

## Antiproliferative Effects of Novel Aliphatic Acetogenin Analogs Against Aggressive Solid Tumor Cell Lines

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**Abstract.** *The antiproliferative effects of three synthetic analogs of aliphatic acetogenins selected from a previous screening were compared to those of the drugs used for the treatment of malignant pleural mesothelioma (MPM) and pancreatic ductal adenocarcinoma (PDAC). Materials and Methods: Four PDAC and three MPM cell lines were used in the study. Cell growth inhibition was determined after 48 h exposure to the drugs. Cell-cycle disruption and apoptosis induction were studied by flow cytometry. The modulation of Akt phosphorylation was studied using a specific ELISA for P-Ser473 Akt. Results: The new compounds inhibited cell growth, induced apoptosis and cell-cycle abrogation in all cell lines. Phosphorylated Akt levels rose after treatment. Conclusion: The results demonstrated better performance of aliphatic acetogenin analogs against PDAC cells when compared to standard anticancer drugs. For MPM cells, the application of the new compounds may play an important role in overcoming the resistance to conventional treatments.*

The recent approval of ixabepilone, temsilorimus and trabectedin illustrates the relevance of natural products for cancer treatment (1). Living organisms provide an abundant source of novel therapeutics for the treatment of human

cancers. Privileged phytochemicals, with their inherent drug-likeness, remain an extremely powerful tool to aid the discovery of potent and selective antitumor drugs for a wide variety of cellular targets (2). Natural dietary agents including fruits, vegetables and spices have shown their ability to suppress or prevent cancer (3, 4). In this particular context, epidemiological studies have demonstrated the health benefits of avocado (*Persea americana*). However, the cellular and molecular cancer prevention mechanisms of avocado phytochemicals are still largely unknown. From the compounds present in avocado, the so-called aliphatic acetogenins represent the most promising molecular scaffold for designing anticancer drugs (5). This group previously reported on the relevance for the antiproliferative activity of the  $\beta'$ -hydroxy- $\alpha,\beta$ -unsaturated ketone moiety present in the molecular structure of aliphatic acetogenins (6, 7). Furthermore, the antiproliferative activity was not limited to drug-sensitive cell lines, but also to more resistant cell lines. These findings motivated this study of aliphatic acetogenins analogs against malignant pleural mesothelioma (MPM) and pancreatic ductal adenocarcinoma (PDAC), for which development of new drugs is urgently needed.

MPM is one of the most lethal human tumors with a median survival of less than one year. MPM is resistant to most chemotherapy regimens (8), radiation therapy (9) and therapy targeted against angiogenesis (10). The only effective chemotherapy regimen is the use of pemetrexed (PTX) and cisplatin in combination (11). However, median survival times range from nine to twelve months and the response rate is lower than 50%. PDAC is also a lethal cancer (12). Usually it is diagnosed at advanced stages and is extremely aggressive. There is a lack of effective therapies leading to a poor prognosis of five-year survival. Only the deoxynucleoside analog gemcitabine (2',2'-difluorodeoxycytidine, dFdC), and

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fluoropyrimidines such as 5-fluorouracil (5-FU) and capecitabine show some therapeutic activity. Nowadays, dFdC is the standard drug for advanced PDAC (13) while drug combinations are hardly more effective than PDAC alone.

This study explored the antiproliferative efficacy of a set of three selected aliphatic acetogenin analogs (Figure 1) against three MPM and four PDAC cell lines. The activities of the compounds were compared to those of the standard anticancer drugs dFdC, 5-FU and PTX. Mechanistic studies focused on the effects of these drugs on cell-cycle abrogation, analysis of signaling pathways and apoptotic cell death.

## Materials and Methods

**Reagents and chemicals.** RPMI-1640 medium was obtained from Cambrex Bio-Sciences (Verviers, Belgium). Fetal calf serum (FCS) was from Greiner BioOne (Frickenhausen, Germany). Trichloroacetic acid (TCA), L-glutamine, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), 5-FU, and sulforhodamine B (SRB) were from Sigma (St Louis, MO, USA). PTX and dFdC were from Eli Lilly (Indianapolis, IN, USA). Synthetic aliphatic acetogenin analogs were obtained from Dr. Juan I. Padrón (La Laguna, Spain) (6, 7).

**Cell culture and plating.** This study used the primary pancreatic cell lines LPc006, LPc028 and LPc067, which were isolated from a total of 79 primary tumors from patients who underwent surgery at the Regional Referral Center for Pancreatic Disease Treatment, University Hospital of Pisa (Pisa, Italy). The American Type Culture Collection (ATCC, Manassas, VA, USA) was the origin of the pancreatic cell line HPAC and the mesothelioma cell lines MSTO211H, H28 and H2452 (ATCC numbers: CRL-2081, CRL-5820, CRL-5946, respectively). Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine in a 37°C, 5% CO<sub>2</sub>, 95% humidified air incubator. Periodic assays were performed to detect mycoplasma contamination. Exponentially growing cells were trypsinized and resuspended in medium containing 100 U/ml penicillin and 0.1 mg/ml streptomycin. Single-cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted and diluted to get the appropriate cell densities for seeding onto 96-well microtiter plates, in a volume of 100 µl/well at densities of 20,000 for all cell lines.

**Chemosensitivity testing.** Pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested at six different dilutions in the range 0.001–100 µM. The drug treatment was started on day one after plating. Drug incubation times were 48 h; subsequently, cells were precipitated with 25 µl ice-cold 50% (w/v) TCA and fixed for 60 min at 4°C. The SRB assay (14) was performed and the optical density (OD) of each well was measured at 492 nm. Values were corrected for background OD from wells only containing medium. The percentage growth (PG) was calculated with respect to untreated control cells at each drug concentration level based on the difference in OD at the start and the end of drug exposure, according to NCI formulas (15). With these calculations, a PG value of 0 corresponds to the number of cells present at the start of drug exposure, while negative PG values denote net cell kill. The effect, defined as 50% growth inhibition

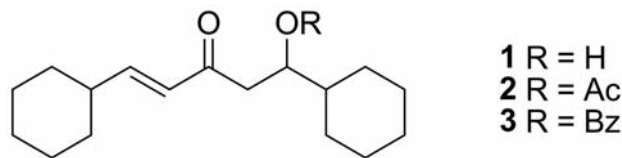


Figure 1. Chemical structure of investigational aliphatic acetogenin analogs.

(GI<sub>50</sub>), represents the concentration at which PG is +50. The GI<sub>50</sub> for each cell line was determined by non-linear least squares curve fitting of the dose–response curves (GraphPad PRISM; Intuitive Software for Science, San Diego, CA, USA).

**Cell-cycle analysis.** Cells were seeded in six-well plates at a density of 2×10<sup>5</sup> cells/well. After 24 h, the drugs were added to the respective wells and cells incubated for an additional period of 24 h. Cells were trypsinized, harvested, transferred to test tubes (12×75 mm; Becton Dickinson, San José, CA, USA) and centrifuged at 1,200 rpm for 10 min at 5 °C. The supernatant was discarded and the cell pellets were resuspended in 500 µl of propidium iodide (PI) buffer (PI 50 µg/ml, 0.1% sodium citrate, 0.1% triton X-100 and 0.1 mg/mL RNase). Flow cytometric determination of DNA content (10,000+ cells/sample) was performed in a FACSCalibur Flow Cytometer using FACSdiva 6.0 software (both from Becton Dickinson).

**Annexin V binding assay.** Cells were seeded in six-well plates at a density of 2×10<sup>5</sup> cells/well. After 24 h the drugs were added to the respective well and incubated for a period of 24 h. Cells were then trypsinized, harvested, transferred to test tubes (12×75 mm) and centrifuged at 1,200 rpm for 10 min. The supernatant was discarded and the cell pellets were resuspended in 100 µl of cold binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl<sub>2</sub>). Annexin V staining protocol was performed according to the manufacturer's protocol (Annexin V-FITC apoptosis detection Kit I; Becton Dickinson), with minor alterations. Cells were stained by the addition of both 5 µl Annexin V-FITC and 5 µl PI. Samples were gently vortexed and incubated for 15 min at room temperature in the dark. Subsequently, 400 µl of binding buffer were added to each tube. Samples were analyzed by flow cytometry in a FACSCalibur Flow Cytometer using FACSdiva 6.0 software.

**Akt phosphorylation.** To study the effect of compounds on the activation of Akt/PKB pathway, LPc006 cells were exposed to the drugs under study. The phosphorylation of Akt was studied using a specific ELISA for P-Ser473 Akt, (BioSource, Camarillo, CA, USA). Expression values were calculated according to the manufacturer's protocol. The results were expressed as U/ng and normalized with the expression levels in control cells, which was set to 100%.

## Results

**Cell growth inhibition.** Growth inhibition and cytotoxicity was studied for compounds 1-3 against a panel of three MPM and four PDAC, including three PDCA primary human solid tumor cell lines. Relatively steep dose–response

curves were obtained for all analogs (data not shown). The compounds produced not only total growth arrest (cytostasis) but also reduction in cell mass, as indicated by negative PG values at high drug concentrations (10–30  $\mu\text{M}$ ). The sensitivities, expressed as  $\text{GI}_{50}$ , are listed in Table I. The compounds under study were active against all cell lines, with  $\text{GI}_{50}$  values in the range 1.7–29  $\mu\text{M}$ .

Taken as whole, the results show that PDAC cells are more sensitive to the drugs than MPM cells. For MPM cells, the antiproliferative activity of analogs **1–3** was similar in all cell lines with  $\text{GI}_{50}$  values in the range 13–29  $\mu\text{M}$ . However, for the chemotherapeutic agent PTX, large differences in activity were found for MPM cells. Interestingly, the new drugs **1–3** showed similar potency with conventional PTX to the most resistant mesothelioma cell line H28. The data on antiproliferative activity against PDAC cell lines revealed differences between the investigational drugs. Thus, compounds **1** and **3** were the most active against LPc006 and PP109 cells. This is a remarkable effect, since in a previous study, compound **2** showed superior performance than derivatives **1** and **3** against the cell lines A2780 (ovarian) and T-47D (breast) (7). In all PDAC cell lines, compounds **1–3** were more active than 5-FU and less active than dFdC.

**Cell-cycle analysis.** Cell-cycle phase distribution was examined by flow cytometry to determine whether cell growth inhibition involved cell-cycle changes. The ability of compounds **1–3** to affect cell cycle was investigated after 24 h of exposure to the drugs. The MPM cell lines H28 and H2452 and the PDAC cell lines LPc006 and HAPC were used for these studies. Compounds were added to treated cells at a dose of 10  $\mu\text{M}$ . The results are summarized in Figure 2. The data show a marked arrest in the  $\text{G}_2/\text{M}$  phase of the cell cycle for the most sensitive cell line LPc006 (Figure 2C). The increase in the  $\text{G}_2/\text{M}$  phase was concomitant with a decrease mainly in the  $\text{G}_1$  compartment. Analogs **1–3** produced a comparable cell-cycle pattern in treated cells. In contrast, LPc006 cells exposed to 50  $\mu\text{M}$  dFdC showed a slight increase in the  $\text{G}_1$  phase (data not shown).

**Apoptosis and cell death.** The induction of apoptosis was studied by the analysis of DNA fragments detected in the sub- $\text{G}_1$  phase after exposure of cells to compounds **1–3** for 24 h. For MPM cell lines, no significant apoptotic cells were observed in the sub- $\text{G}_1$  phase when H28 and H2452 cells were treated with analogs **1–3** at 10  $\mu\text{M}$ . Nor was apoptosis induction detectable in cells exposed to PTX. In contrast, for the PDAC cell line LPc006, compounds **1–3** at 10  $\mu\text{M}$  induced larger apoptosis than 50  $\mu\text{M}$  dFdC (Figure 3).

**Assay of Akt phosphorylation.** Phosphorylated Akt (pAkt) plays a central role in signaling cascades involved in cell-death control and several drugs produce an effect on this

Table I. Growth inhibition of aliphatic acetogenin analogs against MPM and PDAC cells<sup>a</sup>.

Panel	Cell line	Compound					
		<b>1</b>	<b>2</b>	<b>3</b>	PTX	dFdC	5-FU
MPM	MSTO-						
	211H	16 $\pm$ 7.7	29 $\pm$ 15	18 $\pm$ 4.3	0.02 $\pm$ 0.1		
	H2452	18 $\pm$ 5.5	15 $\pm$ 4.4	15 $\pm$ 5.8	2.82 $\pm$ 1.4		
	H28	13 $\pm$ 7.9	14 $\pm$ 9.4	14 $\pm$ 2.4	11 $\pm$ 4.3		
PDAC	HPAC	13 $\pm$ 7.0	19 $\pm$ 7.3	10 $\pm$ 6.7			
	LPc006	2.5 $\pm$ 1.2	15 $\pm$ 3.9	1.7 $\pm$ 0.5		0.07 $\pm$ 1.3	176 $\pm$ 64
	LPc028	3.0 $\pm$ 1.1	23 $\pm$ 6.4	3.6 $\pm$ 1.2		0.01 $\pm$ 0.2	107 $\pm$ 20.2
	LPc067	16 $\pm$ 7.9	19 $\pm$ 1.5	16 $\pm$ 6.1		0.05 $\pm$ 0.1	94.9 $\pm$ 24.9

<sup>a</sup>Antiproliferative activity was evaluated as  $\text{GI}_{50}$  (in  $\mu\text{M}$ ) and compounds **1–3** were compared with three standard drugs used for treatment of mesothelioma (PTX) and pancreatic (dFdC and 5-FU) cancer. Values are given as mean $\pm$ standard deviation of three to five experiments.

pathway. In order to evaluate whether compounds **1–3** also modulated Akt, Akt phosphorylation levels were studied in LPc006 cells. In these experiments, the cells were exposed for 24 h to 5  $\mu\text{M}$  of each compound and  $\text{GI}_{50}$  concentration levels of dFdC. The amount of pAkt in treated cells rose to 143.5%, 121.5% and 144.3% for compounds **1–3**, respectively. In contrast, dFdC exposure slightly reduced pAkt levels.

## Discussion

Natural products remain a source of inspiration for medicinal chemists in the search of new drugs. Previously, this research group found that the  $\beta^1$ -hydroxy- $\alpha,\beta$ -unsaturated ketone fragment present in aliphatic acetogenins of avocado fruit is a plausible molecular scaffold for developing new anticancer drugs (6). Structure–activity relationship studies indicated the relevance of a  $\beta^1$ -acyloxy group in the enhancement of the antitumor activity, regardless of the sensitivity of the cell line (7). These results motivated the present study of the scope and limitations of the promising drug leads against a panel of two of the most resistant solid tumors, MPM and PDAC.

The present study revealed that compounds **1–3** exhibit considerable differences in activity when compared to the standard anticancer drugs PTX, dFdC and 5-FU. Indeed, the new derivatives were found to inhibit cell growth, induce apoptosis and cell-cycle arrest in three MPM cell lines and four PDAC cell lines. These findings are consistent with previous findings in ovarian, non-small cell lung, colon and breast tumor cell lines (7). Cell-cycle arrest in the  $\text{G}_2/\text{M}$  phase has been observed also in breast cancer cells treated with the natural aliphatic acetogenin persin (16). The present results indicated that the compounds damage the cells, causing an accumulation of cells in the  $\text{G}_2/\text{M}$  phase prior to

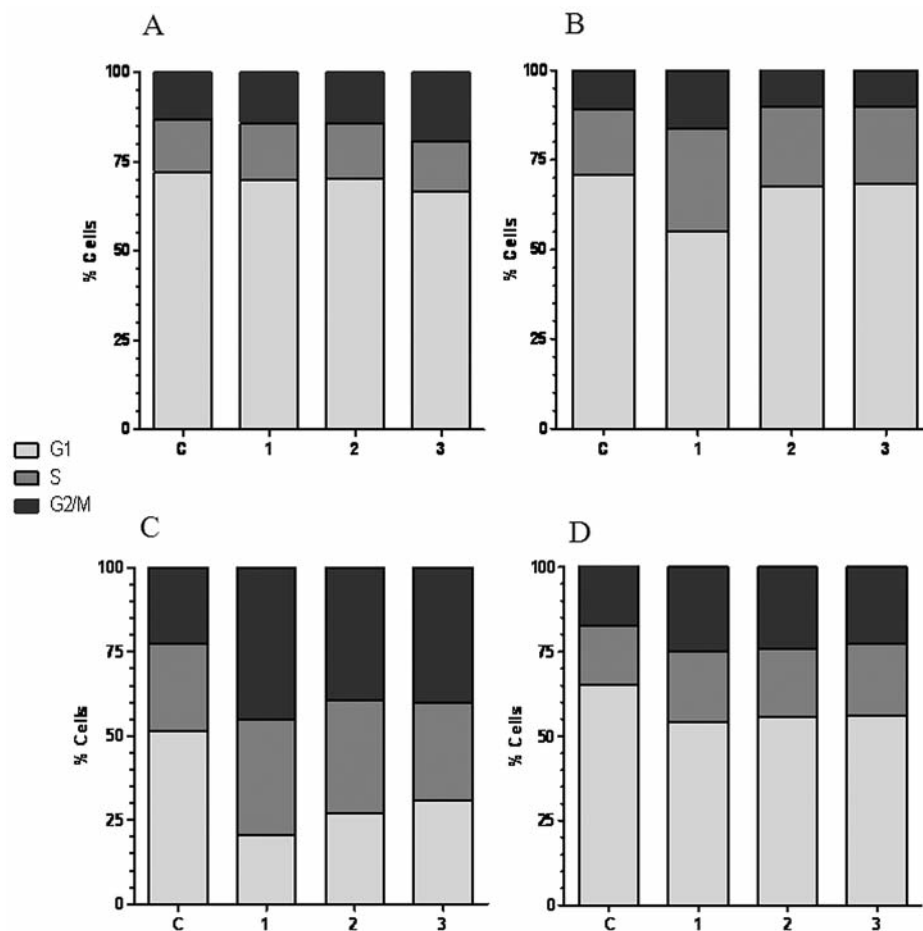


Figure 2. Cell-cycle phase distribution of MPM cell lines H28 (A) and H2452 (B), and PDAC cell lines LpC006 (C) and HAPC (D) exposed to compounds 1-3 at 10  $\mu$ M for 24 h. C: Untreated cells.

cell death. No clear difference was observed between the effects of the drugs 1-3. Furthermore, the GI<sub>50</sub> values of analogs 1-3 were comparable to the GI<sub>50</sub> value of PTX in H28 cells and analogs 1-3 were significantly more active than 5-FU in all PDAC cell lines. Hence, the new compounds showed selective action for PDAC cells. Despite having a low GI<sub>50</sub>, dFdc was less effective than compounds 1-3 in affecting the cell cycle. Thus, dFdc barely affected the cell cycle of PLc006 cells whilst all new drugs showed a pronounced arrest in the G<sub>2</sub>/M phase. These results correlated with the data obtained in the apoptosis assays where analogs 1-3 induced more total cell death (early and late apoptosis) than did dFdc. Finally, the increase in pAkt levels were in line with those previously reported for the human head and neck cancer cell lines UMSCC-1 and UMSCC-6 after exposure to 100 nM of dFdc (17). The apparent inconsistency of reduced pAkt levels observed after dFdc treatment of LpC006 cells can be attributed to higher levels of miR-21 present in this cell line (18). The increase observed

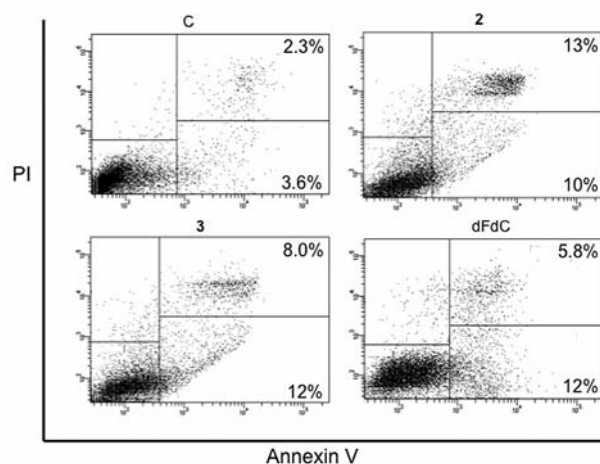


Figure 3. Annexin V and PI staining of LpC006 untreated (control, C) cells and cells exposed to compounds 2-3 (10  $\mu$ M) and dFdc (50  $\mu$ M) for 24 h. Viable cells are Annexin V<sup>-</sup> and PI<sup>-</sup>, early apoptotic cells are Annexin V<sup>+</sup> and PI<sup>-</sup> and late apoptotic cells are Annexin V<sup>+</sup> and PI<sup>+</sup>. Quantification of apoptosis gave the percentage of cells that were apoptotic.

in pAkt levels after treatment with compounds **1-3** was consistent with the findings of G<sub>2</sub>/M arrest, since pAkt is known to play a role in the cell cycle overcoming cell-cycle arrest in G<sub>1</sub> (19) and G<sub>2</sub>/M (20) phases.

Taken as a whole, the results show that the investigational drugs **1-3** perform better than the conventional anticancer drugs dFdC and 5-FU against PDAC cell lines. In addition, for MPM cells, the application of the new compounds seems more plausible in overcoming the resistance to PTX. Although the exact molecular target of drugs **1-3** remains unknown, the results demonstrated that their mechanism of action differs to that of dFdC, 5-FU and PTX.

In summary, the new agents **1-3** showed remarkable biological activity towards two of the most aggressive human cancer cell lines, including cell-cycle arrest and apoptosis induction. More experiments are warranted to validate the usefulness of the new agents either alone or in combination with existing chemo- and radiotherapy. In light of the differences observed in the biological activity of this family of compounds, more detailed studies may be necessary in order to uncover their mechanism of action.

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