Pre-Clinical Evaluation of a Novel RXR Agonist for the Treatment of Neuroblastoma

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Running title: UAB30 decreased neuroblastoma growth in vitro and in vivo

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Abbreviations: 13-cis RA - 13-cis-retinoic acid; ANOVA - analysis of variance; FACS - fluorescence-activated cell sorting; mRNA - messenger ribonucleic acid; EMEM - Eagle’s minimal essential medium; LD$_{50}$ - lethal dose, 50%; RIPA - radioimmunoprecipitation assay; PARP - Poly ADP-ribose polymerase; SDS-PAGE - sodium dodecyl sulfate polyacrylamide; SPF - specific pathogen free

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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.
Introduction

Neuroblastoma, the most common extracranial solid tumor of childhood, is responsible for over 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) [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 Matthay et al demonstrated improvement in three 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 cheilitis, 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 morphological 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 American Type Culture Collection, ATCC, Manassas, VA. 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 five 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 Dulbecco’s modified Eagle’s medium (DMEM, 30-2601, ATCC) containing 10% fetal bovine serum (Hyclone, Suwanee, GA), 4 mM L-glutamine (Thermo Fisher Scientific Inc., Waltham, MA), 1 μM non-essential amino acids and 1 μg/mL penicillin/streptomycin (Gibco, Carlsbad, CA). SK-N-BE(2) and SH-SY5Y cells were maintained in a 1:1 mixture of minimum Eagle’s medium and Ham’s F-12 medium (30-2004, ATCC) with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine (Thermo Fisher Scientific), 1 μM non-essential amino acids and 1 μg/mL penicillin / streptomycin (Gibco). IMR-32 cells were maintained in EMEM (30-2003, ATCC) with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine (Thermo Fisher Scientific), D-glucose 1.0 gm/L and 1 μg/mL penicillin / streptomycin (Gibco). SH-EP and WAC(2) cell lines were maintained in RPMI 1640 medium (30-2001, ATCC) with 10% fetal
bovine serum (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 (9272), anti-phospho AKT (9271), anti-ERK1/2 (9102), anti-MYCN (9450) and mouse monoclonal anti-β-actin, anti-p53 (1C12) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit anti-phospho ERK1/2 (05-797R) and mouse monoclonal GAPDH (6C5) was from Millipore (EMD Millipore, Billerica, MA). Mouse monoclonal anti-RXR and anti-nestin was obtained from Abcam (clones MOK13-17 and 10C, respectively, Cambridge, MA). UAB30 was synthesized as described [10]. 13-cis-retinoic acid (13-cis-RA) was from Sigma (R3255, Sigma-Aldrich, St. Louis, MO).

Antibodies for immunofluorescence were as listed: primary antibodies p53 (Cell Signaling, 1:1000) and RXR (Abcam, 1:1000) and secondary antibody goat anti-mouse Alexa Fluor 594 (A-11045, Thermo Fisher, 1:33 dilution).

**Cellular differentiation** - Neuroblastoma cells were cultured in standard media or with UAB30 (10 μM, 48 hours). Pictures [Photometrics CoolSNAP HQ2 CCD camera (Tucson, AZ) attached to a Nikon Eclipse Ti microscope (Tokyo, Japan)] 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 RIPA supplemented with protease inhibitors (Sigma), phosphatase inhibitors (Sigma) and phenylmethanesulfonylfluoride. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce, Rockford,
IL) and separated by electrophoresis on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Antibodies were used according to manufacturer’s recommendations. Molecular weight markers (Precision Plus Protein Kaleidoscope Standards, Bio-Rad, Hercules, CA) confirmed the expected size of the target proteins. Immunoblots were developed with Luminata Classico or Crescendo ECL (EMD Millipore). Blots were stripped with stripping solution (Bio-Rad) at 37 ºC for 15 minutes and then re-probed 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® assays. 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). Following treatment, 10 μL of alamarBlue® dye (Invitrogen Life Technologies, Carlsbad, CA) was added and the absorbance at 595 nm was measured using microplate reader (BioTek Gen5, BioTek Instruments, Winooski, VT). Viability was reported as fold change. Cell viability / proliferation were also measured with trypan blue staining. Cells (1.5 × 10^3 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 µM, 48 hours). Cells were trypsinized, washed with PBS, and fixed in 100% ethanol. Ethanol was removed, cells stained with solution containing 0.3 µM propidium iodide (Invitrogen) in 0.1% Triton X and RNAse A (Qiagen, Valencia, CA) for 30 minutes at room temperature, and analyzed with fluorescence-activated cell sorting (FACS) using a FACSCalibur™ Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA). Data were analyzed with ModFit LT software (Verity Software House Inc., Topsham, ME). Negative controls were included in each flow cytometry run.

Cellular Invasion Assay – Twelve-well culture plates (TransWell®, Corning Inc., Lowell, MA) 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 × 10^4 cells / well)] were treated with UAB30 and plated into the upper well. All other cell lines were plated at 4 x 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, twelve-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 x 10^4 cells / well) were treated with UAB30, placed into the upper 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 ($4.5 \times 10^5$ 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 (Tucson, AZ) attached to a Nikon Eclipse Ti microscope (Tokyo, Japan)] were obtained at time zero. Cells were treated with 0, 10 or 25 μM 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 $2 \times 10^5$ cells / well. Wounding assays were not performed with IMR-32 or SH-SY5Y cells since 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 μM). Cells were plated on glass chamber slides and allowed to attach. SH-SY5Y 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 hour, cells were fixed with 3% paraformaldehyde, permeabilized with 0.15% Triton X-100, and the primary antibody was added and incubated at room temperature (RT) for 1 hour. The Alexa Fluor 594 secondary antibody was added for 45 minutes at RT. 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 MicroImaging, LLC, Thornwood, NY) using a 63× objective with a zoom of 0.9. MetaMorph® Microscopy Image Analysis
Software (Ver. 7.6, Analytical Technologies, Molecular Devices, Sunnyvale, CA) analyzed the images and detected overlap.

**In Vivo Tumor Growth** – Six week old, female, athymic nude mice were (Harlan Laboratories, Inc., Chicago, IL) 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 × 10^6 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) [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 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 ºC. The staining reactions were developed with VECTASTAIN Elite ABC kit (PK-6100, Vector Laboratories, Burlingame, CA), TSA™ (biotin tyramide reagent, 1:400, PerkinElmer, Inc., Waltham, MA) and DAB (ImmPACT DAB, Vector Laboratories, Burlingame, CA). 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.M.M.) 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].

Data Analysis – Experiments were repeated at least in triplicate, and data reported as mean ± standard error of the mean. Densitometry of western blots was performed using the image histogram analysis feature of Adobe Photoshop® software (Adobe Systems Inc., San Jose, CA). Student’s t-test, Fisher’s 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 6 cell lines used (Fig. 1 B). Further, 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 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 amplified and non-amplified neuroblastoma cell lines showed significantly decreased survival with similar LD_{50} concentrations (Fig. 1E), and these results held true for both non-isogenic and isogenic MYCN cell lines. The LD_{50} for UAB30 ranged from 37.8 to 58.3 µM (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, 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 Data Fig. S1, S2).

**UAB30 resulted in cell differentiation and cell cycle arrest.** Retinoids are known to cause cellular differentiation, so we wished to determine if 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 Data Fig. S3), further indicating differentiation. Since 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 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).

Since UAB30 caused G0/G1 cell cycle arrest, we wished to determine if 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 Data Fig. 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 µM, 72 hours) (Supplementary Data Fig. 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 if 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 µM) and allowed to invade for 48 hours. There was a significant decrease in cellular invasion at 10 µM and this change was even more marked at 25 µM (Fig. 3A). Cellular 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, C, respectively). In the membrane migration, migration by all cell lines except SK-N-BE(2) was significantly decreased after 10 µM, but at 25 µM 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 non-adherent 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 if 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 (Supplemental Data S6, S7). The effects of UAB30 treatment upon p53 and MYCN expression were also studied. Neither of these proteins was altered with UAB30 (Supplemental Data S8, S9, S10) and UAB30 did not result in an increase in p53 movement into the nucleus (Supplemental 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 2249 ± 83 mm³ versus 1031 ± 188 mm³ 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 to 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 eight days post-injection, animals treated with vehicle had significantly larger tumors compared to the UAB30 treated animals. At 28 days, the mean tumor volume in control animals was 1872 ± 259 mm$^3$, versus 362 ± 120 mm$^3$ 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 animals with SK-N-BE(2) xenografts treated with UAB30 had a significant increase in their mean survival compared to control animals (39.2 ± 2.8 vs. 27.3 ± 2.3 days, p ≤ 0.03, UAB30 vs. control) (Fig. 4B). The SK-N-BE(2) tumor xenografts were examined for Ki67 staining to determine cellular proliferation. UAB30 treatment significantly reduced cellular proliferation as shown in the graph and the accompanying representative photomicrographs (Fig. 4C).

Since 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-retinoic acid in vivo. SK-N-AS or SK-N-BE(2) neuroblastoma cells (2.5 × 10$^6$ in Matrigel$^\text{TM}$) 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 to 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 versus 2185 ± 60 mm³, UAB30 vs. control, p ≤ 0.01) and those treated with 13-cis RA (930 ± 320 mm³ versus 2114 ± 212 mm³, UAB30 vs. 13-cis RA, p ≤ 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 to 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 1862 ± 293 mm³, UAB30 vs. control, p ≤ 0.0001) or 13-cis RA treated animals (178 ± 97 mm³ versus 1717 ± 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]. Since 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 (Supplemental Data Fig. S8). Further, 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 (Supplemental Data 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 epidermal growth factor (EGF) [33] or platelet derived growth factor (PDGF) may also be involved. For example, in the non-cancerous Ito cell line, retinoic acid 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 (Supplemental Data 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 et al noted variations in apoptosis in neuroblastoma cell lines that were treated with all-trans-retinoic acid (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 nine 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 et al 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 Data 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 RA 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-RM 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-RM. The reduced toxicity profile and these current results suggest that UAB30 may be useful as maintenance therapy for children with neuroblastoma.

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AI027767) and High Resolution Imaging (grants P30 CA013148 and P30 AR048311) with special thanks to Shawn Williams for his assistance with confocal microscopy.

References


<table>
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<th>Cell Line</th>
<th>G1 (%)</th>
<th>G2 (%)</th>
<th>S (%)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>UAB30</td>
<td>Control</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td></td>
<td></td>
<td></td>
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<td>67.9 ± 0.6</td>
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<td>71.7 ± 1.9</td>
<td>14.6 ± 0.7</td>
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</table>

Table 1. Cell cycle progression following treatment with UAB30 as measured by flow cytometry. Numbers bolded and italicized are statistically significantly from controls (p ≤ 0.05).
Figure Legends

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 µM for 48 hr). 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 ± standard error of the mean. There was a significant decrease in viability in all cell lines following UAB30 treatment starting at 25 µM that reached statistical significance in all cell lines by 50 µM. E The LD_{50} for UAB30 was calculated for all cell lines. This value ranged from 46.1 µM to 78 µM, and did not differ based upon amplification of MYCN. F To determine if 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 μM 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 μM UAB30. Cleaved caspase 3 was not detected by immunoblotting in the SK-N-BE(2) (BE) and SH-SY5Y (SY5Y) cell lines.

**Figure 2. UAB30 induced cellular differentiation in neuroblastoma cell lines.**  **A** Neuroblastoma cell lines were treated with UAB30 at 10 μM for 24 hours and photographs were obtained. Cells were examined for the presence of neurite outgrowths indicating differentiation. In all cell lines, neurite outgrowths (*black arrows*) were seen following UAB30 treatment.  **B** Neurite outgrowths were quantified by counting the number of neurite outgrowths per cell and were reported as fold change neurite outgrowths. UAB30 significantly increased neurite outgrowths in all cell lines. **C** Cells were treated with 10 μM UAB30 for 48 hours and cell cycle was analyzed with propidium iodide staining and flow cytometry. The data for all cell lines in graphic form: black bars represent percentage of cells in G1, dark grey represent percentage of cells in G2, and light grey represents the percentage of cells in S phase. In all cell lines UAB30 treatment resulted in an increase of cells in G1, and in all cell lines except SH-SY5Y there was decrease in S phase. **D** Representative FACS histograms of SH-EP neuroblastoma cells before (*upper panel*) and after (*lower panel*) UAB30, where there was an increase in the percentage of cells in the G1 phase and a decrease in the percentage of cells in the S phase.
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 μM UAB30. **B** Cellular migration was assessed in a similar fashion with the bottom side of the insert coated with collagen Type 1. 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 μM 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 μM UAB30, a concentration well below the LD$_{50}$ for the cell lines.

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 / 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 to 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 to vehicle treated animals (31.6 ± 1.6 vs. 21.4 ± 1.4 days, UAB30 vs. vehicle, p ≤ 0.0001). Animals with SK-N-BE(2) xenografts had similarly increased survival with UAB30 treatment (39.2 ± 2.8 vs. 27.3 ± 2.3 days, p ≤ 0.03, UAB30 vs. vehicle).

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 (upper panel) and UAB30 (lower panel) treated xenografts.

**Figure 5.** UAB30 decreased tumor growth compared to 13-cis-RA treatment. A Human neuroblastoma cells, SK-N-AS (2.5 × 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 (control / vehicle), UAB30-treated (UAB30) or 13-cis-retinoic acid-treated (13-cis RA) chow (n = 10 / group). Tumor growth was measured twice weekly with calipers until 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 tumors that were significantly smaller than both control (930 ± 320 mm³ versus 2185 ± 60 mm³, UAB30 vs. control, p ≤ 0.01) and 13-cis RA treated (930 ± 320 mm³ versus 2114 ± 212 mm³, UAB30 vs. 13-cis RA, p ≤ 0.01) animals. Human neuroblastoma cells, SK-N-BE(2) (2.5 × 10⁶ 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 (control / vehicle), UAB30-treated (UAB30) or 13-cis-retinoic acid-treated (13-cis RA) chow (n = 10 / group). Tumor growth was measured twice weekly with calipers and animals were followed until control tumors reached parameters set forth by the IACUC for euthanasia when all animals were euthanized. Animals with SK-N-BE(2) xenografts treated with UAB30 had significantly smaller tumors than control animals (178 ± 97 mm³ versus 1862 ± 293 mm³, UAB30 vs. control, p ≤ 0.0001) or animals treated with 13-cis RA (178 ± 97 mm³ versus 1717 ± 304 mm³, UAB30 vs. 13-cis RA, p ≤ 0.001).
Figure 1

A. Image of tissue section.

B. Western blot images of RXR and GAPDH.

C. Bar graph showing percent RXR nucleus, with fold change survival.

D. Graph showing fold change survival with concentration of UAB30 (µM).

E. Bar graph showing UAB30 LD50 (uM) with MYCN status.

F. Western blot images of PARP, Cleaved PARP, and β-actin.

G. Western blot images of Caspase 3, Cleaved Caspase 3, and β-actin.

Legend:
- AS
- BE
- SHEP
- WAC
- SY5Y
- IMR
- RXR
- GAPDH
- MYCN -
- MYCN +
Figure 2

A. Control SK-N-AS, UAB30 (10 μM), Control SK-N-BE(2), UAB30 (10 μM)

SH-SY5Y, SK-N-BE(2), IMR-32, SH-EP, WAC2

SH-SY5Y, SK-N-BE(2), IMR-32, SH-EP, WAC2

B. *p ≤ 0.05, Control vs. UAB30

Fold Change Neurite Outgrowths

0 μM UAB30, 10 μM UAB30

C. *p ≤0.05 Control vs. UAB30

Percent

0 20 40 60 80 100

D. SH-EP Control

G1 68.4%
S 21.9%

G1 74.6%
S 11.7%
Figure 3

A. 

Fold Change Invasion

Concentration UAB30 (µM)

* p ≤ 0.05 Control vs. UAB30

** p ≤ 0.01 Control vs. UAB30

B. 

Fold Change Migration

Concentration UAB30 (µM)

* p ≤ 0.02 Control vs. UAB30, except SK-N-BE(2)

** p ≤ 0.01 Control vs. UAB30

C. 

Percentage Scratch Closed

UAB30

* p ≤ 0.05 Control vs. UAB30
**Figure 4**

A. 

- SK-N-AS Control
- SK-N-AS UAB30
- SK-N-AS BE(2) Control
- SK-N-AS BE(2) UAB30

B. 

Mean Survival ± SEM (days)

- SK-N-AS: 21.4 ± 1.4 vs. 31.6 ± 1.6, *p ≤ 0.0001, Vehicle vs. UAB30
- SK-N-BE(2): 27.3 ± 2.3 vs. 39.2 ± 2.8, *p ≤ 0.03, Vehicle vs. UAB30

C. 

*p ≤ 0.02, Control vs. UAB30

*UAB30 Initiated on June 22, 2017. © 2015 American Association for Cancer Research. mct.aacrjournals.org Downloaded from
Figure 5

**A.**

SK-N-AS

* p ≤ 0.01, UAB30 vs. Control
** p ≤ 0.01, UAB30 vs. 13-cis RA
† p ≤ 0.05, 13-cis RA vs. Control

**B.**

SK-N-BE(2)

* p ≤ 0.01, UAB30 vs. Control
** p ≤ 0.01, UAB30 vs. 13-cis RA
† p ≤ 0.05, 13-cis RA vs. Control
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