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

Alicia M. Waters, Jerry E. Stewart, Venkatram R. Atigadda, Elizabeth Mroczek-Musulman, Donald D. Muccio, Clinton J. Grubbs, and Elizabeth A. Beierle

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): 1-11. ©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 Matthy 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 hypertiglyceridemia 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 cell 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 obtained within 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 (30-2001, 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 N-acetyl-L-cysteine (Sigma).
penicillin/streptomycin (Gibco). SH-EP and WAC(2) cell lines were maintained in RPMI1640 medium (30-2001, ATCC) with 10% FBS (Hyclone), 2 mM 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 (29272), anti-phospho-AKT (9271), anti-ERK1/2 (9102), anti-MYC(N) (9450), 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-RRX 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 media 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 phenylmethanesulfonfylfluoride. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce) and separated by electrophoresis on SDS-PAGE gels. Antibodies were used according to manufacturer’s recommendations. Molecular weight markers (Precision Plus Protein Kaleidoscope Standards, Bio-Rad) confirmed the expected size of the target proteins. Immunoblots were developed with Luminata Classic or Crescendo ECL (EMD Millipore). Blots were stripped with stripping solution (Bio-Rad) at 37 °C for 15 minutes and then reprobed with selected antibodies. Equal protein loading was confirmed with immunoblotting with antibody to β-actin or GAPDH.

Cell viability, proliferation, apoptosis assays
Cell viability was measured with AlamarBlue assay. A total of 1.5 × 10^3 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-SY5Y cell lines, activation of caspase-3 was also measured with a caspase-3 activation kit.

Cell-cycle analysis
Cells were plated (1.0 × 10^6 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 × 10^5 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 [SK-N-AS and SK-N-BE(2), (8 × 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-SY5Y cells as they did not propagate in an adherent fashion.

Immunofluorescence
Immunofluorescence staining was utilized to detect movement of RRX and p53 into the nucleus following UAB30 (10 µmol/L). Cells were plated on glass chamber slides and allowed to attach. SH-SY5Y and IMR-32 cells 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. MetaMorph Microscopy Image Analysis Software (Ver. 7.6, Analytical Technologies, Molecular Devices) analyzed the images and detected overlap.

**In vivo tumor growth**

Six-week-old, 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 (140109064) and in compliance with institutional, national, and NIH animal use guidelines. Human neuroblastoma cells, SK-N-AS or SK-N-BE(2) [2.5 ± 10^5 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-RV-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/L 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, rehydrated, and steamed. Sections were quenched with 3% nasia were met, when they were euthanized with CO2 and nativity also leads to cell-cycle arrest (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 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 found to be increased following treatment with UAB30 (Supplementary Fig. S3), further indicating differentiation. As retinoic acid also leads to cell-cycle arrest in neuroblastoma (18), cell-cycle progression was analyzed. UAB30 resulted in an arrest in G0–G1 progression in all cell lines with an increased

**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 percentage of RXR staining in the nucleus of the cells (Fig. 1C), indicating that UAB30 functioned as a 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 amplified 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 found to be increased following treatment with UAB30 (Supplementary Fig. S3), further indicating differentiation. As retinoic acid also leads to cell-cycle arrest in neuroblastoma (18), cell-cycle progression was analyzed. UAB30 resulted in an arrest in G0–G1 progression in all cell lines with an increased

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**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.
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 0.01 to 78 μmol/L for 48 hours. The staining of RXR in the nucleus was calculated with Metamorph and reported as percent nuclear staining. UAB30 resulted in increased nuclear staining of RXR. Representative photomicrographs of SH-EP cells stained for RXR (red) and nucleus (blue) showed increased overlap after UAB30 treatment (white bar, 20 μm).

Figure 1. UAB30 decreased neuroblastoma cell survival and apoptosis. A, immunohistochemistry was performed on 13 formalin-fixed, paraffin-embedded human neuroblastoma specimens for RXR. RXR staining was seen in all samples examined as shown by brown stain. Representative photomicrograph at 10×. Insert left corner shows negative control and right corner is 40× magnification. B, immunoblotting was completed on SK-N-AS, SK-N-BE(2), SH-EP, WAC2, SH-SY5Y, and IMR-32 cell lines for RXR protein, which was detected in all cell lines. C, immunofluorescence staining and confocal microscopy was used to detect RXR staining in the nucleus with and without treatment of UAB30 (10 μmol/L for 48 hours). The staining of RXR in the nucleus was calculated with Metamorph and reported as percent nuclear staining. UAB30 resulted in increased nuclear staining of RXR. Representative photomicrographs of SH-EP cells stained for RXR (red) and nucleus (blue) showed increased overlap after UAB30 treatment (white bar, 20 μm). D, SK-N-AS (AS), SK-N-BE(2) (BE), SH-EP (SHEP), WAC2 (WAC), SH-SY5Y (SY5Y), and IMR-32 (IMR) cell lines were treated with UAB30 at increasing concentrations. After 48 hours of treatment, cell viability was measured with AlamarBlue assays. Data reported as mean ± SEM. There was a significant decrease in viability in all cell lines following UAB30 treatment starting at 25 μmol/L that reached statistical significance in all cell lines by 50% at 50 μmol/L. E, the LD50 for UAB30 was calculated for all cell lines. This value ranged from 46.1 μmol/L to 78 μmol/L, and did not differ based upon amplification of MYCN. F, to determine whether decreased survival was due to apoptosis, immunoblotting for cleavage of PARP and caspase 3 was completed. Cells were treated with UAB30 at increasing concentrations for 48 hours and lysates were examined. There was an increase in cleaved PARP at the 50 μmol/L concentration in all cell lines, and was evident at lower concentrations in some of the cell lines. G, apoptosis was also detected with immunoblotting for cleaved caspase-3. 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 50 μmol/L UAB30. Cleaved caspase-3 was not detected by immunoblotting in the SK-N-BE(2) (BE) and SH-SY5Y (SY5Y) cell lines.

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

UAB30 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 hallmark 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).

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>
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<tbody>
<tr>
<td></td>
<td>G1 (%)</td>
<td>G2 (%)</td>
<td>S (%)</td>
<td>G1 (%)</td>
<td>G2 (%)</td>
<td>S (%)</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>72.5 ± 0.7</td>
<td>12.5 ± 0.7</td>
<td>18.9 ± 1.9</td>
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<tr>
<td>SK-N-BE(2)</td>
<td>55.2 ± 0.7</td>
<td>63.7 ± 1.2</td>
<td>15.3 ± 0.8</td>
<td>63.6 ± 1.1</td>
<td>12.6 ± 0.2</td>
<td>13.6 ± 1.1</td>
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<tr>
<td>SH-EP</td>
<td>68.5 ± 0.4</td>
<td>74.4 ± 0.2</td>
<td>8.7 ± 0.5</td>
<td>74.6 ± 0.2</td>
<td>12.5 ± 0.3</td>
<td>15.3 ± 0.3</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>76.3 ± 0.5</td>
<td>12.5 ± 0.3</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>SH-SYSY</td>
<td>49.6 ± 3.0</td>
<td>51.3 ± 10.3</td>
<td>20.3 ± 1.3</td>
<td>51.4 ± 10.3</td>
<td>13.2 ± 7.0</td>
<td>30.1 ± 4.3</td>
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<tr>
<td>IMR-32</td>
<td>57.9 ± 2.3</td>
<td>71.7 ± 1.9</td>
<td>14.6 ± 0.7</td>
<td>71.8 ± 1.9</td>
<td>12.4 ± 2.6</td>
<td>27.4 ± 1.7</td>
</tr>
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</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 (Fig. 3B). SH-SY5Y and IMR-32 cells were not studied with scratch assay as they propagated in both an adherent and nonadherent fashion, which was prohibitive for the evaluation of migration by scratch methods.

**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.

**Figure 3.**

UAB30 decreased neuroblastoma cell invasion and migration. A, for invasion, cells were plated in TransWell culture plates with Matrigel coating the top side of the insert. Cells were treated with increasing concentrations of UAB30, allowed to invade for 48 hours, and then fixed, stained, and counted. Invasion was reported as fold change. Invasion was significantly decreased 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 LD50 for the cell lines.
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 \((2.5 \times 10^6\) in Matrigel) were injected into the right flank of each mouse \((n = 20/\text{cell line})\). On the day of injection, mice were randomized to receive standard chow (control, vehicle) or chow with UAB30 added \((n = 10/\text{group})\). UAB30 was administered at a dose \((100 \text{ mg/kg body weight})\) 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 \(2,249 \pm 83 \text{ mm}^3\) versus \(1,031 \pm 188 \text{ mm}^3\) 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

Figure 4.
UAB30 decreased tumor growth and increased animal survival in xenograft models of neuroblastoma. A, human neuroblastoma cells, SK-N-AS or SK-N-BE(2) \((2.5 \times 10^6\) cells), were injected into the right flank of female nude mice. On the day of injection (day 0), animals were randomized to receive vehicle-treated or UAB30-treated chow \((n = 10/ \text{group})\). Tumor growth was followed for 28 days and tumors were measured twice weekly with calipers to calculate tumor volumes. Animals with SK-N-AS xenografts treated with UAB30 had significantly smaller tumors beginning at day 7 that continued to day 28 compared with vehicle-treated animals. Similar results were noted with the SK-N-BE(2) xenografts. Animals treated with UAB30 had significantly smaller tumors than those that were treated with vehicle. This difference was significant beginning at day 8 and continued to day 28. B, mice with neuroblastoma xenografts were followed for survival. Animals were euthanized when IACUC parameters were met. Animal survival was compared with log-rank test. Animals with SK-N-AS xenografts treated with UAB30 had a significant increase in survival compared with vehicle-treated animals \((21.4 \pm 1.4 \text{ vs. } 31.6 \pm 1.6, P \leq 0.0001, \text{ vehicle vs. UAB30})\). Animals treated with UAB30 had similarly increased survival with UAB30 treatment \((27.3 \pm 2.3 \text{ vs. } 39.2 \pm 2.8, P \leq 0.03, \text{ vehicle vs. UAB30})\). C, formalin-fixed, paraffin-embedded SK-N-BE(2) tumor xenografts were stained for Ki67 as a marker of cellular proliferation. In tumor xenografts treated with UAB30, there was significantly less Ki67 staining than controls. Representative photomicrographs of Ki67 staining in control (top) and UAB30-treated (bottom) xenografts.
with vehicle-treated controls (31.6 ± 1.6 vs. 21.4 ± 1.4 days, UAB30 vs. control, P ≤ 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 mm³, versus 362 ± 120 mm³ in the UAB30-treated animals (P ≤ 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 graph and the accompanying representative photomicrographs (Fig. 4C).

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 × 10⁶ 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. Animals with SK-N-AS xenografts treated with UAB30 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. 1,285 ± 60 mm³, UAB30 vs. control, P < 0.01) and those treated with 13-cis-RA (930 ± 320 mm³ vs. 2,114 ± 212 mm³, UAB30 vs. 13-cis RA, P < 0.01; Fig. 5A). Similar results were noted with the SK-N-EB(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 vs. 1,862 ± 293 mm³, UAB30 vs. control, P ≤ 0.0001) or 13-cis-RA–treated animals (178 ± 97 mm³ vs. 1,717 ± 304 mm³, UAB30 vs. 13-cis-RA, P ≤ 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 13-cis-RA in children with advanced neuroblastoma failed to show efficacy in the face of active disease (26). As RA was a differentiating agent, it was hypothesized that it may be more effective in the setting of minimal residual disease. Therefore, when subsequently studied in children with high risk neuroblastoma, the RA therapy was administered following autologous bone marrow transplantation (2). In this setting, there was clearly a survival benefit and the use of 13-cis-RA has been incorporated into standard therapy for high risk disease. The issues remaining with 13-cis-RA and other retinoids relate to toxicities. Because of the hypercalcemia, hypertriglyceridemia, skin and mucous membrane issues, the dosage of retinoids is sometimes limited (27). In addition, other formulations such as fenretinide have also demonstrated difficulties in attaining adequate plasma levels and were limited due to formulations that were difficult to administer to young children (28). The absence of known toxicities with UAB30 (7) prompted study of this agent for neuroblastoma.

The potential mechanisms involved in UAB30-induced cellular differentiation remain to be elucidated. Other investigators have found that RA-induced cellular differentiation and apoptosis in neuroblastoma cell lines was dependent upon AKT and ERK activation (29). In contrast, others showed that AKT and not ERK activation was responsible for RA-induced cellular differentiation (19, 30). We were unable to provide evidence that activation of the AKT or ERK pathway was involved in UAB30-induced cellular differentiation in neuroblastoma cell lines.

There are other potential candidates responsible for the cellular changes associated with UAB30, such as p53. It has been reported that p53 was upregulated in tumor cells following retinoid treatment and was responsible for cellular differentiation (31, 32). We investigated p53 expression and found no consistent change by immunoblotting (Supplementary Fig. S8). Furthermore, in p53 wild-type cell lines, UAB30 did not lead to increased p53 in the nucleus, an indicator of its function as a transcription factor (Supplementary Fig. S9). Therefore, activation of p53 by UAB30 was likely not responsible for the phenotypic changes observed in these cell lines. Growth factors such as EGF (33) or PDGF may also be involved. 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-E(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-E(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 instance, Villani and colleagues demonstrated that in neuroblastoma tumor cell lines, 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 xenograft 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.

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References

Authors’ Contributions
Conception and design: A.M. Waters, E.A. Beierle
Development of methodology: A.M. Waters, V.R. Atigadda, E.A. Beierle
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Waters, J.E. Stewart, D.D. Muccio, E.A. Beierle
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Waters, E. Mroczek-Musulman, E.A. Beierle
Writing, review, and/or revision of the manuscript: A.M. Waters, D.D. Muccio, C.J. Grubbs, E.A. Beierle
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.M. Waters, E.A. Beierle
Study supervision: E.A. Beierle
Other (assisted first author with in vivo and in vitro assays and provided scientific support): J.E. Stewart
Other (provided study compound): D.D. Muccio

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