VEGF inhibition and cytotoxic effect of aplidin in leukemia cell lines and cells from acute myeloid leukemia

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Background: Aplidine (APL) is a marine depsipeptide isolated from the Mediterranean tunicate Aplidium albicans that is under clinical phase II development. In contrast to the lack of bone marrow toxicity reported in phase I/II studies, it has been shown to induce cytotoxicity at very low concentration against lymphoblastic leukemia blast, as well as having an impact in the vascular endothelial growth factor (VEGF)/VEGF receptor 1 loop.

Patients and methods: To confirm these findings we investigated APL-related VEGF inhibition and its cytotoxic effect on myeloid leukemic cells lines (K-562, HEL and HL60) and fresh leukemia blasts derived from 30 patients with acute myeloid leukemia (AML). The conventional active 4-demethoxydaunorubicin (idarubicin; IDA) was included as a positive control.

Results: APL was found to be significantly \((P < 0.001)\) more active than IDA in obtaining 50% growth-inhibition in K-562, HEL and HL60 cell lines. Results obtained with AML blast cells were superimposable. \(\text{ID}_{50}\) ranged from 0.024 to 0.610 \(\mu\text{M}\) for IDA (0.200 ± 0.176) and from 0.001 to 0.108 \(\mu\text{M}\) for APL (0.020 ± 0.031). Annexin V tests and cell cycle analysis performed on cell lines confirmed the stronger citotoxic capability of APL as apoptotic inducer and as a G1 blocker. The inhibitory effects of APL on VEGF release and secretion have been confirmed by ELISA tests performed on HEL: the VEGF concentration in cell surnatant was reduced from 169 to 36 pg/ml after 24 h of exposure to a pharmacological concentration of APL.

Conclusions: APL harbors a strong \textit{in vitro} antileukemic activity at a concentration achievable in patients at non-myelotoxic doses. Our data also support the notion of an impact on VEGF secretion. Clinical studies with this new marine-derived compound in relapsed/resistant leukemia are underway.

Key words: aplidine, marine compounds, myeloid leukemia, VEGF inhibiton

Introduction

Aplidin (APL) (PharmaMar, Colmenar Viejo, Madrid, Spain) (Figure 1) is a novel antitumor agent isolated from the Mediterranean tunicate \textit{Aplidium albicans} by Rinehart et al. in 1990 [1, 2]. This depsipeptide presents a chemical structure closely related to didemnin B, another marine agent previously isolated from the Caribbean tunicate \textit{Trididemnin solidum} [3]. The preclinical \textit{in vitro} and \textit{in vivo} data generated evidence of antitumor activity in different tumor types [4–7] with minimal or lack of myelotoxicity in rodents and dogs, and APL has recently entered phase II clinical trials in a variety of solid tumors as it showed a positive therapeutic index, in particular no bone marrow toxicity and promising pharmacological properties, in phase I studies [8–12]. Objective evidence of activity and clinical benefit have been reported in pretreated melanoma, renal cancer, non-Hodgkin’s lymphoma, colorectal and medullary thyroid carcinoma, and other neuroendocrine tumors [13–15].

The cytotoxic effect of APL seems to be the consequence of affecting several cellular processes including the inhibition of protein synthesis, via GTP-dependent elongation factors 1-alpha [16], and ornithine decarboxylase (ODC) activity [10]. Furthermore, APL induces massive and acute p53-independent apoptosis and G1 cell cycle block [17, 18], as well as deregulation of angiogenic-related genes [19]. Recently, it has also been demonstrated that APL inhibits vascular endothelial growth factor (VEGF) secretion and vascular endothelial growth factor receptor-1 (VEGFR-1/flt-1), preventing autocrine stimulation in the human lymphoid leukemic cell line MOLT-4 [19, 20].

In contrast to the lack of bone marrow toxicity, a number of translational studies have demonstrated strong cytotoxic activity at pharmacological concentration against acute lymphoblastic leukemia blasts explanted from pediatric patients, with lack of cross-resistance with conventional antileukemic agents.
On this basis, this study aimed to characterize the cytotoxic activity of APL on myeloid leukemic cell lines and fresh leukemic cells derived from patients with acute myeloid leukemia (AML). Additionally, its effects on the cell cycle and impact on VEGF production and secretion have also been investigated.

Materials and methods

Drug

APL was kindly supplied by PharmaMar (Madrid, Spain) as dry powder. Stock solutions of drug were prepared in DMSO (Sigma, St Louis, MO, USA) (10⁻³ M) and stored at −20°C. Drug stocks were diluted with cell culture media just prior to their use.

Cell cultures

K-562 (human chronic myelogenous leukemia cell line), HEL (human promyelocytic leukemia cell line) and HL60 (human erythroleukemia cell line) cells were grown in RPMI (Gibco-BRL, Paisley, UK), supplemented with 10% fetal calf serum (Gibco-BRL) and antibiotics at 37°C in an atmosphere that contained 5% CO₂.

AML primary blast cells

Bone marrow or peripheral blood samples were obtained at diagnosis from 30 adult patients (16 females and 14 males) with de novo or secondary AML, after obtaining of a signed informed consent. The diagnosis was based on French–American–British (FAB) criteria. Mononuclear cells (MNCs) were isolated from peripheral blood using Ficoll gradient sedimentation (Gibco-BRL), washed in phosphate-buffered saline (PBS) (Gibco-BRL) and treated as described below. All samples contained >80% of leukemic blasts.

WST-1 cytotoxicity test

Cells lines and MNCs were seeded at a concentration of 25 000 and 50 000 cells/100 μl, respectively, in 96-well plates and exposed to increasing concentrations of idarubicin (IDA) and APL (0.001–1 μM). The incubation time was 72 h for cell lines and 48 h for MNCs. Each concentration of the drugs was tested in quadruplicate; four wells were also prepared for untreated cells and medium. Cell viability was determined with a colorimetric assay, using Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany). The test is based on the cleavage of the tetrazolium salt WST-1 in formazan by mitochondrial dehydrogenases in viable cells [23]. The formazan dye was quantified by a scanning multiwell spectrophotometer by measuring the absorbance of the dye at 450 nm. After the incubation period, Cell Proliferation Reagent WST-1 (10 μl/well) was added and the absorbance was measured after 3 h. ID₅₀ values (concentration of drug required to cause 50% growth inhibition of treated cells compared with control cells) were determined by plotting the percentage of cell survival versus the logarithm of anticancer drug concentration.

Apoptosis (Annexin-V-FLUOS staining kit)

Apoptosis was evaluated in cell lines and MNCs after 48 and 24 h, respectively, of incubation with the drugs (10, 50 and 100 nM). The cells were then collected, washed in PBS and incubated for 15 min with Annexin-V–fluorescein [24, 25] and propidium iodide (Roche Diagnostics) to distinguish apoptotic cells from necrotic cells. Samples were analysed by flow cytometry using 488 nm excitation.

Cell cycle analysis

Cell lines were cultured in serum-free RPMI medium for 24 h. Serum and drugs (0.01–1 μM) were then added for 24 h. After incubation, the cells were fixed in 95% cold ethanol and kept at 4°C overnight. DNA was then stained with propidium iodide (50 ng/ml) (Roche Diagnostics) in the presence of Nonidet P40 0.01% and RNase (Sigma). Samples were incubated for 1 h and DNA histograms analyzed using a FACScan flow cytometer (Becton Dickinson, Sparks, MD, USA).

VEGF mRNA and protein levels

HL60 and HEL cells were used to evaluate APL effects on VEGF release. Cells were seeded in 24-well plates at a concentration of 500 000 cells/ml and treated with APL 10, 50 and 100 nM. After different times, cells and supernatants were collected, washed in PBS and treated with APL 10, 20 and 50 nM. VEGF mRNA and protein levels in untreated cells to evaluate VEGF expression by semiquantitative RT–PCR. VEGF/β-actin primers were used in a ratio of 4:1 in PCR mixture [primers: VEGF₅'-CGA AGT GGT GAA GTT CAT GGA TG-3' and VEGFAS 5'- TTC TGT AGT CTT TTC TGG TGA G-3' (three isoforms: 404, 535 and 607 bp) [26]; β-actin BA5 5'-TGG ACT TCG AGC AAG AGA TG-3' and BA3 5'-GAT CTT CAT TGT GCT GGG TG-3' (320 bp) [27]]. Signal intensities of the products were quantified on 3% agarose gel by densitometric analysis of non saturated bands using Chemi Doc acquisition system (Bio-Rad, Baltimore, MD, USA) and Quantity One Quantification Software (Bio-Rad). The variations in cDNA synthesis in the samples were normalized by their relative quantities of β-actin.

Statistical analysis

Results were analyzed by a standard paired Student’s t-test. A P value of <0.01 was considered statistically significant.

Results

In vitro cytotoxicity of APL and apoptosis

The WST-1 colorimetric cell proliferation assay was performed to investigate in vitro APL cytotoxic activity, compared with that of IDA. The growth inhibition induced in leukemic cells after exposure to IDA and APL was expressed as the percentage of cell survival observed in treated cells versus untreated controls. ID₅₀ values, obtained from dose–response curves, showed that APL was significantly more active than IDA in inhibiting the
growth of K-562, HL60 and HEL cells ($P<0.0001$) (Figures 2 and 3). Moreover, IDA ID$_{50}$ values were $0.075 \pm 0.049 \mu M$ in K-562, $0.184 \pm 0.144 \mu M$ in HL60 and $0.122 \pm 0.085 \mu M$ in HEL experiments, while the correspondent APL ID$_{50}$ were $0.015 \pm 0.013 \mu M$, $0.034 \pm 0.028 \mu M$ and $0.085 \pm 0.049 \mu M$. Further evidence of APL potency was obtained by morphologic cytofluorimetric analysis of cells after treatment: the residual population of viable cells appeared strongly reduced after APL compared with after IDA treatment (Figure 4).

Cytotoxicity tests performed on the MNCs of 30 patients with AML confirmed the results obtained in cell-line experiments (Figure 5A). APL dose–response curves lay under the IDA ones in 28 out of 30 patients, showing a better activity of the marine compounds than IDA (Figure 5B). The ID$_{50}$ mean values were $0.200 \pm 0.176 \mu M$ for IDA and $0.020 \pm 0.031 \mu M$ for APL, consistent with significantly stronger citoxic activity of APL ($P<0.0001$). Furthermore, a comparison between the experimental data on IDA and APL demonstrated the lower range of ID$_{50}$ in response to APL ($0.001$–$0.108 \mu M$) than to IDA ($0.024$–$0.610 \mu M$). Two patients samples were not considered sensitive to IDA and APL because the percentage of vital cells was in each instance was $\geq 70\%$ (Figure 5C).

The percentage of apoptotic cells was determined by Annexin-V-FLUOS staining kit in cell lines and primary cells of AML after incubation (48 and 24 h, respectively) with APL and IDA at different concentrations (10, 50 and 100 nM). The apoptotic index demonstrated a better capability of APL in the induction of apoptosis in AML cells and cell lines (Figure 6), with a $P$ value $<0.001$ for 10 and 50 nM concentrations.

Figure 2. Distribution of ID$_{50}$ values (concentration of drug required to cause 50% growth inhibition of treated cells compared with control cells) obtained from K-562, HEL and HLM treated for 72 h with idarubicin (IDA) and aplidine (APL) (0.001–1 \mu M). ID$_{50}$ values were expressed in \mu M. Comparison between the two compounds (Student’s $t$-test) indicated a statistically significant difference ($P=0.0001$).

Figure 3. Survival plots of cell lines treated with idarubicin (IDA) and aplidine (APL). APL (broken line) dose–response curves lay always under IDA ones (continuous line).
The APL-induced apoptosis was found to be concentration-dependent between 10 and 50 nM, while at the suprapharmacological concentration of 100 nM necrosis of cells was massive, and apoptosis not evaluable.

**Cell cycle analysis**

Analysis of MNCs cycle after 24 h of treatment with APL and IDA showed strong cycle perturbations, with cell populations moving from G2 towards G1 phase with increasing drug concentration. A G1 block was evident at the highest concentration used (1 μM), while at intermediate doses, the G1 block was still evident only in APL-treated cells, while IDA induced a G2 block. An S phase delay was observed when low concentrations (0.01 μM) of APL were used (Figure 7).

**VEGF levels**

Figure 8 shows the levels of VEGF secreted in the medium in HL60 and HEL cells treated with different APL concentrations at different time-points. The reduction of VEGF protein release was dose-dependent in both cell lines and already detectable after 12 h of incubation, and strikingly evident after 24 and 36 h. In contrast, when the mRNA levels were measured, we observed a concomitant dose-dependent accumulation of VEGF transcript in both cell lines, especially for 50 nM concentration of APL (Figure 9).

**Discussion**

In the present study we demonstrated and confirmed that APL, a new and promising anticancer agent currently in phase II clinical trials, has potent *in vitro* cytotoxic and apoptotic effects against three human myeloid leukemia cell lines (K-562, HEL and HL60), as well as against 30 AML blast cells explanted from patients. These data support previous results observed on lymphoblastic cells [17, 20]. Moreover, APL appears to be more effective *in vitro* than IDA as a growth inhibitor in leukemic cell lines and MNCs from AML patients. In line with previous results the APL-related *in vitro* effects are achievable with low concentrations in plasma of patients treated with the recommended dose [28]. Moreover, the lower range of APL ID50 in AML cells in comparison with IDA (0.001–0.108 versus 0.024–0.610 μM) suggests that the efficacy of APL in inhibiting cell proliferation is more predictable than that of IDA. Preliminary data indicate that plasma concentrations of APL up to 20 nM are achievable and sustained, and thus the ID50 is achievable in patients.
Further confirmation was provided by apoptotic assays in which APL clearly shows an extremely potent and rapid apoptotic activity in myeloid cells. In 24 h (AML MNCs) and 48 h (cell lines) experiments this effect was dose-dependent up to 50 nM, while at higher concentration the curve was plateauing due to cell necrosis.

As previously demonstrated [17], APL causes important perturbations of the cell cycle, inducing a block at different phases of the cycle, probably activating cell cycle checkpoints, but its mechanism of action is still unclear. In our studies APL was an effective cycle blocker agent, active already at nanomolar concentrations, and the cell cycle arrest was evident and rapid. In fact, after 24 h incubation APL was able to block K-562, HL60 and HEL cells in G1 phase at concentration of 100 nM, while an S phase delay was observed at 10 nM. IDA obtained the same results at concentrations 10 times higher than those of APL.

Finally, our data confirm that APL modulates VEGF release, probably inhibiting VEGF secretion. Broggini et al. [20] previously demonstrated the capability of APL to inhibit VEGF secretion, down-regulate VEGFR-1, and inhibit VEGF mRNA synthesis. Our results support the first hypothesis: in fact APL induces a strong dose-dependent reduction of VEGF protein release in both cell lines used (HEL and HL60), but mRNA levels did not appear reduced by a 24-h incubation. In contrast, VEGF transcript accumulation was observed in cell cytoplasm, suggesting a possible block of extracellular secretion. As previously reported [29], APL probably induces rapidly an oxidative stress in the cell that is linked to glutathione depletion and causes the activation of a series of kinases including the c-Jun terminal kinase (JNK), p38 mitogen-activated kinase, Src and epidermal

Figure 5. Blast cells obtained from 30 acute myeloid leukemia (AML) patients were treated in vitro with idarubicin (IDA) and aplidine (APL) (0.001–1 μM) for 48 h. (A) Distribution of ID50 values (concentration of drug required to cause 50% growth inhibition of treated cells compared with control cells; μM). There was a statistically different activity (standard paired Student’s t-test) between the two compounds (P < 0.0001). (B and C) Dose–response curves obtained from blast cells of two AML patients, considered sensitive and non-sensitive, respectively, treated in vitro for 48 h with IDA and APL. APL curves lay under IDA ones in 28 out of 30 patients, while the percentage of vital cells was in each instance 70% or higher in two out of 30 patients (C).

Figure 6. Apoptosis induced in cell lines (HL60 only reported) and in blast cells from acute myeloid leukemia (AML) patients treated with idarubicin (IDA) and aplidine (APL) for 48 and 24 h, respectively. Three concentrations of the drugs (10, 50 and 100 nM) were tested. The use of the suprapharmacological concentration of 100 nM induced a massive necrosis and apoptosis was not evaluable. *P < 0.0001; †P < 0.001.
growth factor receptor. Sustained activation of JNK has been found to be crucial for the induction of apoptosis by the drug [30]; however, the activation of these kinases must have additional consequence that remains mostly unknown. VEGF is usually synthesized and secreted in response to cellular hypoxia as a result of the stabilization of the transcription factor hypoxia inducible factor (HIF)-α that activates its promoter. The possibility exists that the cellular oxidative status, perhaps mediated at least partially by these enzymes, causes the blockade of VEGF secretion by affecting the secretory pathway, and in a delayed response can also inhibit VEGF expression via the blockade of HIF-α stabilization or action.

In conclusion, this study has shown that APL is an effective agent displaying both cytotoxic and anti-angiogenic activities when tested in vitro against AML cells, confirming previous results reported in pediatric acute lymphoblastic leukemia blasts. The full experimental results generated with APL in leukemia models supports the notion of a therapeutic potential of APL in patients with relapsed acute leukemia; phase I trials in pediatric patients as well as phase II studies in adult relapsed leukemias are underway.
observed in concomitant with VEGF protein release reduction in medium. A dose-dependent accumulation of VEGF mRNA transcript was non-satured bands (Chemi Doc acquisition system; Bio-Rad).

References


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Figure 9. Vascular endothelial growth factor (VEGF) mRNA levels measured by semiquantitative RT-PCR and densitometric analysis of non-satured bands (Chemi Doc acquisition system; Bio-Rad). A dose-dependent accumulation of VEGF mRNA transcript was observed in concomitant with VEGF protein release reduction in medium.

