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Anticancer efficacy of *p*-dodecylaminophenol against high-risk and refractory neuroblastoma cells *in vitro* and *in vivo*

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ABSTRACT

Neuroblastoma is an aggressive and drug-resistant refractory cancer. The human high-risk neuroblastoma cell line, SK-N-AS (non-amplified N-myc) is derived from stromal cells and it is resistant to treatment with retinoic acid (**1**, RA), which is a chemotherapeutic agent used to induce neuronal cellular differentiation of neuroblastomas. We have developed *p*-dodecylaminophenol (**3**, *p*-DDAP), based on *N*-(4-hydroxyphenyl)retinamide (**2**, 4-HPR), a synthetic amide of **1**, since **1** and **2** are associated with the side-effect of nyctalopia. In order to evaluate the effects of **3** on high-risk neuroblastomas, we employed SK-N-AS cells as well as a second high-risk human neuroblastoma cell line, IMR-32, which is derived from neuronal cells (amplified N-myc, drug sensitive). Compound **3** suppressed cell growth of SK-N-AS and IMR-32 cells more effectively than **1**, **2**, *p*-decylaminophenol (**4**, *p*-DAP), *N*-(4-hydroxyphenyl)dodecananamide (**5**, 4-HPDD) or *N*-(4-hydroxyphenyl)decananamide (**6**, 4-HPD). In SK-N-AS cells, **3** induced G_0/G_1 arrest and apoptosis to a greater extent than **1** and **2**. In IMR-32 cells, **3** induced apoptosis to a similar extent as **1** and **2**, potentially by inhibiting N-myc expression. In addition, *i.p.* administration of **3** suppressed tumor growth in SK-N-AS-implanted mice *in vivo*. Since **3** showed no effects on blood retinol concentrations, in contrast to reductions following the administration of **2**, it exhibited excellent anticancer efficacy against high-risk neuroblastoma SK-N-AS and IMR-32 expressing distinct levels of N-myc. Compound **3** may have potential for clinical use in the treatment of refractory neuroblastoma with reduced side effects.

Neuroblastoma is the most common pediatric extracranial solid tumor.[1] It accounts for 15% of pediatric cancer deaths.[2] Greater than 40% of all children with neuroblastoma are designated as high-risk patients, based on adverse features, including ~18 months age at presentation, the presence of disseminated disease, unfavorable histological features, and amplification of the N-myc oncogene.[3] It also has a 3-year event-free survival rate of only 20%.[2] Current treatments for neuroblastoma consist of a coordinated sequence of chemotherapy, surgery, and radiation.[2–5] Only one-third of children with high-risk disease are expected to be long-term survivors when subjected to these regimens. Surviving children remain at risk for additional health problems related to long-term toxicities of chemotherapy. Failures in therapy underscore the need for more effective and less toxic regimens.

Neuroblastomas are heterogeneous in their biological characteristics, tumor stage, and patient age. They exhibit important prognostic factors that strongly correlate with survival, which are used for treatment assignment.[6] Genetic abnormalities, including DNA ploidy and amplification of the N-myc oncogene, are markers in determining the tumor phenotype and predicting treatment outcomes, and these are used to categorize patients into four groups; very low risk, low risk, intermediate risk, and high risk.[7,8] In addition, neuroblastomas have a dependency on angiogenesis, such that high vascularity is characteristic for the progressed tumor stages and poor outcome.[9] In order to assess anti-cancer efficacies of new agents, we have used the human neuroblastoma cell lines, SK-N-AS and IMR-32, which are from cancers in high-risk stages.[10]

Abbreviations: **1**, RA, all-*trans* retinoic acid; **2**, 4-HPR, *N*-(4-hydroxyphenyl) retinamide, fenretinide; **3**, *p*-DDAP, *p*-dodecylaminophenol, 4-(dodecylamino) phenol; **4**, *p*-DAP, *p*-decylaminophenol, 4-(decylamino)phenol; **5**, 4-HPDD, *N*-(4-hydroxyphenyl)dodecananamide, *p*-dodecanoylaminophenol; **6**, 4-HPD, *N*-(4-hydroxyphenyl)decananamide, *p*-decanoylaminophenol; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid; RT-PCR, reverse transcription-polymerase chain reaction; MRP1, multidrug resistance-related protein 1; SD, standard deviation.

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Table 1
Properties of neuroblastoma cell lines.

	SK-N-AS	IMR-32
N-myc	non-amplified	amplified
Chromosome	1p deleted	1p deleted
vs. **1**	resistant	growth inhibition
Derived	6 years old girl	13 months old boy
Type	S (stromal)	N (neuronal)
p53 family	p73 deleted	Normal
MDR	MRP1 (+)	Unknown

MDR (multidrug-resistance).

SK-N-AS cells are derived from stromal cells of a 6-year old girl (S-type),[11] harbor a 1p-deleted chromosome, and overexpress angiogenetic proteins (placenta growth factor and vascular endothelial growth factor)[12] and the multidrug resistance-related protein 1 (MRP1).[13] In addition, SK-N-AS cells are poorly differentiated, do not have an amplified *MYCN*, and are resistant to drugs, including retinoids (Table 1).[14,15] IMR-32 cells are derived from the neuronal cells of a 13-months old boy (N-type), harbor a 1p-deleted chromosome and overexpress angiogenetic proteins (vascular endothelial growth factor etc.). IMR-32 cells are also poorly differentiated, have an amplified bad prognostic factor *MYCN*, and are sensitive to drugs, including retinoids (Table 1).[10,16]

Retinoids are neuronal cell-differentiating agents that serve as anti-tumor agents for neuroblastoma therapy. Among retinoids, the use of 13-*cis*-retinoic acid following autologous bone marrow transplantation improves overall survival in children with high-risk neuroblastoma.[5] However, depression of rod function and dark adaptation are side effects, which are consistent with retinoid metabolite concentrations in the circulation.[1] On the other hand, *N*-(4-Hydroxyphenyl)retinamide (**2**, fenretinide) (Fig. 1), a synthetic amide of all-*trans*-retinoic acid (**1**, RA), is an effective anticancer drug, which is used against a wide variety of tumor types.[17] Compound **2** is currently in clinical trials for the treatment

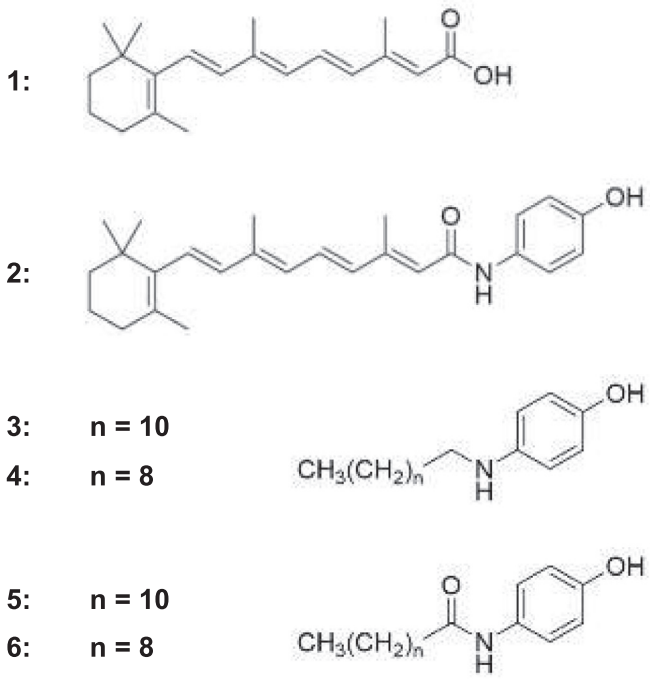

Fig. 1. Chemical structures of **1**, **2**, alkylaminophenol (**3**, **4**), and acylaminophenols (**5**, **6**).

of breast, bladder, renal, and neuroblastoma malignancies.[18–21] However, these studies have shown that treatment with **2** is accompanied by night blindness, due to a decrease in serum retinol levels.[22] It appears that this side effect occurs by the displacement of retinol from serum retinol binding protein (RBP), a regulator of plasma retinol levels, thereby reducing delivery of retinol to the eyes (nyctalopia).[23] In order to maintain potent anticancer activity, while having reduced side effects that would be incurred by treating with **1** and **2**, we have developed agents whose design is based on the assumption that the cyclohexene unit of both **1** and **2**, is responsible for their association with RBP.[24–26] We synthesized four new compounds, which had lengths of carbon side chains similar to that of **2**; *p*-dodecylaminophenol (**3**, *p*-DDAP), *p*-decylaminophenol (**4**, *p*-DAP), *N*-(4-hydroxyphenyl)dodecananamide (**5**, 4-HPDD), and *N*-(4-hydroxyphenyl)decananamide (**6**, 4-HPD) (Fig. 1).[24] Herein, we evaluate these derivatives of **2** (Fig. 1), with particular emphasis on **3**. In the current studies, we examine whether retinoids and aminophenols are effective against SK-N-AS cells and compare them with IMR-32 cells (Table 1) *in vitro*. We determine whether **3** can suppress *in vivo* proliferation of SK-N-AS cells, which are refractory and have significant drug-resistance.

First, we examined the effects of **1**, **2**, and **3** (Fig. 1) on the growth of SK-N-AS cells and IMR-32 cells by treating with various concentrations of **1**, **2**, and **3** for 24 h and 72 h. Cell growth was suppressed by retinoids in dose- and time-dependent manner as shown in Fig. 2A. The growth of SK-N-AS cells incubated for 24 h and 72 h at a concentration of 1 μM, was inhibited approximately 82% and 90% by **3**, 2% and 10% by **2**, and 0% and 3% by **1** as compared with controls, respectively (Fig. 2A). These results indicate that **3** reduced SK-N-AS cell growth drastically at early time periods, while **1** and **2** showed little or no effect, respectively, and that **3** exhibited the most potent anti-proliferative effects among these retinoids against drug-resistant SK-N-AS cells.

On the other hand, growth inhibition of IMR-32 cells incubated with retinoids for 24 h at a concentration of 0.1 μM were approximately 78% for **3**, 4% for **2**, and 9% for **1** (Fig. 2B). In contrast, 72 h-treatment with 0.1 μM and 0.4 μM retinoids, suppressed IMR-32 cell growth approximately 92% and 100% by **3**, approximately 17% and 27% by **2**, and approximately 60% and 65% by **1**, respectively. In converse, at a low concentration of 0.01 μM, cell growth inhibition was approximately 47% for **1** and approximately 15% for **3** and **2**. These results indicate that **3** showed inhibitory effects on IMR-32 cell growth at early time periods, while **1** showed effects at late time period, and that IMR-32 cells are distinguished from SK-N-AS cells by their high sensitivity toward **1**. This suggests that **3** is a potent anti-proliferative agent against both SK-N-AS and IMR-32 cells.

Next, we examined growth inhibition against SK-N-AS cells incubated with other *p*-aminophenols for 24 h and 72 h. Growth inhibition with *p*-alkylaminophenols (**3** and **4**) was much greater