Biosciences). Briefly, cells were harvested after treatment with vehicle (control) or individual drug (TA or RA), or combination of investigational agents for 48 h. Cells were incubated with Annexin-V antibody and 7-AAD for 15 min in 1X binding buffer and cells in various populations (e.g., pre-apoptotic, apoptotic and dead) were analyzed using BD LSR II flow cytometer. Data were analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR).

2.1.5. Caspase activation assay

LA1-55n, SH-SY5Y and SMS-KCNR cells were treated with TA or RA or TA + RA, and the activity of caspase 3/7 was evaluated using Caspase-Glo 3/7 kit (Promega, Madison, WI), according to manufacturer instructions. Briefly, 4000 cells/well were plated in a 96-well white walled clear bottom plate (Lonza, Basel, Switzerland) in two sets (cell viability and caspases 3/7). After 24 h, cells were treated with DMSO (control), TA, RA, or TA + RA (with indicated drug concentrations). Following 48 h post-treatment, the assay (Cell Titer-Glo or Caspse-Glo 3/7) reagent (100 μl/well) was added, mixed and the plates were incubated in the dark for 60 min. Luminescence was measured using a SYNERGY HT microplate reader. The activity of caspases was normalized with cell viability. All the