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ORIGINAL ARTICLE

**Ann M. Straight · Kevin Oakley · Russell Moores
Andrew J. Bauer · Aneeta Patel · R. Michael Tuttle
J. Jimeno · Gary L. Francis**

Aplidin reduces growth of anaplastic thyroid cancer xenografts and the expression of several angiogenic genes

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Abstract *Background*: Anaplastic thyroid cancer (ATC) is one of the most aggressive and highly lethal human cancers. Median survival after diagnosis is 4–6 months despite available radiotherapy and chemotherapy. Additional treatments are needed for ATC. Vascular endothelial growth factor (VEGF) is a potent angiogenic stimulus, which is expressed by ATC. Previously, anti-VEGF antibody was used to block VEGF-dependent angiogenesis in ATC xenografts. This treatment induced partial (56%) but not complete tumor regression. Aplidin (APLD) is a marine derived antitumor agent currently in phase II clinical studies. Multiple activities of this compound have been described which likely contribute to its antiproliferative effect. Notably, APLD has been shown to have antiangiogenic properties which include: inhibition of VEGF secretion, reduction in the synthesis of the VEGF receptor (FLT-1), and blockade of matrix metalloproteinase production by endothelial cells. We hypothesized that Aplidin, with its broad spectrum of action and anti-angiogenic properties, would be a potentially effective drug against ATC. *Methods*: Thirty BALB/c nu/nu mice were injected with ATC cells (ARO-81, 1×10^6) and allowed to implant for 3 weeks. Animals were randomized to receive daily intraperitoneal injections of vehicle, low dose (0.5 mg/kg/day), or high dose (1.0 mg/kg/day) APLD. After 3 days, the animals were killed and the tumors were removed, weighed, and divided for RNA and protein analyses. *Results*: APLD significantly reduced ATC xenograft growth (low dose, 20% reduction, $P=0.01$; high dose, 40% reduction, $P<0.001$). This was associated with increased levels of apoptosis related proteins polyadenosylribose polymerase 85 (PARP-85, 75% increase, $P=0.024$) and caspase 8 (greater than fivefold increase, $P=0.03$). APLD treatment was further associated with lost or reduced expression of several genes that support angiogenesis to include: VEGF, hypoxia inducible factor 1(HIF-1), transforming growth factor-beta (TGFβ), TGFβ receptor 2 (TGFβR2), melanoma growth stimulating factor 1 (GRO1), cadherin, and vasostatin. *Conclusions*: This data supports the hypothesis that APLD may be an effective adjunctive therapy against ATC. The demonstrated molecular impact against angiogenic related genes specifically supports future strategies combining APLD with VEGF interacting agents.

Keywords Thyroid cancer · Aplidin · Angiogenesis

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A. M. Straight · A. J. Bauer · G. L. Francis
Department of Pediatrics, Walter Reed Army Medical Center, Washington, DC, USA

K. Oakley · R. Moores · A. Patel · A. M. Straight
A. J. Bauer · G. L. Francis (✉)
Department of Pediatrics,
Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD, 20814 USA
E-mail: gfrancis@usuhs.mil
Tel.: +1-301-2959716
Fax: +1-301-2953898

R. M. Tuttle
Section of Endocrinology, Memorial Sloan Kettering Cancer Center, New York, NY, USA

J. Jimeno
PharmaMar SAU RD Colmenar Viejo, Madrid, Spain

Introduction

Thyroid cancer affects 6.7 women and 2.5 men/100,000 persons annually [1]. For women, this is similar to the frequency of leukemia (7.3/100,000/year) or cervical cancer (8.6/100,000/year) [35]. Most thyroid cancers are well differentiated and have a low associated

mortality. Anaplastic thyroid carcinoma (ATC) accounts for 3–5% of all thyroid cancers. It develops from the dedifferentiation of papillary or follicular carcinomas in about half of all cases. ATC is one of the most aggressive human cancers. Patients typically have wide spread local invasion and a high frequency of distant metastases at the time of diagnosis. In contrast to the differentiated thyroid cancers, disease specific mortality is almost 100%. Median survival from diagnosis ranges from 4 months to 6 months despite available chemotherapy and external beam radiation therapy [2, 30, 45]. More effective treatments are needed for ATC.

Over the past several decades, there has been intensive research in the area of tumor angiogenesis and in the therapeutic utility of antiangiogenic treatments for solid tumors. It is widely accepted that as tumors grow they exceed the diffusion limit of oxygen from existing blood vessels and must be able to stimulate new blood vessel formation in order for growth to continue and metastasis to occur. In tumor angiogenesis, hypoxia-caused by avascular growth increases transcription of several angiogenic genes and stabilizes the mRNAs of key growth and survival genes [5, 11]. Vascular endothelial growth factor (VEGF) is the most prominent of the pro-angiogenic molecules. VEGF promotes endothelial regeneration, increases vascular proliferation, stimulates the formation of collateral blood vessels, inhibits the function of antigen presenting cells, and is involved in the chemotaxis of monocytes, production of tissue factors, and lymphangiogenesis [16, 29, 50]. Additionally, VEGF may function as an apoptotic protector for newly formed vessels via the phosphatidylinositol 3-kinase/Akt signaling pathway [6, 22].

Recent studies have identified VEGF and the VEGF receptor (Flt-1) as critical for the growth of thyroid carcinomas of all types [19, 29, 46, 47]. Differentiated thyroid carcinomas that express VEGF and Flt-1 have the greatest tumor growth and recurrence risk [19, 29]. VEGF is robustly expressed by ATC [2, 5, 23, 25, 27, 31]. Based on the critical role for VEGF in a variety of thyroid cancers, we previously used systemic VEGF monoclonal antibody (VEGF-MAb) to block VEGF-supported angiogenesis in ATC (ARO-81 cells) xenografts [5]. Tumor growth was reduced by 56%, but was not fully arrested in any of the treated animals. We suspected that blockade of angiogenesis at multiple levels might have a greater effect on tumor growth.

Aplidin, (APLD, PharmaMar, Inc., Madrid, Spain) is a marine-derived antitumor agent isolated from the Mediterranean tunicate *Aplidium albicans* [8, 10, 12, 15]. Specifically, it is a cyclic depsipeptide structurally related to didemnin B (Fig. 1) [40]. APLD has been shown to be cytotoxic against a broad spectrum of tumor types in both preclinical studies and clinical phase I and phase II studies. Several mechanisms of action have been identified, which likely contribute to APLD's anti-proliferative effect. APLD has been shown to: (1) interfere with the cell cycle by inducing a G_1 arrest and a G_2 blockade,

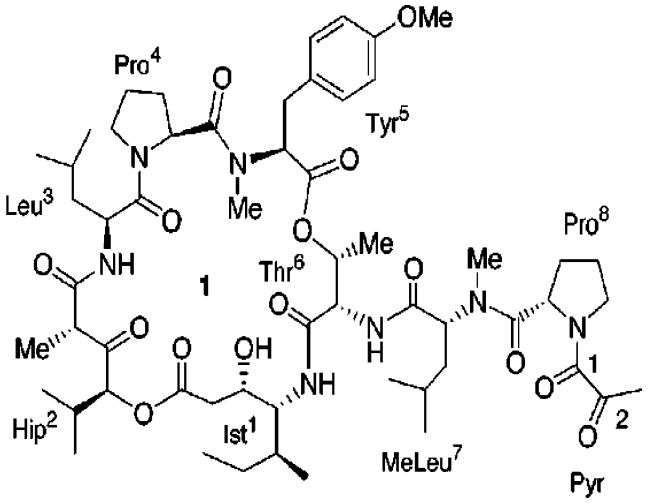

Fig. 1 Chemical structure of Aplidin. Aplidin, (APLD, PharmaMar, Inc., Madrid, Spain) is a marine-derived antitumor agent isolated from the Mediterranean tunicate *Aplidium albicans* [8, 10, 12, 15]. Specifically, it is a cyclic depsipeptide structurally related to didemnin B

(2) inhibit protein synthesis via GTP-dependent elongation factor 1-alpha, (3) inhibit palmitoyil protein thioesterase, involved in signal transduction pathways associated with cell proliferation, and (4) inhibit ornithine decarboxylase, the rate-limiting enzyme in the biosynthesis of polyamines and a protein involved in the signaling pathway downstream the Ras proto-oncogen [13, 14, 18, 48]. Importantly, APLD also has been shown to have antiangiogenic activity in in vivo and in vitro models at clinically relevant concentrations. APLD appears to act at multiple levels of the angiogenic cascade. In vitro it has been shown to: inhibit secretion of VEGF by the human leukemia cells MOLT-4, to block the expression of the VEGF receptor, Flt-1, and to inhibit endothelial functional responses to angiogenic stimuli [8, 44].

Aplidin has completed phase I trials with evidence of a positive therapeutic index. The dose limiting toxicities (DLTs) reported included: muscular toxicity, asthenia, skin rash, and diarrhea [28]. The associated muscular toxicity was characterized by muscle pain and muscular weakness with late increases in creatine kinase [28]. L-carnitine was given as a 24-h pretreatment or co-administered to prevent myotoxicity. Co-administration of L-carnitine was proven to be able to improve the recovery of the drug-induced muscular toxicity and allowed for dose escalation of APLD [39]. Safety data demonstrated a lack of associated myelotoxicity except for mild lymphopenia [28]. Notably, there appears to be no or very little cross-resistance between APLD and other chemotherapeutic agents [7]. APLD is currently being given in phase II trials at a dose of 5 mg/m^2 every other week delivered by intravenous infusion over 3 h [28].

Aplidin appears to be an excellent and potent inhibitor of neo-vascularization and cell division. Based on

the favorable biochemical profile of APLD, we designed this study to determine the effect of APLD on ATC xenografts.

Materials and methods

Grant support

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Cell cultures

ARO-81 cells (a generous gift from Dr. G. Juillard, University of California, Los Angeles, CA, USA) were maintained in Dulbecco's modified Eagle Media supplemented with 10% fetal bovine serum, sodium bicarbonate (0.375%), glutamine (2 mM), nonessential amino acids (1X), and gentamycin (0.01 mg/ml) in an atmosphere of 5% CO_2/air (95% humidity, 37°C).

Animal procedures

Following the approval by the Investigational Animal Care and Use Committee (IACUC), Uniformed Services University, Bethesda, MD, 30 BALB/c nu/nu mice were anesthetized and injected sub-cutaneously with 1×10^6 ARO-81 cells over the dorsal scapula [4, 5]. Cells were allowed to implant for 3 weeks. Mice were then randomized ($n=10$ for each treatment) to receive one of the following treatments for 3 days.

(a) Negative control—vehicle only (Phosphate buffered saline, PBS)
(b) Low dose APLD—(0.5 mg/kg/day)
(c) High dose APLD—(1.0 mg/kg/day)

All vehicle, low dose APLD, and high dose APLD treatments were administered intraperitonelly. Tumor size was measured daily with skin calipers, and calculated using the formula for the volume of an ellipse [(mm^3) = length×height×width/2]. Animals were allowed unrestricted access to food and water and were sacrificed after 3 days. Tumors were removed, weighed, and divided. One portion was homogenized in RNA-Later (Ambion, Inc. Austin, TX, USA, 1 ml) and stored (−70°C) for microarray hybridizations. The other was fresh frozen (−70°C) for protein extraction and Western blots.

Angiogenesis-gene expression

To determine if APLD treatment had an effect on the expression of angiogenesis-promoting genes, RNA was extracted from control and APLD-treated tumors (Trireagent, 1 ml, 0°C) and used for cDNA microarray comparisons. Biotin-16-deoxy-UTP-labeled single strand cDNA probes were generated according to the manufacturer's instructions (Super Array, Inc., Frederick, MD, USA). Briefly, total RNA (5 μg) was reverse transcribed using MMLV reverse transcriptase, labeled with biotin-16-dUTP and amplified by linear polymerase chain reaction. The probes were then hybridized overnight with GE Array Q Series Human Angiogenesis Gene Array (HS-009) membranes containing cDNA fragments from 96 genes involved in angiogenesis, five housekeeping genes, and a negative control. Following hybridization, the membranes were incubated with alkaline phosphatase conjugated streptavidin and chemiluminescence was visualized by autoradiography. Signal intensities were calculated as a ratio of each angiogenic gene compared to β-actin, and were considered significantly different if there was more than a twofold difference in the ratio between treated and control tumors. Additionally, this system has the capability to detect a target gene product at final concentration of approximately 10 fM.

Western blot procedures

Tumor sections were homogenized in RIPA buffer (0°C) and equal aliquots (10 g) from control and treated tumors were resolved by electrophoresis and visualized by Western blot. Gels were run (200 V, 45 min) and proteins were transferred onto nitrocellulose membranes (30 V, 1 h) using the NOVEX System (Invitrogen Life Technologies, Carlsbad, CA, USA). Membranes were blocked (1% bovine serum albumin) and probed with antibodies specific for the 85-kD cleavage product of polyadenosylribosephosphate polymerase (PARP-85, 1:1,000 dilution, Promega Corporation, Madison, WI, USA), caspase 8 (1:1,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:10,000 dilution, Sigma Chemical Company, St. Louis, MO, USA). Immunoblots were developed using streptavidin-conjugated secondary antibodies (Amersham, Inc. Buckingham, UK) and enhanced chemiluminescent substrate. Chemiluminescence was detected using the BioRad Photo Documentation System (BioRad, Inc., Hercules, CA, USA).

Methods of data analysis

All statistical analyses were performed using SPSS for Windows 95 (version 7.5, SPSS Inc., Chicago, IL, USA). Final tumor volume and the intensity of gene and protein expression were compared between treatments using ANOVA. Nonparametric analyses were performed using χ^2 test as indicated.

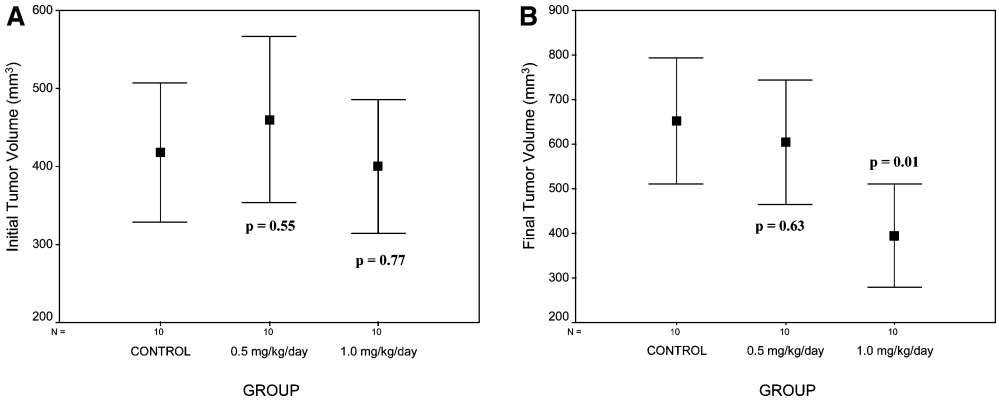

Fig. 2 Volumes of control and treated xenografts. Animals received daily intraperitoneal injections of vehicle (control), low dose (0.5 mg/kg/day) or high dose (1.0 mg/kg/day) Aplidin for 3 days. Initial (Fig. 2a) and final (Fig. 2b) mean tumor volumes ± SEM are shown

Results

Mean tumor volumes (initial and final) for each group are shown in Fig. 2. There were no differences in the initial tumor volumes (Fig. 2a), but final tumor volumes (Fig. 2b) were significantly reduced (40%) in the high dose group (395 ± 58 mm^3) compared to control (653 ± 71 mm^3, $P = 0.01$). The change in tumor size for individual animals was evaluated. When the ratios of final/initial tumor volumes were compared with controls, there were significant reductions in tumor growth for the low dose (20% reduction, $P = 0.01$) and high dose (40% reduction, $P < 0.001$) groups. Furthermore, in six of the high dose animals (6/10, 60%), there was not only a reduction in tumor growth rate but actually a reduction in tumor volume compared to the pretreatment value. These reductions in size ranged up to 29% and are consistent with a partial response. None of the low dose group had a reduction in tumor volume when compared to the pretreatment values but there were six animals (6/10, 60%) in which tumor growth was slowed to less than 25% over the treatment period. By contrast, none of the control animals had a reduction in tumor volume when compared to pretreatment values, and only one animal had a tumor growth rate under 25% (1/10, 10%, growth rate = 17%). The proportion of animals showing a reduction in tumor volume (partial response) or a tumor growth rate less than 25% was significantly greater in the treatment groups compared to control ($P < 0.001$, χ^2).

The effect of APLD treatment on gene expression is shown in Fig. 3. A representative microarray from a control animal (Fig. 3a) is compared with that of a high dose treatment animal (Fig. 3b). Positive control hybridizations (glyceraldehyde-3-PO_4-dehydrogenase and β-actin) were equally visualized in the control and treatment samples. Control ATC xenografts expressed twenty (20) of the ninety-six (96) angiogenic genes screened for by microarray analysis. Notably, APLD treated xenografts retained expression of only 4 of the 20 angiogenic genes expressed by control ATC xenografts. These four genes included: fibroblast growth factor 6 (FGF6, ratio/β-actin = 0.78), transforming growth factor-β receptor 3 (TGFβR3, ratio/β-actin = 0.87), secreted phosphoprotein 1 (SPP1, ratio/β-actin = 0.79) and VEGF D (VEGF-D, ratio/β-actin = 0.78) (Table 1). When expression of each of these four genes was compared between groups, the intensity of expression was similar for FGF6, TGFβR3, and SPP1. However, in the example shown in Fig. 3, the intensity of VEGF-D expression was markedly reduced in the treated xenograft (open arrow, ratio/β-actin = 0.78) compared to the control (solid arrow, ratio/β-actin = 1.05). This suggests that APLD treatment does interfere with VEGF-D expression. All treated animals completely lost expression of tissue inhibitor of metalloproteinase 2, GRO 1 or melanoma growth stimulating factor 1, cadherin, and vasostatin (Table 2). In addition, all but one of the treated animals completely lost expression of tissue inhibitor of metalloproteinase 1, VEGF, TGFβR2, TGFβ1, *Homo sapiens* secreted protein, *Homo sapiens* restin, midkine, *Homo sapiens* macrophage scavenger, hypoxia inducible factor 1, *Homo sapiens* endoglin, prothrombin, and tumor necrosis factor α (Table 3). The reduction in these gene products, but not the positive controls or the retained genes outlined above, indicates a specific effect of APLD that is not a result of generalized inhibition of gene transcription.

Western blot hybridizations were performed for PARP-85, caspase 8, and β-actin. High dose APLD therapy significantly increased the level of important proteins involved in apoptosis when compared to control (Fig. 4). Specifically, the intensity of hybridization was similar for β-actin, but significantly increased for PARP-85 (75% increase, $P = 0.024$) and the 50 kD form of caspase 8 (greater than fivefold increase, $P = 0.03$).

Discussion

Anaplastic thyroid cancer is the most aggressive form of thyroid cancer [2, 31]. The vast majority of patients succumb to disease within months, and no previous treatments have induced remission [2, 31]. Among new drugs available for possible use against ATC, APLD

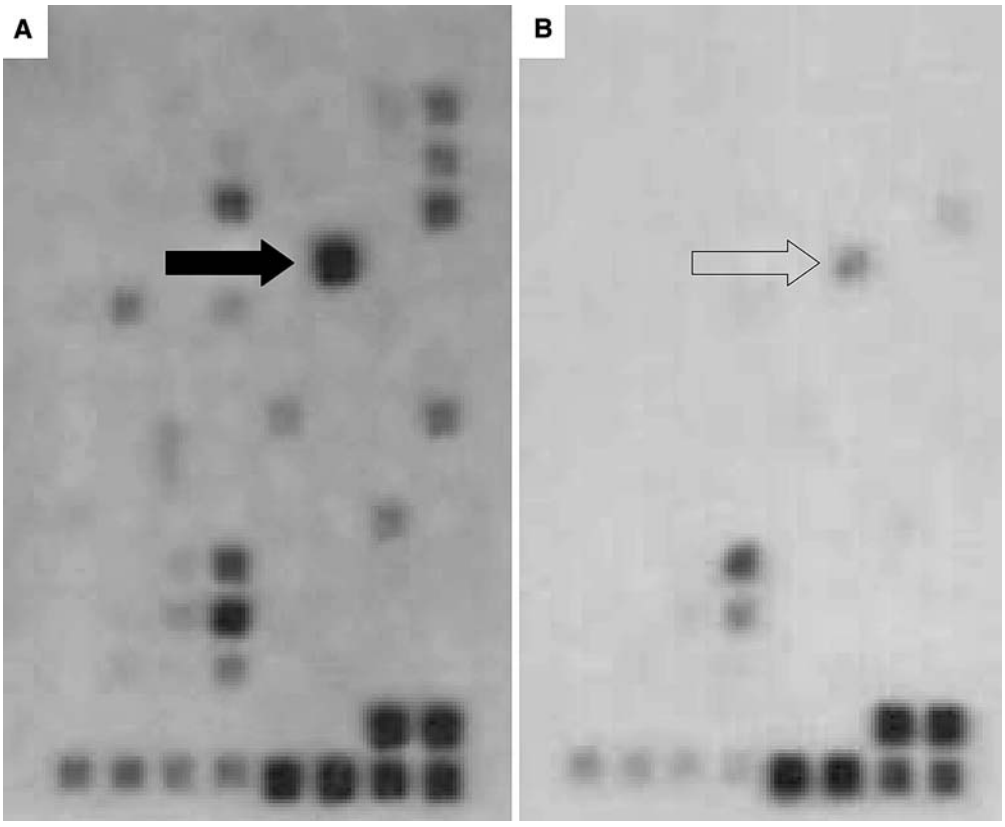

Fig. 3 Effect of Aplidin on angiogenic gene expression. Representative hybridizations are shown for control (Fig. 3a) and Aplidin-treated (Fig. 3b) xenografts. Positive controls (GAPDH and β-actin) are shown along the *bottom two rows*. The intensity of VEGF-D expression was markedly reduced in the treated xenograft (*open arrow*) compared to the control (*solid arrow*)

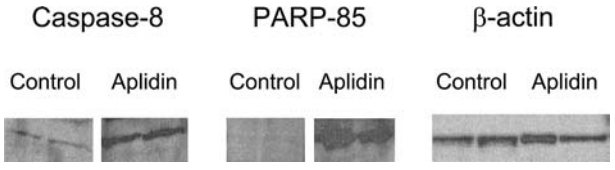

Fig. 4 Expression of apoptosis related proteins by Western blot analysis. Results of Western blot hybridizations for PARP-85, caspase 8, and β-actin are shown in duplicate for control and Aplidin treated xenografts. The intensity of hybridization was similar for β-actin, but significantly increased for PARP-85 (75% increase, $P = 0.024$) and the 50 kD form of caspase 8 (greater than fivefold increase, $P = 0.03$)

has several features that suggest promise. APLD treatment appears to interfere with angiogenesis by reducing the secretion of VEGF, by blocking the expression of the VEGF receptor, Flt-1, and by inhibiting endothelial functional responses to angiogenic stimuli [4, 8, 12, 15, 18, 21, 33, 34, 37, 38, 40, 42]. In addition, APLD inhibits protein synthesis and induces apoptosis in many different cell lines. Based on these effects, we hypothesized that APLD might be effective for the treatment of ATC and tested this against ATC xenografts in nude mice.

We found significant reductions in tumor volume over a 3-day treatment course with either 0.5 mg/kg/day

Table 1 Angiogenic genes retained in Aplidin treated ATC xenografts

Gene name	Function	Intensity[a]
Fibroblast growth factor 6 (FGF6)	Growth factor	0.78
Transforming growth factor β receptor 3 (TGFBR3)	Growth factor receptor	0.87
Secreted phophoprotein 1 (Osteopontin) (SPP1)	Tumor growth suppressor	0.79
Vascular endothelial growth factor D (VEGF)	Vascular growth factor	0.78

[a]Intensity ratio/β-actin

Table 2 Angiogenic genes completely lost from Aplidin treated ATC xenografts

Gene name	Function
Tissue inhibitor of metalloproteinase 2 (TIMP2)	Collagenase inhibitor
GRO1, Melanoma growth stimulating activity (GRO1)	Growth factor
Cadherin (CDH5)	Vascular epithelium adhesion molecule
Vasostatin (CHGA)	Angiogenesis inhibitor

Table 3 Angiogenic genes expressed in trace amounts by only one Aplidin treated xenograft

Symbol	Gene name	Function
TIMP1	Tissue inhibitor of metalloproteinase 1	Collagenase inhibitor
VEGF	Vascular endothelial growth factor	Vascular growth factor
TGFBR2	Transforming growth factor β receptor 2	Growth factor receptor
SPARC	Homo sapiens secreted protein	Cytokine/chemokine
TGFB1	Transforming growth factor, beta 1	Growth factor
RSN	Homo sapiens restin	Microtubule binding protein
MDK	Midkine, neurite growth-promoting factor 2	Cytokine/chemokine
MSR1	Homo sapiens macrophage scavenger receptor 1	Macrophage scavenger receptor
HIF1A	Hypoxia-inducible factor 1	Transcription factor
ENG	Homo sapiens endoglin	Transforming growth factor receptor
F2	Human prothrombin	Coagulation factor
TNFalpha	Tumor necrosis factor alpha	Cytokine/chemokine

or 1.0 mg/kg/day. Of note, several animals in the high dose group not only showed arrested tumor growth but also an actual reduction in tumor volume when compared to their own pretreatment tumor volume. Overall 60% of the high dose group showed a reduction in tumor volume that ranged up to 29%. By contrast, none of the control animals had a reduction in tumor volume and the slowest growth rate was 17% over the 3-day period. These findings directly support our hypothesis that APLD would be more effective against ATC than our previous treatment with VEGF-MAb [5]. In the former experiments using VEGF-MAb, we found an overall 56% reduction in tumor growth rate, but none of the treated animals showed a reduction in tumor volume compared to pretreatment size [5]. These findings also suggest that APLD treatment may be more effective than treatment with combretastatin A4 phosphate (CA4P). CA4P is a tubulin-binding agent, which possesses tumor vascular-targeting behavior [17]. Treatment of ATC xenografts with CA4P has been reported to be associated with a reduced tumor growth rate but not a reduction in tumor volume compared to the pretreatment size [17].

During our investigation into the mechanisms of APLD's action, we found that APLD reduced the expression of many genes that support angiogenesis. In the control tumors, we found moderate (ratio/ß-actin = 0.5–0.7) or intense (ratio/ß-actin = 0.7–1.0) expression of 20 different genes that support angiogenesis. Only 4 of the 20 angiogenic genes (FGF-6, TGFβR3, SPP1 and VEGF-D) were retained with similar intensity in the APLD-treated tumors. The relative importance FGF-6 and SPP1 in the pathogenesis of ATC is unknown. VEGF-D is a lymphangiogenic factor as well as a stimulator of angiogenesis; however, its role in ATC has also not been defined. TGF-β is a known inhibitor of cellular proliferation [25]. TGF-β and the TGFβR2 have previously been identified in papillary, follicular, and anaplastic thyroid cancers [26]. Little is known about the role of TGFβR3. Studies have suggested that an interruption of the TGF-β signaling pathway, either by loss of TGF-β, TGFβR, or Smad expression, may play a role in thyroid tumor progression [26, 49]. In our study TGF-β and its receptors, TGFβR2 and TGFβR3, were expressed by control mice. TGF-β and TGFβR2 expression was lost in all but one of the APLD treated mice. TGFβR3 expression was retained. This data suggests that interference with the TGF-β signaling pathway may be an additional property of APLD.

Aplidin treatment completely blocked detectable expression of the following genes: tissue inhibitor of metalloproteinase 2 (TIMP-2), melanoma growth stimulating factor 1 (GRO1), cadherin (CDH5) and vasostatin (CHGA). The lower limit of sensitivity for the array hybridizations is approximately 10 fM. Each of these gene products is important in tumor angiogenesis, proliferation, or cell adhesion. TIMP-2 is a 21-kD protein with regulatory binding sites for transcription factor Sp1 (Sp1), transcription factor AP2 (AP2), AP1, and others [23]. TIMP-2 appears to activate pro-matrix metalloproteinase 2 [36]. Melanoma growth stimulating factor or growth regulated oncogene (GRO1) is encoded by a gene belonging to the family of chemotactic cytokines known as chemokines (CXC) [41]. GRO is an autostimulatory growth factor for melanoma cells but is also expressed by fibroblasts, endothelial cells, synovial cells, and several tumor cell lines. Cadherin is a calcium ion-dependent cell adhesion molecule that appears to have important roles in transformation of epithelial cells and tumor invasion [9]. Vasostatin is derived from the N-terminus of chromogranin A [1]. Both proteins have vasoinhibitory activity [1]. The protein is released from human polymorphonuclear neutrophils and may represent a component of innate immunity [32]. Vasostatin has been shown to induce adhesion and spreading of fibroblasts and to play a role in the local control of cell adhesion [20].

Vascular endothelial growth factor expression was detected in control tumors, and was lost following APLD treatment of all but one of the treated tumors. This observation is consistent with previous research showing that VEGF is robustly expressed by ATC [2, 5, 23, 25, 27, 30, 31] and extends the results of previous studies that show a reduction in VEGF secretion in response to APLD treatment. If VEGF expression is not completely blocked by APLD treatment, the question arises as to whether treatment of tumors with APLD plus a VEGF-Mab, or another VEGF interacting agent, may further limit or reduce tumor growth.

Aplidin treatment has been previously associated with reduced the expression of the VEGF-receptor, Flt-1 [8, 44]. In our study, the VEGF receptor was not detected in either control or treated tumors. This precludes us from being able to determine if APLD has any effect on Flt-1 expression.

Treatment with APLD was associated with increased the levels of apoptosis promoting proteins including PARP-85 (75% increase) and caspase 8 (greater than fivefold increase). These data are supportive of previous findings in which APLD induced apoptosis in a variety of cell lines, but provide additional insight into the mechanism of APLD's action against ATC [8, 18]. The data suggest that APLD could induce apoptosis directly in ATC xenografts, or might induce apoptosis through the blockade of angiogenesis and the resultant tumor hypoxia that would ensue.

In conclusion, our data show that APLD reduces the in vivo growth of ATC xenografts, inhibits the expression of multiple angiogenic genes, and increases the levels of pro-apoptotic peptides. These findings provide additional information about the mechanism of action for this class of drug, provide rationale for the use of APLD against a wide array of solid tumors, and suggests that combining APLD treatment with a VEGF interacting agents may be of clinical benefit.

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