Preclinical Evaluation of a Novel RXR Agonist for the Treatment of Neuroblastoma

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Abstract

Neuroblastoma remains a common cause of pediatric cancer deaths, especially for children who present with advanced stage or recurrent disease. Currently, retinoic acid therapy is used as maintenance treatment to induce differentiation and reduce tumor recurrence following induction therapy for neuroblastoma, but unavoidable side effects are seen. A novel retinoid, UAB30, has been shown to generate negligible toxicities. In the current study, we hypothesized that UAB30 would have a significant impact on multiple neuroblastoma cell lines in vitro and in vivo. Cellular survival, cell-cycle analysis, migration, and invasion were studied using AlamarBlue assays, FACS, and Transwell assays, respectively, in multiple cell lines following treatment with UAB30. In addition, an in vivo murine model of human neuroblastoma was utilized to study the effects of UAB30 upon tumor xenograft growth and animal survival. We successfully demonstrated decreased cellular survival, invasion, and migration, cell-cycle arrest, and increased apoptosis after treatment with UAB30. Furthermore, inhibition of tumor growth and increased survival was observed in a murine neuroblastoma xenograft model. The results of these in vitro and in vivo studies suggest a potential therapeutic role for the low toxicity synthetic retinoid X receptor selective agonist, UAB30, in neuroblastoma treatment. Mol Cancer Ther; 14(7): 1559–69. ©2015 AACR.

Introduction

Neuroblastoma, the most common extracranial solid tumor of childhood, is responsible for more than 15% of pediatric cancer deaths (1). Despite aggressive multimodal therapies, children diagnosed with high-risk neuroblastoma continue to have a poor outcome. Current high-risk treatment regimens include chemotherapy, surgical resection, autologous stem cell transplantation, and radiation followed by maintenance therapy with immunotherapy and 13-cis-retinoic acid (13-cis-RA, isotretinoin; ref. 2).

Neuroblastomas may undergo spontaneous maturation but most tumors show little neural differentiation (3). Retinoic acid therapy has been demonstrated to induce differentiation and growth arrest (4), and Matthey and colleagues demonstrated improvement in 3-year event-free survival in high-risk patients treated with maintenance 13-cis-RA therapy after stem cell transplantation (2). However, toxicities associated with 13-cis-RA occasionally limit its dosage. A phase I study of 13-cis-RA concluded the dose-limiting toxicity to be hypercalcemia with cheilosis, mucositis, and hypertriglyceridemia also being common (5).

A novel retinoid, 9-cis-UAB30 (UAB30), has been developed that has minimal toxicity. UAB30 is a synthetic analog of 9-cis-RA that selectively activates retinoid X receptors (6). Toxicity studies in rodents and dogs showed no treatment related toxicities (7). A pilot clinical trial study in humans has demonstrated a favorable toxicity profile with primarily hepatic metabolism and no significant increase in serum triglycerides (8).

We hypothesized that UAB30 would induce cell-cycle arrest, inhibit cellular migration and invasion, and lead to apoptosis and cell death in neuroblastoma cell lines in vitro and impede tumor growth in vivo. To confirm, we illustrated the significant cellular and morphologic effects of UAB30 on multiple neuroblastoma cell lines and demonstrated decreased neuroblastoma xenograft growth in vivo.

Materials and Methods

Cells and culture

The human neuroblastoma cell lines SK-N-AS (CRL-2137), SK-N-BE(2) (CRL-2271), SH-SY5Y (CRL-2266), and IMR-32 (CCL-127) were obtained from the ATCC. SH-EP and WAC(2) human neuroblastoma cell lines were a kind gift from M. Schwab (Deutsches Krebsforschungszentrum, Heidelberg, Germany), and have been described in detail (9). Cell lines were maintained in the past 5 years and authentication was not performed. All cell lines were maintained under standard conditions at 37°C and 5% CO₂. SK-N-AS cells were maintained in DMEM (30-2601, ATCC) containing 10% FBS (Hyclone), 4 mmol/L L-glutamine (Thermo Fisher Scientific Inc.), 1 μmol/L nonessential amino acids, and 1 μg/mL penicillin/streptomycin (Gibco). SK-N-BE(2) and SH-SY5Y cells were maintained in a 1:1 mixture of minimum Eagle medium and Ham F-12 medium (30-2004, ATCC) with 10% FBS (Hyclone), 2 mmol/L L-glutamine (Thermo Fisher Scientific), 1 μmol/L nonessential amino acids and 1 μg/mL penicillin/streptomycin (Gibco). IMR-32 cells were maintained in EMEM (30-2003, ATCC) with 10% FBS (Hyclone), 2 mmol/L L-glutamine (Thermo Fisher Scientific), d-glucose 1.0 g/mL, and 1 μg/mL...
penicillin/streptomycin (Gibco). SH-EP and WAC(2) cell lines were maintained in RPMI1640 medium (30-2001, ATCC) with 10% FBS (HyClone), 2 mmol/L L-glutamine (Thermo Fisher Scientific) and 1 μg/ml penicillin/streptomycin (Gibco).

**Antibodies and reagents**

Rabbit polyclonal anti-PARP (9542S), anti-cleaved caspase-3 (9664), anti-AKT (9272), anti-phospho AKT (9271), anti-ERK1/2 (9102), and mouse monoclonal anti-β-actin, anti-p53 (1C12) antibodies were obtained from Cell Signaling Technology. Rabbit anti-phospho ERK1/2 (05-797R) and mouse monoclonal GAPDH (6C5) was from Millipore (EMD Millipore). Mouse monoclonal anti-RXR and anti-nestin was obtained from Abcam (clones MOK13-17 and 10C, respectively). UAB30 was synthesized as described (10). 13-cis-RA was from Sigma (R3255, Sigma-Aldrich).

Antibodies for immunofluorescence were as listed: primary antibodies p53 (Cell Signaling Technology, 1:1,000) and RXR (Abcam, 1:1,000) and secondary antibody goat anti-mouse Alexa Fluor 594 (A-11045, Thermo Fisher Scientific, 1:33 dilution).

**Cellular differentiation**

Neuroblastoma cells were cultured in standard medium or with UAB30 (10 μmol/L, 48 hours). Pictures (Photometrics CoolSNAP HQ2 CCD camera attached to a Nikon Eclipse Ti microscope) were obtained and the number of neurite outgrowths per cell were counted and reported as fold change neurite outgrowths (11).

**Immunoblotting**

Western blots were performed as previously described (12). Whole-cell lysates were isolated using radioimmunoprecipitation assay supplemented with protease inhibitors (Sigma), phosphatase inhibitors (Sigma) and phenylmethylsulfonylfluoride. Protein concentrations were determined using BCA Protein Assay. Antibodies and reagents

**Cell viability, proliferation, apoptosis assays**

Cell viability was measured with AlamarBlue assays. A total of 1.5 × 10^5 cells/well were plated on 96-well culture plates, allowed to attach, and treated with 9-cis-UAB30 (UAB30) at increasing concentrations (48 hours). After treatment, 10 μL of AlamarBlue dye (Invitrogen Life Technologies) was added and the absorbance at 595 nm was measured using microplate reader (BioTek Gen5, BioTek Instruments). Viability was reported as fold change. Cell viability/proliferation were also measured with Trypan blue staining. Cells (1.5 × 10^5 cells per well) were plated, allowed to attach and treated with UAB30 for 48 hours. Cells were counted with a hemacytometer and reported as fold change in cell count and fold change in the ratio of dead to live cells.

Cellular apoptosis was detected with two methods, immunoblotting for cleavage of PARP and caspase-3, and a colorimetric caspase-3 activation kit (KHZ0022, Invitrogen). Increasing intensity of bands for cleaved products combined with decreasing intensity of bands for total products indicated apoptosis. In the SK-N-BE(2) and SH-SYSY cell lines, activation of caspase-3 was also measured with a caspase-3 activation kit.

**Cell-cycle analysis**

Cells were plated (1.0 × 10^5 cells), allowed to attach overnight, and then treated with UAB30 (10 μmol/L, 48 hours). Cells were trypsinized, washed with PBS, and fixed in 100% ethanol. Ethanol was removed, cells stained with solution containing 0.3 μmol/L propidium iodide (Invitrogen) in 0.1% Triton X and RNase A (Qiagen) for 30 minutes at room temperature, and analyzed with FACS using a FACSCalibur Flow Cytometer (Becton Dickinson Biosciences). Data were analyzed with ModFit LT software (Verity Software House Inc.). Negative controls were included in each flow cytometry run.

**Cellular invasion assay**

Twelve-well culture plates (TransWell, Corning Inc.) with 8 μm micropore inserts were used. The top side of the insert was coated with Matrigel (BD Biosciences; 1 mg/mL, 50 μL for 4 hours at 37 °C). Neuroblastoma cells [SK-N-AS and SK-N-BE(2), (8 × 10^5 cells / well)] were treated with UAB30 and plated into the top well. All other cell lines were plated at 4 × 10^4 cells/well. After 48 hours, inserts were fixed with 3% paraformaldehyde, stained with crystal violet, and cells counted with a light microscope and invasion reported as fold change.

**Migration assays**

Similar to invasion, 12-well culture plates (TransWell, Corning) with 8 μm micropore inserts were used. The bottom side of the insert was coated with collagen Type I (10 mg/mL, 50 μL for 4 hours at 37°C). Neuroblastoma cells (4 × 10^5 cells/well) were treated with UAB30, placed into the top well, and after 24 hours, inserts were fixed with 3% paraformaldehyde, stained with crystal violet, and migrated cells counted with a light microscope. Migration was reported as fold change.

Cellular migration was also measured utilizing cell monolayer wounding (scratch) assay. SK-N-BE(2) cells (4.5 × 10^5 cells/well) were plated and allowed to attach overnight. A 200 μL pipette tip created a uniform scratch in the near-confluent cell layer and photos [Photometrics CoolSNAP HQ2 CCD camera attached to a Nikon Eclipse Ti microscope] were obtained at time zero. Cells were treated with 0, 10, or 25 μmol/L UAB30 (24 hours) and photos repeated. The area of the scratch was quantified by measuring the pixel count of the scratched area and comparing it to the pixel count of the same plate at time zero and reported as fold change in scratch closure. Assays with the other cell lines used 2 × 10^5 cells/well. Wounding assays were not performed with IMR-32 or SH-SYSY cells as they did not propagate in an adherent fashion.

**Immunofluorescence**

Immunofluorescence staining was utilized to detect movement of RXR and p53 into the nucleus following UAB30 (10 μmol/L). Cells were plated on glass chamber slides and allowed to attach. SH-SYSY and IMR-32 cell lines did not grow well on these slides because they propagate both floating and adherent and were not analyzed. After 48 hours, cells were fixed with 3% paraformaldehyde, permeabilized with 0.15% Triton,
X-100, and the primary antibody was added and incubated at room temperature for 1 hour. The Alexa Fluor 594 secondary antibody was added for 45 minutes at room temperature. Prolong Gold antifade reagent with DAPI (P36931, Invitrogen) was used for mounting. Imaging was performed with a Zeiss LSM 710 Confocal Scanning Microscope with Zen 2008 software (Carl Zeiss Microlmaging, LLC) using a 63× objective with a zoom of 0.9. Meta Morph Microscopy Image Analysis Software (Ver. 7.6, Analytical Technologies, Molecular Devices) analyzed the images and detected overlap.

In vivo tumor growth
Six-week-old, female, athymic nude mice were (Harlan Laboratories, Inc.) maintained in the SPF animal facility with standard 12-hour light/dark cycles and allowed chow and water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) parameters for euthanasia with retro-orbital bleeding and a 2-ml overdose of Ketamine and Xylazine. Human neuroblastoma cells, SK-N-AS or SK-N-BE(2) [2.5 × 10^6 cells in Matrigel (BD Biosciences)] were injected into the right flank. On the day of injection, mice were randomized to vehicle-treated, UAB30-treated (100 mg/kg), or 13-cis-RA-treated (53 mg/kg; ref. 13) chow (n = 10 per group). The tumor volumes were measured with calipers and calculated with the standard formula of (width^2 × length) / 2 where length is the largest measurement. For survival studies, animals were followed until International Animal Care and Use Committee (IACUC) parameters for euthanasia were met, when they were euthanized with CO2 and bilateral thoracotomy.

Immunohistochemistry
Formalin-fixed, paraffin-embedded human neuroblastoma specimens and xenograft tumor specimens were cut into 6 μm sections, baked at 70 °C for 1 hour, deparaffinized, rehydrated, and steamed. Sections were quenched with 3% hydrogen peroxide and blocked with blocking buffer (BSA, powdered milk, Triton X-100, PBS) for 30 minutes at 4°C. The primary RXR antibody (ab2815, Abcam) or anti-Ki67 rabbit polyclonal antibody (ab15580, Abcam) was added 1:200 dilution and incubated overnight at 4°C. After washing with PBS, the secondary antibody (mouse SuperPicture Polymer HRP, Invitrogen or donkey anti-rabbit, 1:400, Jackson Immunoresearch Laboratories) was added for 1 hour at 22°C. The staining reactions were developed with VECTASTAIN Elite ABC kit (PK-6100, Vector Laboratories), TSA (biotin tyramide reagent, 1:400, PerkinElmer, Inc.) and DAB (ImmPACT DAB, Vector Laboratories). Slides were counterstained with hematoxylin. Negative controls [mouse IgG (1 μg / mL, Invitrogen) or rabbit IgG (1 μg / mL, EMD Millipore)] were included with each run. A board-certified pathologist (E. Mroczek-Musulman) blinded to the treatment groups completed the quantification of Ki67. The area chosen for analysis was the area of greatest immunoreactivity. Five hundred cells were counted and the ratio of immunopositive to total cells reported as percent positive staining. All immunopositive cells were counted without regard to stain intensity (14, 15).

Statistical analysis
Experiments were repeated at least in triplicate and data were reported as mean ± SEM. Densitometry of Western blots was performed using the image histogram analysis feature of Adobe Photoshop software (Adobe Systems Inc.). Student t test, Fisher exact test, ANOVA, or log-rank was used as appropriate to compare data. Statistical significance was determined at the P < 0.05 level.

Results
UAB30 led to neuroblastoma cell death and apoptosis
The expression of RXR receptors in human neuroblastoma specimens and in the cell lines to be utilized in the study was evaluated. Immunohistochemistry demonstrated RXR staining in all 13 human neuroblastoma specimens examined (Fig. 1A). There was no difference in staining between stage or MYCN amplification (data not shown). Immunoblotting detected RXR expression in all six cell lines used (Fig. 1B). Furthermore, following treatment with UAB30, there was an increase in the ratio of RXR staining in the nucleus of the cells (Fig. 1C), indicating that UAB30 functioned as an RXR agonist, leading to movement of the RXR into the nucleus. AlamarBlue assays were used to determine the effect of UAB30 upon cell survival. UAB30 resulted in significant cell death in all six cell lines (Fig. 1D). These results were not dependent upon MYCN amplification, as both MYCN and nonamplified neuroblastoma cell lines showed significantly decreased survival with similar LD_{50} concentrations (Fig. 1E), and these results held true for both nonisogenic and isogenic MYCN cell lines. The LD_{50} for UAB30 ranged from 37.8 to 58.3 μmol/L (Fig. 1E). To determine whether UAB30-induced cell death was apoptotic in nature, immunoblotting was performed for cleavage of PARP and caspase-3. As demonstrated by increased PARP and caspase-3 cleavage (Fig. 1F and G, respectively), the UAB30-induced cell death was via apoptosis. In the SK-N-BE(2) and SH-SY5Y cell lines, the changes in cleaved caspase-3 by immunoblotting were not clear, therefore evaluation of caspase-3 activation in these two cell lines was determined using a caspase-3 activation assay. This assay demonstrated a significant increase in caspase-3 activation following treatment with UAB30 in both cell lines (Supplementary Figures S1 and S2).

UAB30 resulted in cell differentiation and cell-cycle arrest
Retinoids are known to cause cellular differentiation; so we wished to determine whether UAB30 would induce differentiation in neuroblastoma cells. Differentiation in neuroblastoma cell lines is marked by outgrowths of neurites (16). For these experiments, concentrations of UAB30 were chosen below the calculated LD_{50} to show early morphologic changes rather than cell death. After UAB30, cellular differentiation was demonstrated in all cell lines as seen by neurite outgrowths (Fig. 2A, closed arrows). The UAB30-induced neurite outgrowth was quantified by counting the number of neurite outgrowths per cell (11) and reporting as fold change, and UAB30 led to significantly increased neurite outgrowths in all cell lines (Fig. 2B). Expression of nestin protein has been associated with retinoic acid–induced neuronal differentiation in neuroblastoma (17). Nestin protein expression was examined and reporting as fold change, and UAB30 led to significantly increased expression in all cell lines used (Fig. 2B). Expression of nestin protein has been associated with retinoic acid–induced neuronal differentiation in neuroblastoma (17). Nestin protein expression was examined and reported as fold change, and UAB30 led to significantly increased expression in all cell lines used (Fig. 2B). Expression of nestin protein has been associated with retinoic acid–induced neuronal differentiation in neuroblastoma (17). Nestin protein expression was examined and reported as fold change, and UAB30 led to significantly increased expression in all cell lines used (Fig. 2B).
There was an increase in cleaved caspase-3 in the SK-N-AS (AS), SH-EP (SHEP), WAC2 (WAC), and IMR-32 (IMR) cell lines at concentrations of UAB30. Cleaved caspase-3 was not detected by immunoblotting in the SK-N-BE(2)(BE) and SH-SY5Y (SY5Y) cell lines.

percentage of cells in G1 phase and decreased percentage of cells in S-phase (Fig. 2C). Representative FACS histograms for the SH-EP cell line are presented (Fig. 2D). UAB30 increased the percentage of SH-EP cells in G1 phase from 68.4% to 74.6% while the percentage of cells in S-phase decreased from 21.96% to 11.7%. The complete data are shown in both graphic (Fig. 2C) and tabular form (Table 1). Only one cell line, SH-SY5Y, did not significantly decrease the percentage of cells in S-phase, but these cells did show a significant increase in G1 phase with UAB30 treatment (Fig. 2C; Table 1).

As UAB30 caused G0–G1 cell-cycle arrest, we wished to determine whether proliferation of the cells was also affected. Trypan blue exclusion assays were performed. There was not a significant increase in the total number of cells in any of the cell lines (Supplementary Figure S4), and in all cell lines, except SH-SY5Y, there was a significant increase in the ratio between cells that were dead versus those that were viable following exposure to UAB30 (10 μmol/L, 72 hours; Supplementary Figure S5). Taken together, these data indicated that UAB30 diminished cellular proliferation.
UAB30 decreased cell invasion and migration in lower concentrations caused cellular differentiation, so we wished to determine whether these changes would also manifest as changes in other phenotypic features. Aggressive tumor cells are hallmarked by their ability to migrate and invade therefore; we investigated the effects of UAB30 upon cellular invasion and migration. Cells were treated with UAB30 (10 μmol/L) and allowed to invade for 48 hours. There was a significant decrease in cellular invasion at 10 μmol/L and this change was even more marked at 25 μmol/L (Fig. 3A). Cellular

### Table 1. Cell-cycle progression following treatment with UAB30 as measured by flow cytometry

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>UAB30</th>
<th>Control</th>
<th>UAB30</th>
<th>Control</th>
<th>UAB30</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>G1 (%)</td>
<td>G2 (%)</td>
<td>S (%)</td>
<td></td>
<td>G1 (%)</td>
<td>G2 (%)</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>67.9 ± 0.6</td>
<td>72.3 ± 0.7</td>
<td>13.3 ± 1.0</td>
<td>12.5 ± 0.7</td>
<td>13.8 ± 1.2</td>
<td>15.2 ± 1.2</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>53.2 ± 0.7</td>
<td>63.7 ± 1.2</td>
<td>15.3 ± 0.8</td>
<td>13.6 ± 1.1</td>
<td>13.6 ± 1.1</td>
<td>31.1 ± 11</td>
</tr>
<tr>
<td>SH-EP</td>
<td>68.5 ± 0.4</td>
<td>74.4 ± 0.2</td>
<td>8.7 ± 0.5</td>
<td>13.6 ± 0.2</td>
<td>22.8 ± 0.3</td>
<td>11.9 ± 0.2</td>
</tr>
<tr>
<td>WAC2</td>
<td>72.2 ± 0.4</td>
<td>76.2 ± 0.5</td>
<td>8.6 ± 0.1</td>
<td>12.5 ± 0.3</td>
<td>19.1 ± 0.3</td>
<td>11.3 ± 0.2</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>49.6 ± 3.0</td>
<td>51.3 ± 10.3</td>
<td>20.3 ± 1.3</td>
<td>13.2 ± 7.0</td>
<td>30.1 ± 4.3</td>
<td>35.5 ± 16.9</td>
</tr>
<tr>
<td>IMR-32</td>
<td>57.9 ± 2.3</td>
<td>71.7 ± 1.9</td>
<td>14.6 ± 0.7</td>
<td>12.4 ± 2.6</td>
<td>27.4 ± 1.7</td>
<td>15.9 ± 0.7</td>
</tr>
</tbody>
</table>

NOTE: Numbers in bold are statistically significantly different from controls (P ≤ 0.05).
Migration was also studied and there was a significant decrease in migration both across a porous membrane and across a monolayer scratch injury following UAB30 (Fig. 3B and C, respectively). In the membrane migration, migration by all cell lines except SK-N-BE(2) was significantly decreased after 10 μmol/L, but at 25 μmol/L UAB30, migration was significantly inhibited in all cell lines beginning at 10 μmol/L UAB30. B, cellular migration was assessed in a similar fashion with the bottom side of the insert coated with collagen Type I. Cell lines were treated with increasing concentrations of UAB30 and allowed to migrate for 24 hours. The cells were then fixed, stained, and counted and migration reported as fold change. Migration was significantly inhibited in all cell lines at 25 μmol/L UAB30. C, migration was further examined with monolayer wounding assays. Cells were plated, allowed to attach, and a standard scratch was performed. Cells were treated with increasing concentrations of UAB30 for 24 hours and photographs of the plates were obtained. The area of the scratch was quantified by measuring the pixel count of the scratched area and comparing it to the plate at time zero. Data were reported as percentage of scratch area closed after 24 hours. All four cell lines studied showed significant decrease in the ability to migrate across a wound after treatment with 25 μmol/L UAB30, a concentration well below the LD<sub>50</sub> for the cell lines.

**UAB30 did not alter AKT or ERK phosphorylation**

Various pathways have been implicated as the mechanism of action of retinoids. For example, it has been shown that retinoic acid activated both AKT- and ERK-dependent pathways in neuroblastoma cell lines (19, 20). Therefore, we wished to determine whether these kinases were phosphorylated by UAB30. Neuroblastoma cells were treated with UAB30 for 48 hours and immunoblotting was performed to detect total and phosphorylated AKT and ERK. There was no demonstrable change in the phosphorylation of these kinases with UAB30 treatment and all, changes in phosphorylation corresponded to changes in total protein (Supplementary Data S6 and S7). The effects of UAB30 treatment upon p53 and MYCN expression were also studied. Neither of these proteins was altered with UAB30 (Supplementary Data S8, S9, S10) and UAB30 did not result in an increase in p53 movement into the nucleus (Supplementary Data S9). These findings imply that the changes seen in differentiation and cellular survival induced by UAB30 likely did not involve these pathways and that other mechanisms may be involved.
UAB30 decreased in vivo tumor growth in a nude mouse model of neuroblastoma.

An in vivo model of neuroblastoma tumor growth following UAB30 treatment was employed using female athymic nude mice. SK-N-AS or SK-N-BE(2) neuroblastoma cells (2.5 × 10^6 in Matrigel) were injected into the right flank of each mouse (n = 20/cell line). On the day of injection, mice were randomized to receive standard chow (control, vehicle) or chow with UAB30 added (n = 10/group). UAB30 was administered at a dose (100 mg/kg body weight) previously shown to be well tolerated by this species (21). Tumors were measured for 28 days. The tumors in the SK-N-AS control-treated animals grew rapidly, and these animals required euthanasia by 28 days (Fig. 4A). The animals with SK-N-AS tumors treated with UAB30 had significantly smaller tumors than the control animals beginning at day 7 (Fig. 4A). At 28 days when all control animals had expired, the average tumor size in controls was 2,249 ± 83 mm^3 versus 1,031 ± 188 mm^3 in the UAB30-treated animals (P < 0.001). After 28 days, the remaining UAB30-treated animals were followed for survival until euthanasia parameters dictated by IACUC were reached. Kaplan–Meier curves were constructed and animal survival compared with log-rank test (Fig. 4B). The UAB30-treated animals had significantly increased mean survival compared
with vehicle-treated controls (31.6 ± 1.6 vs. 21.4 ± 1.4 days, UAB30 vs. control, \(P \leq 0.0001\); Fig. 4B).

Similar results were noted with the SK-N-BE(2) xenografts. By 8 days postinjection, animals treated with vehicle had significantly larger tumors compared with the UAB30 treated animals. At 28 days, the mean tumor volume in control animals was 1,872 ± 259 mm\(^3\), versus 362 ± 120 mm\(^3\) in the UAB30-treated animals (\(P \leq 0.0001\); Fig. 4A). The remaining control and UAB30-treated animals were followed for survival and Kaplan–Meier curves constructed. Data were evaluated with log-rank test and the Meier curves generated. The mice bearing the SK-N-AS tumors had significantly smaller tumors after 13-cis-RA treatment compared with controls up until 25 days following tumor cell injection (Fig. 5A).

As current regimens for high-risk neuroblastoma utilized 13-cis-RA in the setting of MRD, we wished to determine whether UAB30 treatment was as effective as 13-cis-RA in vivo. SK-N-AS or SK-N-BE(2) neuroblastoma cells (2.5 × 10\(^6\) in Matrigel) were injected into the right flank of female nude mice (n = 30/cell line). On the day of injection, mice were randomized to receive standard chow (control, vehicle), chow with UAB30 added (100 mg/kg), or chow with 13-cis-RA added (13-cis RA, 53 mg/kg; n = 10/group); both doses previously shown to be well tolerated (13, 21). Tumors were measured until the control tumors reached parameters set forth by the IACUC for euthanasia when all animals were euthanized. The mice bearing the SK-N-AS tumors had significantly smaller tumors after 13-cis-RA treatment compared with controls up until 25 days following tumor cell injection (Fig. 5A).
However, those animals treated with UAB30 had significantly smaller tumors than both controls (930 ± 320 mm³ vs. 2,185 ± 60 mm³, UAB30 vs. control, \( P \leq 0.001 \)) and those treated with 13-cis-RA (930 ± 320 mm³ versus 2,114 ± 212 mm³, UAB30 vs. 13-cis RA, \( P \leq 0.01 \); Fig. 5A). Similar results were noted with the SK-N-BE(2) xenografts. Early in the experiment, animals treated with vehicle alone had significantly larger tumors compared with the 13-cis-RA–treated animals (Fig. 5B). However, the animals treated with UAB30 had significantly smaller tumors than both the control (178 ± 97 mm³ versus 1,862 ± 293 mm³, UAB30 vs. control, \( P \leq 0.0001 \)) or 13-cis-RA–treated animals (178 ± 97 mm³ versus 1,717 ± 304 mm³, UAB30 vs. 13-cis-RA, \( P \leq 0.001 \); Fig. 5B). Animals in this experiment were not followed for survival.

**Discussion**

The investigation of retinoids for cancer treatment has been ongoing for over 25 years. In 1983, Flynn and colleagues published their findings that treatment of bone marrow promyelocytes from a patient with acute promyelocytic leukemia with 13-cis-RA resulted in cellular maturation and cessation of proliferation in vitro and maturation of bone marrow cells in vivo (22). Subsequent studies demonstrated efficacy of 13-cis-RA treatment in cutaneous and cervical squamous cell carcinoma (23). Phase I and II trials have also shown efficacy of 13-cis-RA in combination with chemotherapeutic agents (24) or interferon therapy (25) in the treatment of advanced head and neck squamous cell carcinoma. In the treatment of neuroblastoma, the initial studies of neuroblastoma cell lines. For example, in the noncancerous Ito cell line, RA treatment inhibited PDGF-induced cellular proliferation. In tumor cells, Tabata showed that all-trans-retinoic acid (ATRA) reduced PDGF-dependent migration of malignant pleural mesothelioma cells (34). Further, Palomares demonstrated that PDGF and EGF-induced cellular proliferation of rhabdomyosarcoma cells was blocked by ATRA (35). Expression of MYCN was also examined in this study as other retinoids have been shown to downregulate MYCN mRNA (4), but MYCN protein expression was unchanged by UAB30 (Supplementary Fig. S10). Clearly, the exact mechanisms involved in UAB30-induced cellular alterations are not entirely apparent, but will be the subject of future studies.

In these studies, we noted various degrees of apoptosis with UAB30 treatment depending upon the cell lines treated. Although PARP cleavage was noted in all six cell lines by immunoblotting, cleavage of caspase-3 was not clearly seen via immunoblotting in the SK-N-BE(2) and SH-SY5Y cell lines, but was found to be increased when evaluated with a caspase-3 activation kit. These findings are not unique. Celay and colleagues noted variations in apoptosis in neuroblastoma cell lines that were treated with ATRA that were not only cell line dependent, but also time dependent. In their study, the SK-N-BE(2) cell line took up to 9 days of treatment before showing significant changes in TUNEL assay and the SH-SY5Y cell line showed no change in TUNEL assay even after 9 days of treatment (32). We had similar results with the SH-SY5Y cell line when cell cycle was studied. There was a significant increase in the number of cells in G1 and a decrease in S-phase in all cell lines except SH-SY5Y. This cell line demonstrated an increase in cells in G1, but the number of cells in S-phase was not significantly decreased. Other investigators have seen retinoid-induced changes in cell cycle that were not only cell line dependent, but dependent upon the type of retinoid studied (36). For example, Villani and colleagues demonstrated that in neuroblastoma tumor cells, fenretinide, had minimal effect on the G1 phase of the cell cycle, and its metabolite significantly affected the accumulation of cells in the G2–M phase (37). In contrast, studies by Di Marino found an accumulation of neuroblastoma tumor cells in the G0–G1 phase following treatment with retinoic acid (18).

The dose of UAB30 chosen for the in vivo studies was based upon previous experience from our laboratory. The animals were given UAB30 mixed into their food at dose of 400 mg/kg diet which translated to 100 mg/kg body weight per day (31). The animals tolerated this dose without significant changes in mucous membranes or skin. They did not, however, gain weight as quickly as their control counterparts; but their smaller size did not result in a difference in tumor:body weight ratios (Supplementary Fig. S11), indicating that the differences in animal growth were not responsible for the decreased tumor size. We chose to use a model of minimal residual disease (MRD) for this study because current standard of care for...
neuroblastoma is to administer 13-cis-RA in a state of MRD. This administration is used because initial studies of 13-cis-RA in children with active neuroblastoma failed to demonstrate efficacy (26), but when administered following stem cell transplant, led to significantly increased survival (2). In addition, as our data for UAB30 and others’ data for retinoic acid have demonstrated, retinoids are superior at affecting cellular differentiation versus cytotoxicity. Finally, we felt that it would be important to demonstrate that UAB30 was efficacious in the setting of MRD, especially when comparing it to 13-cis-RA to demonstrate its future translational possibilities.

In the current study, we demonstrated that neuroblastoma cell survival was decreased by a novel retinoid, UAB30. In addition, this agent led to alterations in cellular phenotype that resulted in cellular differentiation, cell-cycle arrest and decreased migration and invasion in vitro. Most notable was the decreased tumor growth in in vivo graft models and results that were comparable or better than the currently employed agent, 13-cis-RA. The reduced toxicity profile and these current results suggest that UAB30 may be useful as maintenance therapy for children with neuroblastoma.

Disclosure of Potential Conflicts of Interest

V.R. Atigadda and D.D. Muccio have ownership interest in patent 11/661,030. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

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References


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