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Research Paper

Induction of Resistance to Aplidin® in a Human Ovarian Cancer Cell Line Related to MDR Expression

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KEY WORDS

Aplidin®, marine natural compound, resistant cell line, Pgp expression, flow cytometry

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ABSTRACT

Aplidin®-resistant IGROV-1/APL cells were derived from the human ovarian cancer IGROV-1 cell line by exposing the cells to increasing concentration of Aplidin® for eight months, starting from a concentration of 10 nM to a final concentration of 4 μM. IGROV-1/APL cell line possesses five fold relative resistance to Aplidin®. IGROV-1/APL resistant cell line shows the typical MDR phenotype: (1) increased expression of membrane-associated P-glycoprotein, (2) cross-resistance to drugs like etoposide, doxorubicin, vinblastine, vincristine, taxol, colchicin and the novel anticancer drug Yondelis™ (ET-743). The Pgp inhibitor cyclosporin-A restored the sensitivity of IGROV-1/APL cells to Aplidin® by increasing the drug intracellular concentration. The resistance to Aplidin® was not due to the other proteins, such as LPR-1 and MRP-1, being expressed at the same level in resistant and parental cell line.

The finding that cells over-expressing Pgp are resistant to Aplidin® was confirmed in CEM/VLB 100 cells, that was found to be 5-fold resistant to Aplidin® compared to the CEM parental cell line.

INTRODUCTION

Aplidin®, is a marine depsipeptide isolated from the Mediterranean tunicate *Aplidium albicans* and is structurally related to didemnin B (see Fig. 1). Aplidin® is active against both human haematological and solid tumour cell lines growing in vitro.[1-6] On fresh child-derived B-cell-precursor acute lymphocytic leukaemia (ALL) cells Aplidin®-induced cytotoxicity and apoptosis at very low concentrations.[6,7] Aplidin® was shown to be active against in vivo murine B16 melanoma and in several human tumours growing in nude mice.[8,9]

Several biochemical effects of didemnins have been reported. Amongst these, inhibition of protein, DNA and RNA syntheses in different cell lines.[10,11] Inhibition of protein synthesis via GTP-dependent elongation factor 1-alpha in vitro[12] and inhibition of the ornithine decarboxylase activity by didemnins have been previously described.[1,5] However, the relevance of these biochemical effects for the anti-tumor activity of Aplidin® has not been determined. Aplidin® inhibits the progression of cells from the G_1 to the S phase of the cell cycle and caused a G_2/M blockade.[5] On Molt-4 cells Aplidin® causes an inhibition of VEGF secretion and a downregulation of flt-1.[13] However, Aplidin® cytotoxicity against other ALL cell lines such as Reh, ALL/MIK and Tom-1 was not related to VEGF inhibition suggesting that others mechanisms (e.g., inhibition of other growth factors) might be responsible for the cytotoxic effects.[6] Experiments performed by using a stroma –supported immunocytometric assay showed that cells from children with genetic abnormalities such as t(9;22) and t(4;11) translocations, associated with an inferior treatment outcome, were sensitive to Aplidin® to the same extent as that observed in other BCP-ALL cases[6]. Recently it was found, by using microarray technique, that genes involved in different pathways, such as growth factors, signal transduction or transcription factors were modulated by Aplidin®.[14]

In Phase I and phase II studies that are in progress Aplidin®-induced no neutropenia and only a moderate lymphopenia,[15-17] being myalgia the dose-limiting toxicity.

Considering the pharmacological and clinical interest in this compound, we have attempted to obtain human cell line resistant to Aplidin®. In order to investigate the mechanisms of Aplidin® action and resistance, we have induced Aplidin® resistance in the human ovarian cancer cell line, IGROV-1. In this paper we report the results of these studies.

MATERIALS AND METHODS

Cells and culture conditions. The human ovarian carcinoma IGROV-1 cell line was grown as a monolayer in RPMI-1640 medium supplemented with 10% FBS and 2 mM glutamine (Sigma-Aldrich, St. Louis, MI, USA) at 37°C in a humidified atmosphere containing 5% CO_2 in T25 cm^2 tissue culture flasks (IWAKI, Bibby Sterilin, Staffordshire, UK).

Aplidin®-resistant IGROV-1 cells, IGROV-1/APL, were derived from the cell line by stepwise selection with Aplidin® (kindly supplied by PharmaMar S.A., Colmenar Viejo, Madrid, Spain). Cells were treated for 1h repeatedly for 8 months with increasing concentrations of the drug, starting from a concentration of 10 nM to a final concentration of 4 μM.

CEM, CEM/VM-1 and CEM/VLB 100 were grown at 37°C in a humidified atmosphere containing 5% CO2 in MEM (Sigma) supplemented with 10% Hyclone FBS (Hyclone Laboratories, Inc., Logan, UT, USA), 2 mM glutamine and 1% MEM-vitamins (Sigma) in T25 cm^2 tissue culture flasks (IWAKI).

Clonogenicity test. Resistance to Aplidin® was evaluated using a standard clonogenic assay. Exponentially growing IGROV-1 and IGROV-1/APL cells were treated for 1h with different concentrations of Aplidin®, Yondelis™(ET-743), doxorubicin, melphalan, VP-16, cis-DDP, Oxaliplatin, Taxol, Camptothecin, MNNG, vinblastine or vincristine.

Cells were seeded in 6-well plates at a concentration of 1.000 cells/well. Forty-eight h after seeding, the cells were treated for 1 h with different concentrations of the drugs. After the treatment period, the cells were washed twice with PBS and incubated with fresh drug-free medium. Colonies were allowed to develop for 7–8 days. Plating efficiency of the exponentially growing control IGROV-1 cells was between 40–50%. Colonies were stained with 1% crystal violet solution in 20% methanol and counted using the Entry Level image analysis system (Immagini & Computer, Bareggio, Milan, Italy). A background correction was done and the control cell colony size of colonies having at least 50 cells was established as the minimum for setting the cut-off point.[18]

We have also investigated whether Aplidin® cytotoxicity could be modulated by the mdr phenotype reversing agent cyclosporin-A. For this purpose, both IGROV-1 and IGROV-1/APL cell lines were treated with different concentrations of Aplidin®, alone or in combination with 10 μM of cyclosporin-A. The cytotoxicity was evaluated by the colony assay previously described.

Growth inhibition assay. Leukemia cells in exponentially growing phase were treated for 1 h with different concentrations of Aplidin®. After treatment, cells were washed with PBS and incubated with fresh drug-free medium; viable cells number was estimated by means of a Coulter Counter (Beckman Coulter Corp., Hialeath, FL) at different time intervals after drug-washout.

Flow cytometric analyses of multidrug-related proteins. Pgp MDR-1, MRP-1 and LRP-1 proteins were evaluated using indirect immunofluorescence flow cytometry techniques. Briefly, cells were detached from the tissue culture surface by trypsinization, washed with PBS and then fixed in 70% ethanol. 10^6 fixed cells were washed with PBS containing 2% PHS at room temperature for 10 min. Cells were then incubated for 1h in different sets with the following antibodies: (a) with anti-Pgp antibody, clone MRK16 (catalog n°. MC-012) diluted 1:50 in PHS, (b) anti-MRP-1 antibody, clone MRPr1, (catalog n°. MC-201) diluted 1:30 in PHS, (c) anti-LRP-1 antibody, clone LRP-56, (catalog n°. MC-069) (Kamiya Biochemical, Seattle, WA, USA) diluted 1:30 in PHS. After the incubation, the cells were washed with PHS and incubated with a fluorescein FITC-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgG (Jackson Immuno Research Lab. Inc., West Grove, PA, USA) diluted 1:50 or goat anti-rat diluted 1:50 for MRP-1 for 1h at room temperature in the dark.

MRP-1 and LRP-1 were evaluated in cells after permeabilization in 1% (v/v) lysing solution G (Becton Dickinson, San Jose, CA, USA). Cells were resuspended in PBS and the flow cytometric analyses were performed on at least 20,000 cells for each sample by the FACS Calibur cytometry system (Becton Dickinson) equipped by 488 nm laser and the FITC green fluorescence pulse was detected at 530 nm. A2780 and LoVo/DX cells were used as positive controls.[18]

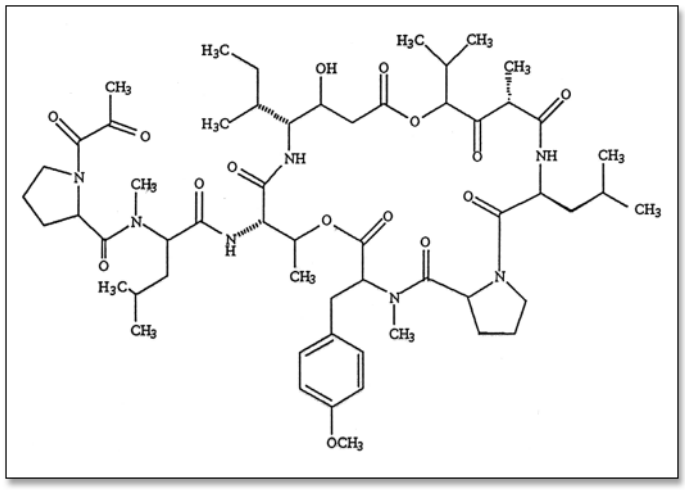

Figure 1. Aplidin® chemical structure.

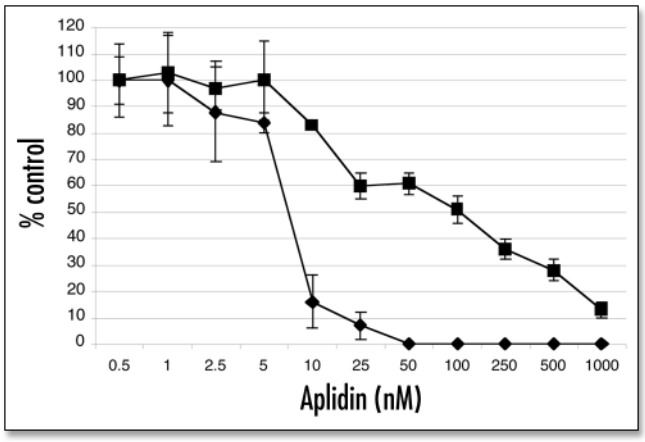

Figure 2. Sensitivity to Aplidin® of IGROV-1 (◆) and IGROV-1/APL (■) cell lines evaluated by colony assay. Each point is the mean of six independent replicates; bars represent S.D.

Cell sorting. In order to better select $Pgp^+$and Pgp^-cell population in the IGROV-1/APL cell line, sorting was performed under aseptic conditions in relation to their Pgp content by using a FACS Vantage SE cytometry system (Becton Dickinson). The two cell populations obtained were then seeded in culture.

Flow cytometric cell cycle analysis. IGROV-1 and IGROV-1/APL cell lines were treated for 1 h with different concentrations of Aplidin®. At different time intervals after drug-washout, the cells were detached and the number of cells was counted by using Coulter Counter and fixed in 70% ethanol. The fixed cells (1–2 x 10^6) were washed with PBS and stained with 1 ml of a solution containing 10 μg/ml PI and 12.5 μl of RNAse 10.000 U (1mg/ml in water) overnight at 4°C in the dark. The flow cytometric analysis were performed by using a FACS Calibur equipped by 488 nm laser and the PI red fluorescence pulse was detected at 620 nm and the distribution of the cells in the different cell cycle phases was calculated.[5]

Measurement of the intracellular concentration of Aplidin®. Liquid chromatography-tandem mass spectrometry analyses were performed using a previously reported method, slightly modified for the analysis of cell pellets. Briefly, IGROV-1 and IGROV-1/APL cells were treated for 1 h with 10 nM Aplidin® alone or in combination with 10 μM cyclosporin-A. At 0, 6 and 24 h after drug washout the cells were harvested and the pellets were stored at -20°C until extraction.

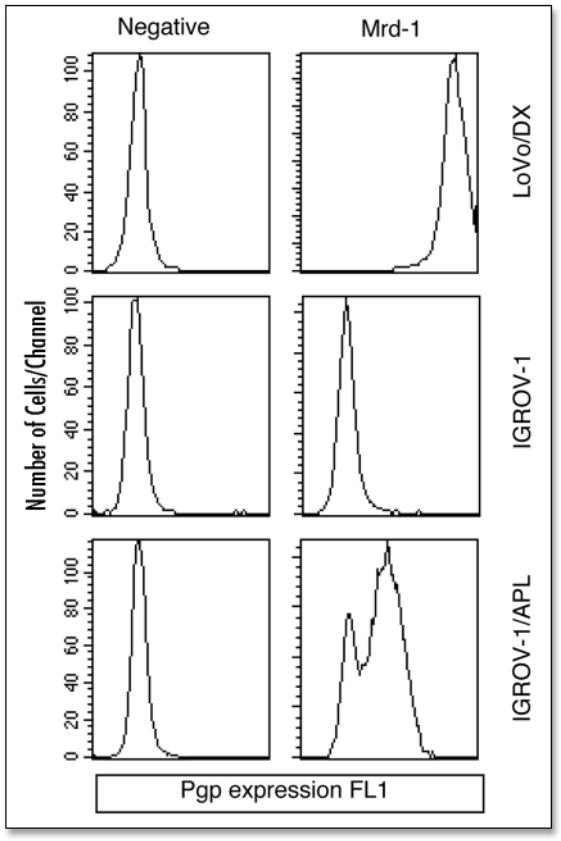

Figure 3. Flow cytometric analysis of MDR-1 expression in IGROV-1 and IGROV-1/APL and cell lines evaluated by flow cytometry. LoVo/DX cell line was used as positive standard for P-gp expression.

Cell pellets were resuspended in 500 µl of drug-free medium. Fifty microliters of the internal standard solution (didemnin B 400 ng/ml in drug-free medium) and 2 ml of acetonitrile containing 1% formic acid were added. After vortexing (5 min) and centrifugation at 2.500 g (10 min), the supernatant was transferred to a new tube. Two ml of chloroform were added. Vortexing and centrifugation were repeated. The upper aqueous phase was discarded. The organic phase was evaporated to dryness under nitrogen flow. The dried residue was stored at -20°C and reconstituted just before analysis with 200 µl of water/acetonitrile/formic acid (49.5:49.5:1, v/v/v) (Carlo Erba, Milan, Italy).

Calibration curves were prepared by serial dilution in drug-free medium of a 2 µM Aplidin® solution to obtain standards ranging from 500 nM to 5 nM. Liquid chromatographic and mass spectrometric conditions, as well as data handling, are described in detail by Celli et al.[19]

Statistical procedures. Statistical analyses were performed by using SAS/STAT software. After assessing the type of distribution followed by the different sets of data, simple regression analysis were performed to evaluate the efficacy of the treatment of the different drugs on both the cell lines. The homogeneity of the regression curves slopes between the two cell lines were evaluated by means of the test of parallelism, in order to show different effect of the drugs on the two cell lines.

RESULTS

Selection and characterization of cells. Figure 2 shows the effect of Aplidin® on the clonogenicity of IGROV-1 and of the subline selected for resistance to the drug, IGROV-1/APL. Resistant cells selection lasted eight months and was obtained by repeatedly treating cells for 1h with increasing concentrations of Aplidin®, ranging from 10 nM to 4 µM.

By using flow cytometry we have characterized the IGROV-1/APL cell line for Pgp expression. We found that the multi-drug resistance protein Pgp was revealed in about the 80% of the IGROV-1/APL cells (Fig. 3). In order to evaluate if the resistance to Aplidin® was due to the expression of the Pgp, we have sorted the IGROV-1/APL resistant cells for their Pgp expression, which were then tested for resistance to Aplidin®. We found that the IC_{50} was 10nM for IGROV-1 and Pgp^-cells and was equal to 50 nM for Pgp^+cells and this value was maintained stable over time, at least one year.

The morphology, growth characteristics and DNA content of IGROV-1/APL resistant cell line were consistent to those of the parental IGROV-1 cell line.

As shown in Figure 4, the expression of both MRP-1 and LRP-1 proteins were similar in both IGROV-1 and IGROV-1/APL cell lines suggesting that they do not play a role in the resistance of IGROV-1/APL to Aplidin®.

Cell cycle analysis. Cells were treated for 1 h with different concentrations of Aplidin® and the cell cycle perturbations induced by the treatment were evaluated at 0, 6, 24, 48 and 72 h after drug washout by flow cytometric cell cycle analysis. Figure 5A shows the growth kinetics of both cell lines: the growth of the

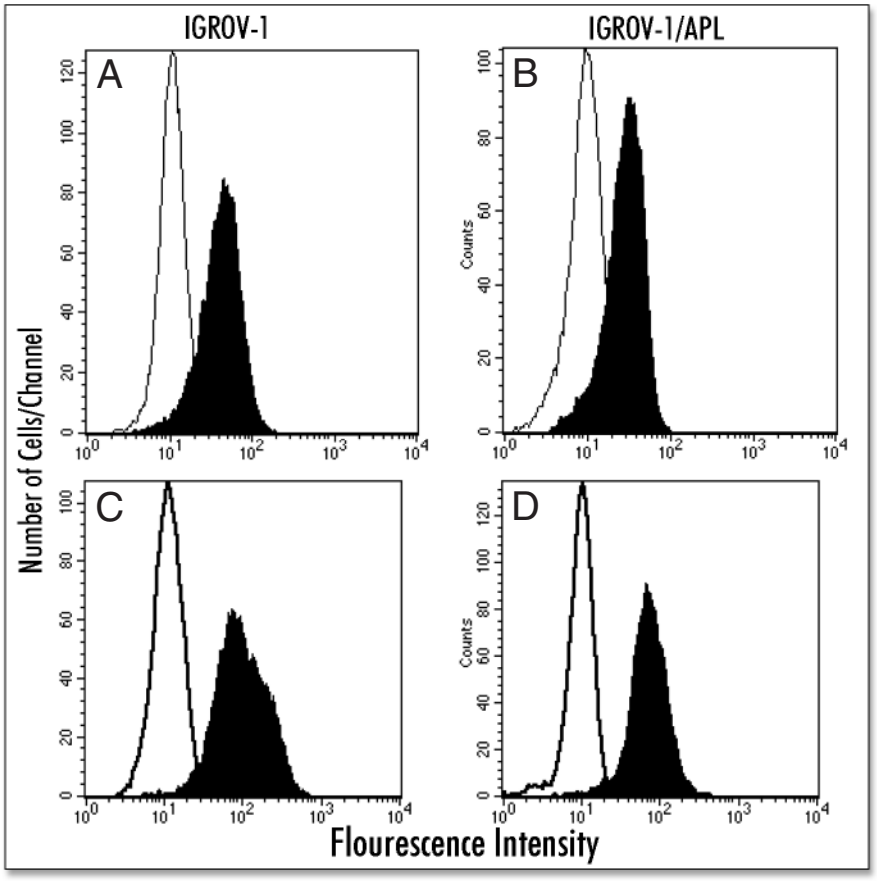

Figure 4. Flow cytometric analysis of LRP-1 (A and B) and MRP-1 (C and D) expression in IGROV-1 and IGROV-1/APL cell lines evaluated by flow cytometry. White histograms refer to the negative fluorescence.

IGROV-1 cell line was affected by Aplidin® treatment even at 10 nM, while the IGROV-1/APL cell line appeared to be slightly sensitive to Aplidin® treatment only during the first 24 h after drug-washout. The DNA histograms (B), refers to the flow cytometric analysis performed at 72 h after drug washout, show that only the parental IGROV-1 and not the IGROV-1/APL cells were blocked in G_1 phase of the cell cycle in a dose dependent manner.

Cross-resistance pattern. We compared the pattern of sensitivity and cross-resistance of the IGROV-1 and IGROV-1/APL cell lines for a panel of anticancer drugs. As shown in Figure 6, IGROV-1/APL cells were cross-resistant to drugs which are known to be a substrate of Pgp such as VP-16, doxorubicin, vinblastine, vincristine, taxol and colchicin. The sensitivity to the novel anticancer drug Yondelis™ (ET-743), which is a substrate of Pgp too,[18] was also reduced. No cross-resistance was observed for melphalan, cis-DDP, Oxaliplatin, MNNG and camptothecin.

Reversal of resistance and Aplidin® uptake. IGROV-1 and IGROV-1/APL cells were treated with Aplidin® alone or in combination with the Pgp inhibitor cyclosporin-A. In IGROV-1/APL line 50 nM Aplidin® caused 50% inhibition of clonogenicity. However, when Aplidin® was added in combination with cyclosporin-A it caused 85-90% inhibition of clonogenicity suggesting that cyclosporin-A, used at the subtoxic concentration of 10 μM, was able to reverse Aplidin® resistance. No differences were observed in the IGROV-1 parental cell line when the clonogenic assay was performed after treatment with or without cyclosporin-A.

The intracellular Aplidin® concentration in IGROV-1 and IGROV-1/APL cells was evaluated by means of liquid chromatography-tandem mass spectrometry after 1 h treatment with 10 nM Aplidin® alone or in combination with 10 μM cyclosporin-A and harvested at 0, 6 and 24 h after drug washout. Table 1 shows that in IGROV-1 cell line cyclosporin-A was completely unable to influence the intracellular concentration of Aplidin® while, in IGROV-1/APL cells, it greatly enhanced cellular drug uptake. At 24 h after drug-washout the intracellular concentration of Aplidin® was 0.18 $x10^{-4}$nMol in IGROV-1/APL cells treated with Aplidin® alone and 0.53 x 10^{-4} nMol in cells treated with the combination Aplidin® and cyclosporin-A and this difference was statistically significant ($p > 0.05$).

Effects on CEM, CEM/VM-1 and CEM/VLB100 cell lines. To corroborate our data about Pgp-mediated Aplidin® resistance, we treated CEM cell lines and the two subclones CEM/VM-1 and VLB100 with Aplidin®.

CEM/VM-1 holds a mutation in the topoisomerase II gene that confers them an atypical multidrug resistance, showing cross resistance to other topoisomerase II poison but not cross-resistant to vinca alkaloids, while CEM/VLB100 is characterized by classical mdr. Figure 7 shows only CEM/VLB100 cell line was resistant to treatment with Aplidin® up to a concentration of 80 nM. On the contrary, CEM VM-1 cells were extremely sensitive to Aplidin® even higher than the parental CEM cell line.

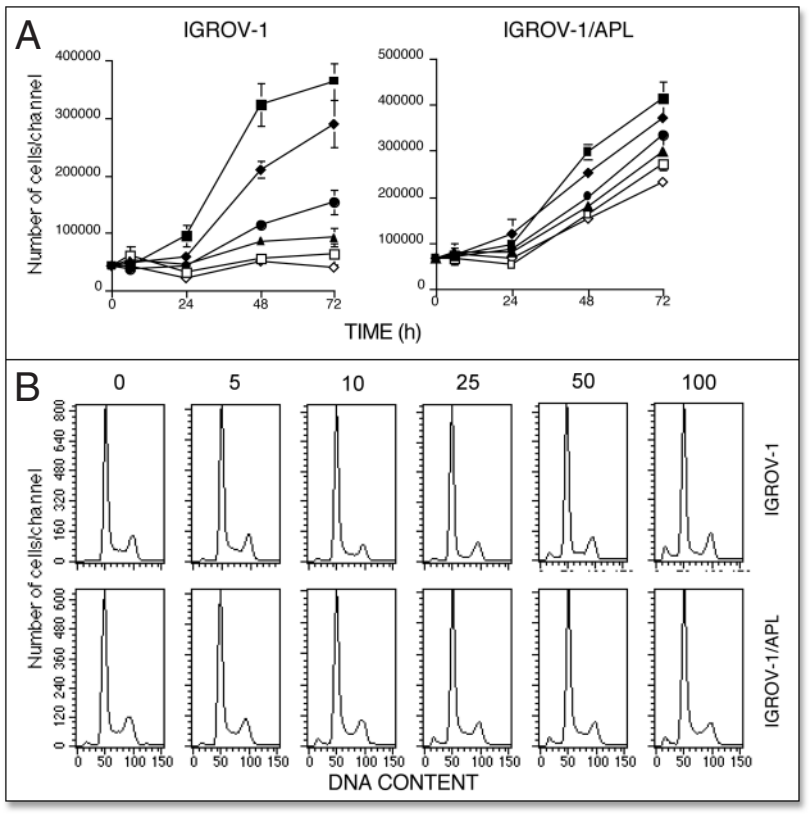

Figure 5. Growth inhibitory effect (A) and cell cycle perturbations (B) induced on IGROV-1 and IGROV-1/APL cells treated for 1 h with 0, 5, 10, 25, 50 or 100 nM Aplidin®. The growth inhibition, evaluated by counting the number of cell by Coulter Counter, and the flow cytometric cell cycle analysis were performed at different time after drug-washout. ■, Control; ◆, 5 nM; ●, 10 nM; ▲, 25 nM; □, 50 nM; ◇, 100 nM.

DISCUSSION

In this work we describe a successful isolation of an Aplidin®-resistant cell line IGROV-1/APL, from the human ovarian cancer cell line IGROV-1, by treating for 1 h repeatedly for eight months with increasing concentrations of Aplidin®.

The main difference between IGROV-1/APL and the parental cell line was the expression of the multidrug transporter Pgp.[20] Aplidin® is not the first peptide to be a substrate for Pgp. It was demonstrated that several hydrophobic peptides (both linear and cyclic) interact with Pgp. It has been reported by Sharma et al.[21] that a CHO cell line resistant to the tripeptide ALLN showed the classical mdr phenotype with the overexpression of Pgp. On the interaction between peptides and Pgp Sarkadi et al.[22] have reported that different hydrophobic peptides stimulate the Pgp ATPase activity. Sharom et al.[23] showed that a radiolabeled tripeptide was transported by Pgp into both membrane vesicles and reconstituted proteoliposomes. Several peptides can reverse the Pgp-mediated resistance to cytotoxic drugs: both cyclic peptides such as cyclosporin-A, its non-immunosuppresive derivate PSC833, or linear peptides, pepstatin A, were reported to antagonize Pgp-mediated drug transport.[18,22-25]

IGROV-1/APL resistant cell line shows the typical mdr phenotype: (1) cross-resistance to drugs like VP-16, doxorubicin, vinblastine, vincristine, taxol and colchicin and the sensitivity to the novel anticancer drug Yondelis™ (ET-743) was reduced;[18] (2) increased expression of membrane-associated Pgp, with consequent decreased intracellular drug retention. Also the observation that the Pgp inhibitor cyclosporin-A[26] could restore the sensitivity of IGROV-1/APL cells to Aplidin® by increasing drug-intracellular concentration is consistent with the conclusion that Aplidin® is a substrate for Pgp. The experiments performed with CEM cell lines further confirmed our results: CEM/VLB100 cells, over-expressing Pgp, were resistant to Aplidin®, while CEM/VM-1 cells, which hold an atypical multi-drug resistance involving mutation of DNA topoisomerase II[27] were not.

That one of the mechanism of resistance to Aplidin® is related to Pgp overexpression was confirmed also by the sensitivity studies performed on the Pgp-positive and Pgp-negative cell populations

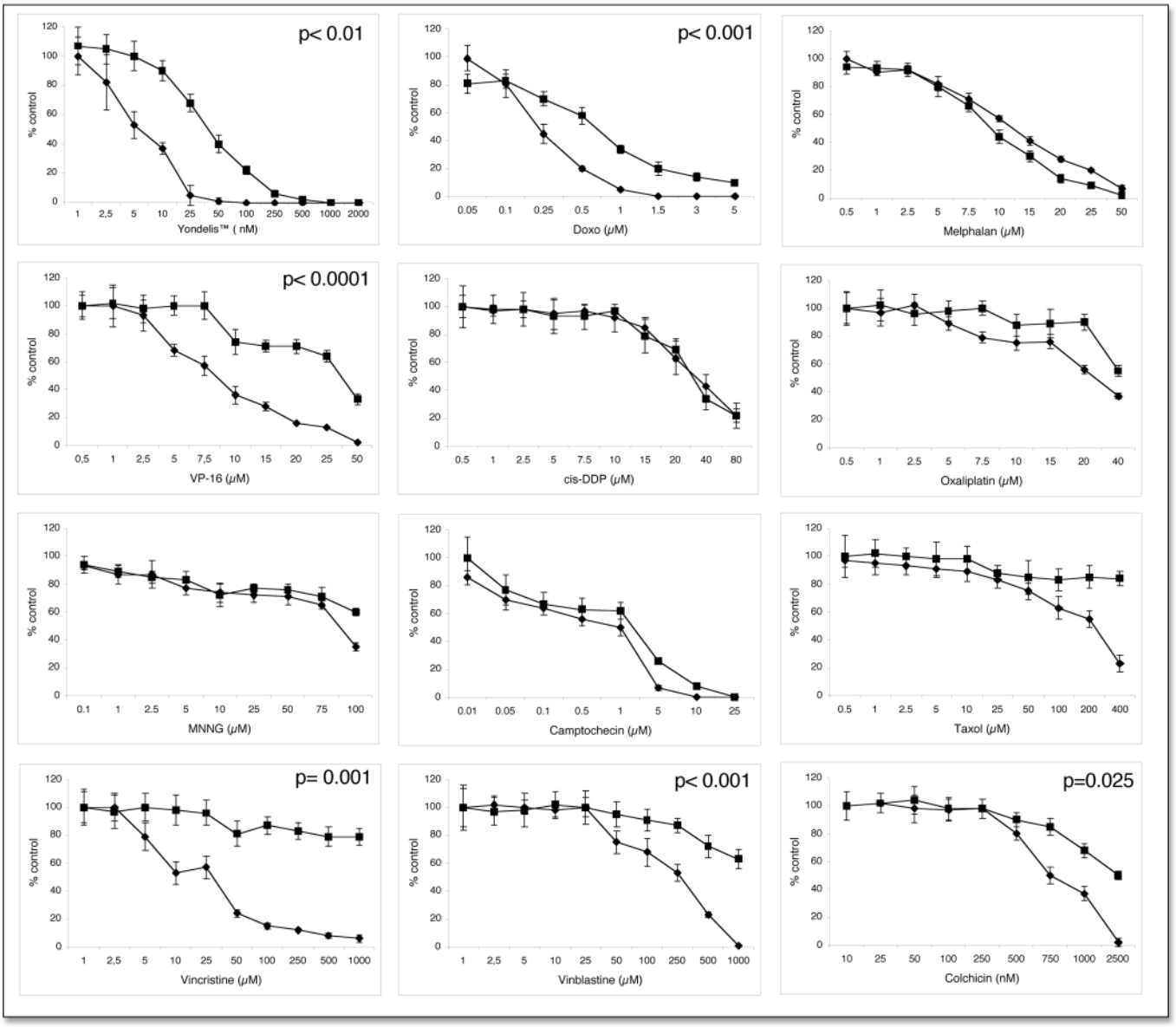

Figure 6. Sensitivity of IGROV-1 (◆) and IGROV-1/APL (■) cell lines to different drugs evaluated by colony assay. Each points is the mean of six independent replicates; bars represent S.D.

Table 1 **Mean cellular concentration (± SD; n = 3) of Aplidin® (10 nM 1 h treatment) evaluated in IGROV-1 and IGROV-1/APL cells at 0, 6 and 24 h after drug washout**

	IGROV-1 (10^{-4} nMol)	IGROV-1/APL (10^{-4} nMol)
0 h	1.06 ± 0.13	0.65 ± 0.09
0 h + cyclosporin-A	1.02 ± 0.10	0.72 ± 0.09
6 h	1.30 ± 0.16	0.38 ± 0.03
6 h + cyclosporin-A	1.34 ± 0.35	0.70 ± 0.14
24 h	1.03 ± 0.22	0.18 ± 0.02
24 h + cyclosporin-A	0.98 ± 0.02	0.53 ± 0.05

obtained after cell sorting of the IGROV-1/APL resistant cells for their Pgp expression: i.e., the resistant cells were only those expressing Pgp.

Recently Losada and coworkers,[28] established an Aplidin®-resistant cell line, derived from the cervical carcinoma HeLa cells, more than 1.000-fold relative resistant to the drug. HeLa-APL resistant cell line did not express mdr-1 and no cross-resistance was observed with anticancer agents related with the Pgp expression. Uptake studies by using fluorescent Aplidin® have shown that the drug accumulated to the same extent in parental and resistant cell line showing that the resistance to Aplidin® was not related to decreased drug transport in Hela/APL cells. In resistant Hela-APL cells Aplidin® only induced a weak and transient activation of JNK and p38 MAPK, while in parental cell line a rapid and dramatic activation of these two MAPKs, leading to apoptosis was found.

Therefore it appears that also for Aplidin® as for many anticancer drugs there are different mechanisms of resistance. The clinical relevance of Pgp expression in relation to the efficacy of anti-cancer

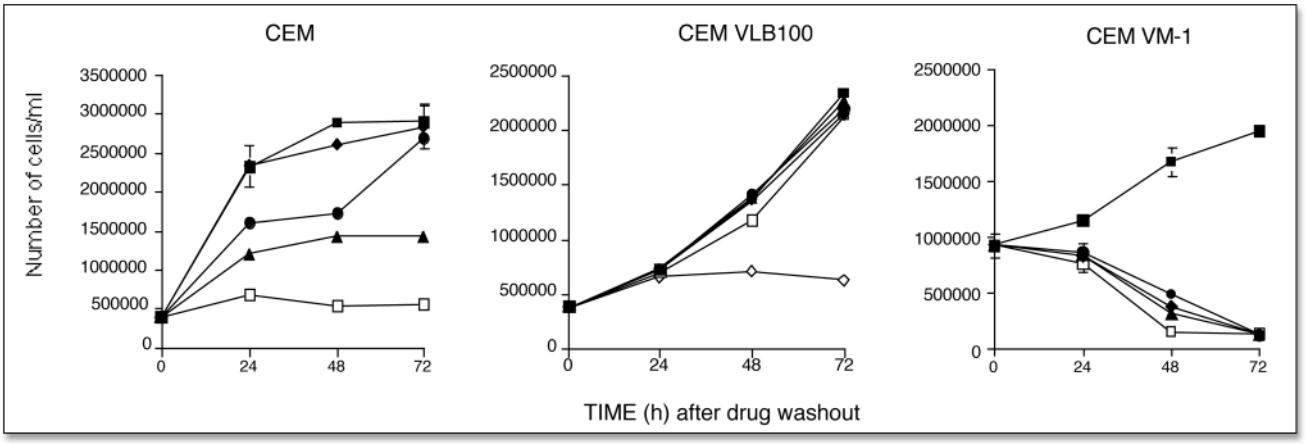

Figure 7. Growth inhibitory effect on CEM, CEM/VLB100 and CEM/VM-1 cells induced by 1 h treatment with 0,10, 20, 40, 80 or 160 nM Aplidin®. The growth inhibition was evaluated at different time after drug-washout by counting the number of cell by Coulter Counter. Each points is the mean of 3 independent replicates; bars represent S.D. ■, Control; ◆, 10 nM; ●, 20 nM; ▲, 40 nM; □, 80 nM; ◇, 160 nM.

treatment is still questionable in spite of the large number of studies performed in this field. On the other hand to know that an anticancer drug is a substrate of Pgp is important in relation to its pharmacokinetic properties. It is in fact demonstrated that the low oral bioavailability or the low penetration in the CNS of several compounds that are substrate of Pgp are related to the expression of this protein in the intestinal epithelium and in the endothelial cells regulating the blood brain barrier.

Therefore the data shown in the present paper indicating that Aplidin® is a substrate for Pgp, provides potentially useful pharmacological information.

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