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MOLECULAR MECHANISM OF PE5-INDUCED CYTOTOXICITY AND GENERATION OF NEW CYTOTOXIC NUCLEAR-DIRECTED

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PhD Thesis

Molecular mechanism of PE5-induced cytotoxicity and generation of new cytotoxic nuclear-directed ribonuclease variants

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Moltes gràcies a tots els que m'heu acompanyat.

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ABC ..... ATP-binding cassette
ACACA ..... acetyl-CoA carboxylase alpha
ACC ..... acetyl-CoA carboxilase
AKR1A1 ..... aldo-keto reductase family 1 member A1
ARRDC4 ..... arrestin domain containing 4
ASAH1 ..... N-acylsphingosine amidohydrolase (acid ceramidase) 1
ASK1 ..... apoptosis signal-regulating kinase 1
ASNS ..... asparagine synthetase
ATF3 ..... activating transcription factor 3
Bax ..... bcl-2-associated X protein
BCAT1 ..... branched chain amino acid transaminase 1
Bcl-2 ..... B-cell lymphoma 2
BCL2L11 ..... BCL2-like 11 (apoptosis facilitator)
BCRP ..... breast cancer resistant protein
BNIP3L ..... BCL2/adenovirus E1B 19kDa interacting protein 3-like
BS-RNase ..... bovine seminal ribonuclease
C>p ..... cytidine 2',3'-cyclic monophosphate
CLU ..... clusterin
CREB5 ..... cAMP responsive element binding protein 5
DHCR24 ..... 24-dehydrocholesterol reductase
DMTF1 ..... cyclin D binding myb-like transcription factor 1
EDTA ..... ethylenediaminetetraacetic acid
EFEMP1 ..... EGF containing fibulin-like extracellular matrix protein 1
EGFR ..... epidermal growth factor receptor
EGR1 ..... early growth response 1
ENO1 ..... enolase 1 (alpha)
ERK ..... extracellular-signal-regulated kinase
FBS ..... fetal bovine serum
G6PD ..... glucose-6-phosphate dehydrogenase
GADD45A ..... growth arrest and DNA-damage-inducible, alpha
GAPDH ..... glyceraldehyde 3-phosphate dehydrogenase
GPC6 ..... glypican 6
GPX3 ..... glutathione peroxidase 3
GST-π ..... glutathione S-transferase-π

HADHA ..... hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/ enoyl-CoA hydratase (trifunctional protein), alpha subunit
HIPK2 ..... homeodomain interacting protein kinase 2
HP-RNase ..... human pancreatic ribonuclease
IC ..... inhibitory concentration
IDH2 ..... isocitrate dehydrogenase 2 (NADP+), mitochondrial
IL24 ..... interleukin 24
IL6 ..... interleukin 6
JAK-STAT ..... janus kinase-signal transducer and activator of transcription
JNK ..... c-Jun N-terminal kinase
KEGG ..... Kyoto Encyclopedia of Genes and Genomes
LDHA ..... lactate dehydrogenase A
M ..... $\log_2$ red/green intensity ratio
MA ..... $\log_2$ red/green intensity ratio versus average $\log_2$ intensity
MAPK ..... mitogen-activated protein kinase
MDR ..... multidrug resistance
ME3 ..... malic enzyme isoform 3
MET ..... met proto-oncogene (hepatocyte growth factor receptor)
MRP1 ..... multidrug resistance associated protein 1
MSMO1 ..... methylsterol monooxygenase 1
MTT ..... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MXD1 ..... MAX dimerization protein 1
NF-κB ..... nuclear factor of kappa light polypeptide gene enhancer in B-cells
NFKBIA ..... nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NLS ..... nuclear localization signal
OXPHOS ..... oxidative phosphorylation
P-gp ..... P-glycoprotein
$p16^{INK4A}$ ..... cyclin-dependent kinase inhibitor 2A
$p21^{WAF1/CIP1}$ ..... cyclin-dependent kinase inhibitor 1A
$p27^{KIP1}$ ..... cyclin-dependent kinase inhibitor 1B
p53 ..... tumor protein p53
PBS ..... phosphate buffered saline
PCA ..... principal component analysis

PCCB ..... propionyl CoA carboxylase, beta polypeptide
PCK2 ..... phosphoenolpyruvate carboxykinase 2 (mitochondrial)
PDGFRB ..... platelet-derived growth factor receptor, beta polypeptide
PGAM1 ..... phosphoglycerate mutase 1 (brain)
PGM1 ..... phosphoglucomutase 1
PHGDH ..... phosphoglycerate dehydrogenase
PI ..... propidium iodide
PPP ..... pentose phosphate pathway
PPP1R15A ..... protein phosphatase 1, regulatory subunit 15A
PTEN ..... phosphatase and tensin homologue
PYCR1 ..... pyrroline-5-carbox1ylate reductase 1
RG ..... red and green signal
RHOB ..... ras homolog gene family, member B
RI ..... ribonuclease inhibitor
RIN ..... RNA integrity number
RLE ..... relative $\log_2$ expression
RMA ..... robust multi-array average
RNase ..... ribonuclease
RNase A ..... bovine pancreatic ribonuclease
ROS ..... reactive oxygen species
SE ..... standard error
SPTLC3 ..... serine palmitoyltransferase, long chain base subunit 3
TCA ..... tricarboxylic acid
TGM2 ..... transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
T_m ..... thermal denaturation midpoint
TM7SF2 ..... transmembrane 7 superfamily member 2
TOP2A ..... DNA topoisomerase II, alpha isozyme
TXNIP ..... thioredoxin interacting protein
TXNRD2 ..... thioredoxin reductase 2
WWOX ..... WW domain containing oxidoreductase
XIAP ..... X-linked inhibitor of apoptosis

Publication arising from this thesis

Vert, A., Castro, J., Ruiz-Martínez, S., Tubert, P., Escribano, D., Ribó, M., Vilanova, M., Benito, A. (2012). Generation of new cytotoxic human ribonuclease variants directed to the nucleus. *Mol Pharm* **9**(10): 2894-2902.

Cytotoxic ribonucleases are promising agents to be used in the treatment of cancer, and among them, onconase has reached phase II/III of clinical trials against different kinds of cancer. Our group previously described a cytotoxic human pancreatic ribonuclease variant, named PE5, which carries a nuclear localization signal. *In vitro* studies demonstrated that this protein is routed into the nucleus, where it cleaves nuclear RNA inducing the apoptosis of cancer cells. In the present work, the molecular mechanism of PE5-induced cytotoxicity has been investigated and compared to that of onconase using global gene expression and miRNA microarrays. The results indicate that PE5 inhibits the growth and development of tumor cells by causing pleiotropic effects and regulating the expression of numerous genes and miRNAs. In particular, this ribonuclease downregulates multiple genes implicated in glucose, lipid and amino acid metabolism, quenching of reactive oxygen species, and drug resistance. In addition, it decreases the expression of several oncogenes and increases that of tumor suppressors involved in cell proliferation, metastasis, and apoptosis. PE5 also downregulates diverse miRNAs that exert an oncogenic activity, promote drug resistance, or contribute to abnormal glucose metabolism and oxidative phosphorylation, whereas it upregulates tumor suppressor miRNAs and miRNAs related to inhibition of drug resistance. Onconase causes pleiotropic effects different from those of PE5, and affects the expression of distinct and more limited number of genes and miRNAs. Specifically, it increases the expression of various genes that encode transcription regulators with tumor suppressor functions implicated in cell proliferation, cell cycle progression, apoptosis, and response to stress. Onconase also downregulates oncogenic miRNAs.

On the other hand, in this work we have improved the properties of PE5 as an antitumor drug candidate. Two goals have been pursued and attained. First, in order to reduce the potential immunogenicity of the protein we have produced PE10, a variant as cytotoxic as PE5 in which half of the residues mutated in PE5 are back-mutated to wild type human pancreatic ribonuclease. And second, in an effort to obtain a more cytotoxic enzyme we have constructed NLSPE5, which carries the nuclear localization signal of SV40 large T-antigen at its N-terminus and exhibits 6-14 times higher cytotoxicity than PE5. Both PE10 and NLSPE5 cleave nuclear RNA and share the same cytotoxic mechanism than PE5. Treatment of cancer cells with these ribonucleases increases two-fold the levels of $p21^{WAF1/CIP1}$ and cyclin E,

similarly increases the accumulation of doxorubicin inside multidrug resistant cells, induces the same proportion of early and late apoptotic cells, and generates a comparable pattern of procaspase-3, -8, and -9 activation.

Les ribonucleases citotòxiques són proteïnes amb un gran potencial per ser utilitzades en el tractament del càncer. De fet, l'onconasa ha assolit fases II/III d'assaigs clínics per al tractament de diferents tipus de càncer. El nostre grup va descriure una variant citotòxica de la ribonucleasa pancreàtica humana, anomenada PE5, que incorpora un senyal de localització nuclear. Varis estudis *in vitro* van demostrar que aquesta proteïna es dirigeix al nucli, on degrada RNA nuclear induint així l'apoptosi de les cèl·lules tumorals. En aquest treball s'ha investigat el mecanisme de citotoxicitat de PE5 i s'ha comparat amb el de l'onconasa utilitzant microarrays globals d'expressió gènica i de miRNAs. Els resultats obtinguts indiquen que PE5 inhibeix el creixement i el desenvolupament de les cèl·lules tumorals causant efectes pleiotròpics i regulant l'expressió de nombrosos gens i miRNAs. En concret, aquesta ribonucleasa disminueix l'expressió de múltiples gens que participen en el metabolisme de la glucosa, de lípids i d'aminoàcids, que formen part de sistemes antioxidants que eviten el dany provocat per les espècies reactives de l'oxigen o que afavoreixen la resistència a fàrmacs. A més, redueix l'expressió de varis oncogens i augmenta la de gens supressors de tumors implicats en proliferació cel·lular, metàstasi i apoptosi. PE5 també disminueix l'expressió de miRNAs que presenten activitat oncogènica, que promouen la resistència a fàrmacs o que contribueixen a la desregulació del metabolisme de la glucosa i de la fosforilació oxidativa, mentre que incrementa els nivells de miRNAs supressors de tumors i miRNAs relacionats amb la inhibició de la resistència a fàrmacs. L'onconasa provoca efectes pleiotròpics diferents dels de PE5 i regula l'expressió d'un nombre més limitat de gens i miRNAs. Concretament, augmenta l'expressió de diversos gens que codifiquen per proteïnes reguladores de la transcripció, les quals realitzen funcions supressores de tumors i estan implicades en proliferació cel·lular, progressió del cicle cel·lular, apoptosi i resposta a l'estrès. L'onconasa també minva l'expressió de miRNAs oncogènics.

D'altra banda, en aquest treball s'han millorat les propietats antitumorals de PE5. Les millores s'han dissenyat per tal d'assolir dos objectius. Primer, amb la finalitat de reduir la potencial resposta immunològica s'ha produït PE10, una variant tan citotòxica com PE5 en la qual la meitat dels residus mutats a PE5 s'han substituït pels de la ribonucleasa pancreàtica humana salvatge. En segon lloc, per tal d'obtenir un enzim més citotòxic s'ha construït NLSPE5. Aquesta variant incorpora el senyal de localització nuclear de l'antigen T-llarg de SV40 al seu extrem N-terminal i exhibeix

una citotoxicitat 6-14 vegades superior a PE5. Tan PE10 com NLSPE5 degraden RNA nuclear i presenten el mateix mecanisme de citotoxicitat que PE5. Així, el tractament de les cèl·lules tumorals amb aquestes ribonucleases augmenta 2 vegades els nivells de $p21^{WAF1/CIP1}$ i ciclina E, provoca un increment similar de l'acumulació de doxorubicina a l'interior de cèl·lules resistents a múltiples drogues, indueix la mateixa proporció de cèl·lules en les fases inicial i final de l'apoptosi i genera un patró d'activació de les procaspases -3, -8 i -9 molt similar.

Las ribonucleasas citotóxicas son proteínas con un gran potencial para ser utilizadas en el tratamiento del cáncer. De hecho, la onconasa ha llegado hasta fases II/III de ensayos clínicos para el tratamiento de diferentes tipos de cáncer. Nuestro grupo describió una variante citotóxica de la ribonucleasa pancreática humana, denominada PE5, que incorpora una señal de localización nuclear. Varios estudios *in vitro* demostraron que esta proteína se dirige al núcleo, donde degrada RNA nuclear induciendo así la apoptosis de las células tumorales. En este trabajo se ha investigado el mecanismo de citotoxicidad de PE5 y se ha comparado con el de la onconasa utilizando microarrays globales de expresión génica y de miRNAs. Los resultados obtenidos indican que PE5 inhibe el crecimiento y el desarrollo de las células tumorales causando efectos pleiotrópicos y regulando la expresión de numerosos genes y miRNAs. En concreto, esta ribonucleasa disminuye la expresión de múltiples genes que participan en el metabolismo de la glucosa, de lípidos y de aminoácidos, que forman parte de sistemas antioxidantes que evitan el daño provocado por las especies reactivas del oxígeno o que favorecen la resistencia a fármacos. Además, reduce la expresión de varios oncogenes y aumenta la de genes supresores de tumores implicados en proliferación celular, metástasis y apoptosis. PE5 también disminuye la expresión de miRNAs que presentan actividad oncogénica, que promueven la resistencia a fármacos o que contribuyen a la desregulación del metabolismo de la glucosa y de la fosforilación oxidativa, mientras que incrementa los niveles de miRNAs supresores de tumores y miRNAs relacionados con la inhibición de la resistencia a fármacos. La onconasa provoca efectos pleiotrópicos diferentes de los de PE5 y regula la expresión de un número más limitado de genes y miRNAs. Concretamente, aumenta la expresión de diversos genes que codifican por proteínas reguladoras de la transcripción, las cuales realizan funciones supresoras de tumores y están implicadas en proliferación celular, progresión del ciclo celular, apoptosis y respuesta al estrés. La onconasa también disminuye la expresión de miRNAs oncogénicos.

Por otra parte, en este trabajo se han mejorado las propiedades antitumorales de PE5. Las mejoras se han diseñado para conseguir dos objetivos. Primero, con la finalidad de reducir la potencial respuesta inmunológica se ha producido PE10, una variante tan citotóxica como PE5 en la cual la mitad de los residuos mutados en PE5 se han sustituido por los que presenta la ribonucleasa pancreática humana salvaje. En segundo lugar, para obtener una enzima más citotóxica se ha construido NLSPE5.

Esta variante incorpora la señal de localización nuclear del antígeno T-largo de SV40 en su extremo N-terminal y exhibe una citotoxicidad 6-14 veces superior a PE5. Tanto PE10 como NLSPE5 degradan RNA nuclear y presentan el mismo mecanismo de citotoxicidad que PE5. Así, el tratamiento de la células tumorales con estas ribonucleasas aumenta 2 veces los niveles de $p21^{WAF1/CIP1}$ y ciclina E, provoca un incremento similar de la acumulación de doxorubicina en el interior de las células resistentes a múltiples drogas, induce la misma proporción de células en las fases inicial y final de la apoptosis y genera un patrón de activación de las procaspasas -3, -8 y -9 muy similar.

Introduction

Cancer is a broad group of diseases, all characterized by uncontrolled cell division resulting in the growth of a mass of cells known as a tumor. Cancerous tumors can arise in almost any tissue or organ and cancer cells, if not eradicated, spread or metastasize throughout the body.

Cancer treatment depends on the type of cancer and the stage of disease progression. Generally, it includes surgical removal of localized tumors accompanied by radiation treatment and adjuvant drug treatment to kill residual cancer cells. Chemotherapy is the most widely used alternative therapy to surgery and it is the main treatment for tumors that are likely to, or have, metastasized. Chemotherapy involves the administration of drugs that interfere processes like DNA synthesis and cell division. They are designed to kill cancer cells, affect the growth of tumors or reduce bloodflow to tumors, in an effort to reduce or eliminate cancerous tumors.

Most of the anticancer chemotherapeutic drugs that are broadly and successfully used today are DNA-damaging agents. Targeting DNA is a powerful way to control gene expression and it has been proven to enable relatively effective and selective destruction of cancer cells. However, the clinical potential of DNA-damaging agents is limited by the number of disadvantages they have. First, they are not as effective as would be required to cure cancer, second, they provoke significant adverse side effects, and third, they can cause mutagenic effects and increase the risk of secondary cancers, defined as cancers not related to the primary cancer that appear 10-15 years after successful elimination of the primary disease (Reviewed in Gurova, 2009). Accordingly, researchers are interested in developing alternative therapies to kill cancer cells.

RNA is the intermediate in the flow of biochemical information from genes to proteins. Therefore, acting at RNA level is a successful way to control gene expression without affecting DNA, and it represents an alternative approach to DNA-damaging chemotherapy. Indeed, targeting RNA for degradation may have similar effectiveness in cancer treatment than targeting DNA, with the advantage that it does not cause mutagenic effects. There are a variety of available technologies for targeting RNA with therapeutic potential (Reviewed in Tafech et al., 2006). The use of ribonucleases (RNases) is one of them.

RNASES AS POTENTIAL ANTITUMOR DRUGS

RNases are a highly heterogeneous group of enzymes that catalyze the cleavage of phosphodiester bonds in RNA. They are found in all domains of life and participate in a diversity of biological activities. While some RNases are involved in basic degradation processes such as the digestion of dietary RNA, housekeeping RNases are implicated in maturation, quality control and turnover of cellular RNA, and play a central role in the control of gene expression by determining the levels of functional RNA in the cell. Other RNases also mediate special biological actions. These actions are diverse and include cell maturation, allergy induction of some pollen species, promotion of blood vessel formation, host defense and stress response. Among these special actions, some RNases have antitumor activity, as they are cytotoxic enzymes capable of selectively kill cancer cells (Reviewed in Schein, 1997).

Most of the described antitumor RNases belong to the bovine pancreatic RNase (RNase A) superfamily, also known as vertebrate secretory RNase superfamily. Proteins of this family are homologous to RNase A and share elements of structure and catalytic properties. Most of them are endoribonucleases, so they hydrolyze RNA internally, have the ability to degrade RNA with no sequence specificity, and are pyrimidine specific RNases, since they initiate the cleavage of the phosphodiester bonds in the 3'-OH of a pyrimidine nucleotide (Reviewed in Beintema and Kleineidam, 1998).

Antitumor RNases could be used, alone or conjugated to ligands or antibodies, as therapeutic agents for cancer treatment. In contrast to most chemotherapeutic drugs, which interfere with DNA synthesis and cell division, antitumor RNases are non-mutagenic agents that exert their effects by interfering with RNA functions such as protein synthesis or gene regulation, and are able to kill non-dividing cells. In the recent years, several natural and engineered cytotoxic RNases have been described to be selective against cancer cells, and they constitute an alternative to conventional DNA-damaging cancer therapy.

The molecular basis of the cytotoxicity of these RNases is not fully understood, but a general mechanism of action is widely accepted. Cytotoxicity requires that RNases

initially interact with the cell membrane and then internalization proceeds by endocytosis. At some point in the endocytic pathway, cytotoxic RNases are translocated to the cytoplasm where they must evade the RNase inhibitor (RI) and cleave cellular RNA, inducing apoptosis. How efficiently a particular RNase carries out each of these steps determines its potency as a cytotoxin (Reviewed in Benito et al., 2005).

NATURAL ANTITUMOR RNASES

Among the natural antitumor members of the pancreatic RNase A superfamily, the most known are bovine seminal RNase from bovine sperm and onconase from the oocytes of *Rana pipiens*, as well as amphinase and jSBL and cSBL lectins, that also have an amphibian origin.

Onconase

Onconase is a natural cytotoxic RNase originally isolated from oocytes and early embryos of the Northern Leopard frog (*Rana pipiens*). It is the smallest member of the pancreatic RNase A superfamily, consisting in a basic single-chain of 104 amino acid residues long with a molecular weight of 11.8 KDa. It has an amino acid sequence about 30% identical to RNase A (Ardelt et al., 1991) and its general fold is very similar to the classic two lobules, V or kidney shaped topology of RNase A, although onconase is more compact than RNase A (Mosimann et al., 1994). Because of its compact structure and the presence of 4 disulfide bonds, 3 of which are conserved in RNase A, onconase is an exceptionally stable protein and is highly resistant to proteolysis. Indeed, the midpoint of thermal denaturation of this enzyme has been calculated to be around 90ºC, almost 30ºC higher than that of RNase A (Notomista et al., 2000). Onconase is 10^2-10^5 - fold less active against polymeric substrates and single-stranded RNA than RNase A (Ardelt et al., 1991; Boix et al. 1996) although both enzymes share the same key catalytic residues (Ardelt et al., 1991). A peculiar feature of onconase is the presence of an N-terminal pyroglutamic residue, which is essential for optimal enzymatic and cytotoxic activities (Newton et al., 1998).

Onconase exhibits aspermatogenic, embryotoxic and immunosuppressive activities (Matousek et al., 2003), but also selective cytotoxic and cytostatic activities against tumor cells. Cytotoxicity has been demonstrated *in vitro* against several cancer cell lines (Darzynkiewicz et al., 1988) and *in vivo* in different tumor models (Mikulski et al., 1990; Lee et al., 2007a). It has also been described that onconase enhances the cytotoxicity of several chemotherapeutic agents such as tamoxifen, lovastatin, cisplatin (Mikulski et al., 1992) vincristine (Rybak et al., 1996), tumor necrosis factor α (Deptala et al., 1998), doxorubicin (Mikulski et al. 1999), interferons (Tsai et al., 2002) and ionizing radiation (Lee et al., 2007b).

The mechanism of the antitumor activity of onconase has been widely studied, but it is not completely understood. There is a controversy about the way onconase enters into the cell. It has been suggested that this enzyme interacts with a cell-surface receptor, as it follows the same internalization pathway than transferrin (Rodríguez et al., 2007). This result is in agreement with a previous work (Wu et al., 1993). However, an onconase variant labeled with Oregon Green binds in a non-saturable manner to the cell surface in HeLa cells, indicating an absence of specific receptors (Haigis and Raines, 2003). It has also been suggested that onconase, due to its cationic nature, binds to the cell surface by electrostatic interactions (Johnson el al. 2007; Sundlass et al., 2013). This binding mechanism could explain, at least in part, its selectivity to cancer cells, since the surface of most cancer cells display a greater electronegativity compared to normal cells. Early studies (Wu et al., 1993; Wu et al., 1995) and more recent ones (Haigis and Raines, 2003) indicate that internalization occurs by energy-dependent endocytosis. Indeed, onconase enters to the cell using AP-2/clathrin mediated endocytosis and then it is routed to the recycling endosomes (Rodríguez et al., 2007), even though a previous work indicated that the entry was not dynamin dependent (Haigis and Raines, 2003). The apparent contradiction might be related to the use of transiently or stably transfected cell lines expressing a dynamin-K44A dominant-negative mutant. From the recycling endosomes, onconase translocates to the cytosol (Rodríguez et al., 2007), where it evades the cytosolic RI and degrades intracellular RNA. RI is a protein that binds to some pancreatic-type RNases inhibiting their activity. It is found in the cytosol where it is thought to act as a safeguard against extracellular RNases that could accidentally reach the compartment (Lee and Vallee, 1993). The inhibition occurs because some of the

residues of the RNase that are important for the RI binding are involved in the ribonucleolytic activity of the protein (Kobe and Deisenhofer, 1996). It has been described that onconase is not inhibited by the RI since it has a low affinity for it. In fact, the dissociation constant of the complex formed between onconase and RI is about 10^7 - fold greater than that of the RNase A-RI complex (Boix et al., 1996). This lower affinity has been attributed to a reduction of the length of some exposed loops responsible for the interaction with the RI (Kobe and Deisenhofer, 1996). Also, it may be due to a reduction of the C30/C75 disulfide bond of onconase once in the cytosol (Torrent et al., 2008).

All cytotoxic RNases degrade intracellular RNA but preferences for the different RNA species have been detected. Several studies have demonstrated that *in vivo* onconase degrades tRNA, leaving rRNA and mRNA unaffected (Iordanov et al., 2000a; Saxena et al., 2002). It has been postulated that this degradation of tRNA leads to indiscriminate inhibition of protein synthesis, inducing cell cycle arrest and apoptosis. Some observations, however, are incompatible with this mechanism. First, apoptosis induced by onconase presents features different from those of an indiscriminate translation inhibition (Iordanov et al., 2000a), second, some proteins involved in regulation of cell cycle progression are upregulated after treatment with onconase (Juan et al. 1998), and third, several genes that affect apoptosis, transcription, inflammation and immune response are also upregulated by onconase (Altomare et al. 2010). Accordingly, it has been proposed that onconase targets the RNA interference system involved in gene expression regulation via siRNA and miRNA (Ardelt et al., 2003). In fact, it has been demonstrated that onconase is able to degrade double-stranded RNA (Saxena et al., 2009). Moreover, the silencing of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene by a specific siRNA in A549 cells has been reported to be prevented by onconase (Zhao et al., 2008), indicating that siRNA is a target of onconase. It has also been shown that onconase increases hsa-miR-17* and decreases hsa-miR-30c levels in malignant pleural mesothelioma cells (Goparaju et al., 2011), providing evidence that one of the targets of onconase is miRNA.

Specifically targeting miRNA may help explain both the high selectivity of onconase against tumor cells and the synergisms encountered with other antitumor agents that

have different mechanisms of action. The development of many tumors has been associated with early alterations at the level of miRNA genes, which are located in the genome hot spots associated with cancer (Reviewed in Hernando, 2007). Onconase may kill tumor cells preferentially over normal cells by specifically cleaving these miRNAs. Targeting miRNA may also explain the synergisms of onconase with other antitumor agents, since microRNA may mediate the acquisition of tumor resistance to chemotherapeutics (Reviewed in Allen and Weiss, 2010). In this sense, the increase of hsa-miR-17* and decrease of hsa-miR-30c levels in malignant pleural mesothelioma cells treated with onconase have been reported to directly result in downregulation of NFKB1 gene, which encodes the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) subunit p50 (Goparaju et al., 2011). The reduced expression of NF-κB could sensitize cells to other antitumor agents, because this reduction could suppress the translation of survival genes that inhibit apoptosis and generates the resistance of tumor cells to treatment (Wang et al., 1996).

Onconase induces cell death by apoptosis, although the apoptotic effects of this enzyme are very dependent on the cell type. The activation of the stress-activated c-Jun N-terminal kinase (JNK) has been described as an early event in the induction of apoptosis by onconase in HeLa cells (Iordanov et al., 2000b), although no changes in the expression level of JNK have been detected in NCI/ADR-RES cells (Castro et al., 2011a). The difference could be related to the status of tumor protein p53 (p53), since JNK exerts an anti-apoptotic activity in p53-deficient tumor cells (Reviewed in Liu and Lin, 2005). Onconase seems to promote the activation of procaspase-9, -3, and -7 but not of procaspase-8 in HeLa cell line (Iordanov et al., 2000a). Other studies with the HL-60 leukemic cell line show the activation of serine proteases along with these caspases (Grabarek et al., 2002). However, in neuroblastoma cell lines onconase leads to a caspase-independent apoptosis with features similar to autophagy (Michaelis et al., 2007). Moreover, it is not clear to what extent the mitochondrial apoptotic pathway is involved. Whereas little release of cytochrome c has been observed in HeLa cells (Iordanov et al., 2000a), treatment with onconase has been shown to enhance cytochrome c-induced caspase activation in HeLa S100 extracts (Mei et al., 2010). It has also been reported that onconase decreases the expression of anti-apoptotic B-cell lymphoma 2 (Bcl-2), while it increases that of pro-apoptotic bcl-2-associated X protein (Bax) in the HL-60 human leukemia cell line

(Ardelt et al., 2007a). However, the levels of Bcl-2 and Bax remain unaltered in NCI/ADR-RES (Castro et al., 2011a). In addition, apoptosis triggered by onconase is independent of the p53 status of the cell line (Iordanov et al., 2000a). This is an interesting feature of onconase due to many cancer cells lack p53 or express a defective p53 protein that reduces or eliminates the apoptotic response induced by several chemotherapeutic drugs.

The effect of onconase on the cell cycle is also dependent on the cell line used in the study. A general consensus in the literature indicates that onconase arrests proliferation in the G_0/G_1 cell cycle phase (Darzynkiewicz et al., 1988; Juan et al., 1998; Halicka et al., 2000), but there are exceptions. Onconase arrests NIH/3T3 cells in the G_2/M cell cycle phase (Smith et al., 1999), while it does not alter the cell cycle phase distribution in Jurkat cells (Tsai et al., 2004) or NCI/ADR-RES cells (Castro et al., 2011a). The effect of onconase on the expression of different key regulators of the cell cycle has been also investigated. Treatment of lymphoma U-937 cells by onconase decreases cyclin D3 protein levels, increases cyclin-dependent kinase inhibitor 1B ($p27^{KIP1}$), cyclin-dependent kinase inhibitor 2A ($p16^{INK4A}$), and cyclin-dependent kinase inhibitor 1A ($p21^{WAF1/CIP1}$) levels, and decreases the amount of the phosphorylated form of retinoblastoma protein (Juan et al., 1998).

Onconase was the first antitumor RNase tested in human clinical trials. Although it shows renal toxicity at high concentrations (Vasandani et al., 1999), different Phase II clinical studies have demonstrated that it is effective as an anticancer drug in the treatment of non-small-cell lung cancer (Mikulski et al., 1995), pancreatic cancer (Chun et al., 1995), breast cancer (Puccio et al., 1996) and malignant mesothelioma (Mikulski et al., 2002). The major advances have been in the treatment of unresectable malignant mesothelioma, since onconase reached a confirmatory Phase IIIb clinical trial for the treatment of this disease. The combined effect of onconase and doxorubicin was compared to that of doxorubicin alone in patients. Results demonstrated that such a combination significantly improves the survival of patients who failed one prior chemotherapy regimen, compared to doxorubicin alone (Reck et al., 2009). Nevertheless, Alfacell Corporation (at present Tamir Biotechnology Inc.), the company that performed the clinical studies, decided not to continue further clinical trials for the treatment of unresectable malignant mesothelioma and focus

their efforts on other types of cancer. At present, onconase is tested in Phase II clinical trials on patients affected by non-small cell lung cancer, in combination with pemetrexed and carboplatin. It is also tested in preclinical trails against pathogenic viruses such as Yellow fever, Dengue fever, SARs, CMV and HPV.

Bovine seminal RNase

Bovine seminal RNase (BS-RNase) is a dimeric protein from bovine sperm with aspermatogenic, embryotoxic and immunosuppressive activities, as well as antitumor activity *in vitro* and *in vivo* (Vescia et al., 1980; Laccetti et al., 1992; Laccetti et al., 1994). BS-RNase is the only natural dimeric member of the pancreatic RNase A superfamily. The enzyme is composed by two identical subunits that are held together by covalent and non-covalent interactions. Two dimeric forms coexist in nature, one with the N-terminal domains swapped between the monomers (M x M form) and the other in which each monomer conserves its own N-terminal domain (M = M form). This structural difference correlates with differences in cytotoxicity (Cafaro et al., 1995). The M = M form is less cytotoxic because under the reducing conditions of the cytosol, the intersubunit disulfide bonds are probably reduced and the M = M dimer dissociates into monomers, which are captured by the RI. The M x M form is maintained by non-covalent interactions and it is probably not inhibited.

The dimeric structure of BS-RNase is necessary for its cytotoxicity as it prevents RI binding by steric hindrance (Murthy et al., 1996), allowing the enzyme to degrade intracellular rRNA (Mastronicola et al., 1995). This degradation leads to inhibition of protein synthesis and, finally, induction of apoptosis (Marinov and Soucek, 2000). Moreover, the dimeric structure of BS-RNase seems to be required to cross the lipid bilayer (Mancheño et al., 1994) and it is maintained during its internalization (Bracale et al., 2003).

BS-RNase is endocytosed in malignant and non-malignant cells to the same extent, but it is much more toxic for cancer cells (Vescia et al., 1980; Mastronicola et al., 1995). This difference has been attributed to the fact that the internalization of BS-RNase is different in each type of cell. Only the internalization pathway of tumor cell

lines effectively induces cell death. Like onconase, ribonucleolytic activity of BS-RNase is essential for its antitumor effect (Kim et al., 1995).

Amphinase

Like onconase, amphinase is a natural cytotoxic RNase isolated from oocytes of *Rana pipiens*. In fact, four variants of this enzyme with a highly similar amino acid sequences were found in the oocytes of this specie (Singh et al., 2007). Amphinase is more basic than onconase and is the largest among the known amphibian RNases. It is cytotoxic and cytostatic against human cancer cells and, although amphinase is considerably less active than onconase, its cytotoxicity is similar to that of onconase (Singh et al., 2007; Ardelt et al., 2007b). Amphinase does not interact with the RI, one of the key factors responsible of its cytotoxicity, and ribonucleolytic activity is required for the antitumor effect of this enzyme (Singh et al., 2007). Amphinase arrests proliferation in the G_1 cell cycle phase and induces cell death by apoptosis, which involve activation of endonucleases, caspases, serine proteases and transglutaminases (Ardelt et al., 2007b).

jSBL and cSBL lectins

jSBL and cSBL lectins are natural cytotoxic RNases isolated from *Rana catesbeiana* and *Rana japonica* oocytes, respectively. They show lectin activity towards cells with sialic acid-rich glycoproteins (Nitta et al., 1987; Sakakibara et al., 1979). Both lectins are selectively cytotoxic to cancer cells and are capable of agglutinating a large variety of tumor cells (Liao et al., 1996; Nitta et al., 1994a). Like onconase, jSBL lectin evades the RI (Nitta et al., 1993) and its ribonucleolytic activity is essential for its cytotoxicity (Huang et al., 1998). However, it has been reported that prolonged exposure of cancer cells to this enzyme contributes to the development of receptor-based resistance (Nitta et al., 1994b).

ENGINEERED CYTOTOXIC RNASES

Knowledge of the molecular basis of the cytotoxicity of RNases has led to the development of new RNase variants with cytotoxic activity, which constitute an alternative to conventional DNA-damaging cancer therapy. Human pancreatic RNase (HP-RNase) is not cytotoxic to cells, probably as a result of the inhibition exerted by the RI once the protein has reached the cytosol. This protein does not produce renal accumulation *in vivo* (Vasandani et al., 1999) and shows a 10^3-10^4 - fold increase in RNase activity compared to onconase (Leland et al., 2001). Thus, generating cytotoxic variants of the HP-RNase would undoubtedly provide potentially useful therapeutic agents that would be expected to have lower immunogenicity and renal toxicity than onconase. This explains why many of the described cytotoxic variants have been constructed onto the HP-RNase scaffold (Reviewed in Rybak and Newton, 1999).

For the construction of these cytotoxic HP-RNase variants, researchers have used different strategies. One of them is avoiding the RI inhibition by decreasing the affinity of the RNase for it. This effect has been achieved either by using site-directed mutagenesis to introduce selected changes in the RNase which create steric or electrostatic incompatibilities in the binding with the RI (Gaur et al., 2001; Leland et al., 2001) or, alternatively, by inserting those residues necessary to generate a variant able to adopt a dimeric structure spontaneously (Piccoli et al., 1999). In this sense, QBI-139 is an engineered HP-RNase variant designed to evade the RI (EVade™ RNase Technology from Quintessence Biosciences Inc.) that is now in Phase I human clinical trial (Strong et al., 2012). It is also possible to endow HP-RNase with cytotoxic activity by increasing the protein cationization, since more cationic RNases are more efficiently delivered into cells. Provided that enough RNase reaches the cytosol, all free RI could be depleted, enabling the new molecules reaching the cytosol to degrade RNA even if they could be inhibited *in vitro* by the RI. This cationization could be achieved either by site-directed mutagenesis (Johnson et al., 2007) or by chemical modification (Futami et al., 2001). An alternative strategy to ensure an efficient and specific internalization of RNases is to either chemically or genetically link them to tumor-associated ligands. Transferrin, growth factors and antibodies are some of the ligands used (Rybak et al., 1992; Tada et al., 2004). Finally,

routing the RNase to the nucleus, where the RI is not present, is another effective manner of avoiding RNase inhibition (Bosch et al., 2004).

PE5 variant

PE5 is an HP-RNase variant that, despite being sensitive to the RI, exhibits cytotoxic activity against a panel of diverse human tumor cell lines, with a 50% inhibitory concentration (IC_{50}) only 5-15 times higher than onconase (Bosch et al., 2004: Castro el al., 2011b). This variant carries a non-contiguous extended bipartite nuclear localization signal (NLS) constituted by at least three basic regions of the protein, comprising Lys1 and the Arg clusters 31-33 and 89-91. Although these regions are separated by more than 90 residues in the primary structure, they are close in the three-dimensional structure of the protein and their topological disposition is equivalent to that of a classical bipartite NLS (Rodríguez et al., 2006). Once PE5 has been internalized and reaches de cytosol, this NLS is recognized by α-importin (Rodríguez et al., 2006), which drives the protein into the nucleus, specifically into the nucleolus, in an energy-dependent and Ran-dependent manner (Bosch et al., 2004). In the nucleus, or at least in the nucleolus, the RI is not present (Roth and Juster, 1972; Furia et al., 2011) and PE5 cleaves nuclear RNA, leaving cytosolic RNA undamaged. The replacement of the critical residues of the NLS significantly reduces the cytotoxicity of PE5, indicating that the NLS endows this HP-RNase variant with cytotoxic activity (Tubert et al., 2011).

PE5 is recognized by α-importin and by RI, and the regions of the enzyme implicated in the binding to both proteins are overlapped. This means that once PE5 has internalized and reaches the cytosol, it can interact with both proteins but not at the same time, and therefore a competence is established between α-importin and RI to bind with PE5. Since the concentrations of α-importin and RI are similar in the cytosol (Haigis et al., 2003; Görlich et al., 1994), the affinity of PE5 for each protein would have to determine to which one it mainly binds. The strength of the binding between RI and HP-RNase is one of the highest described (Boix et al., 1996). However, some faint bands of cleaved RNA are observed in the RI binding assay of PE5, indicating that this variant weakly evades the RI (Bosch et al., 2004).

Accordingly, there are minute amounts of cytosolic PE5 not bound with RI that are captured by α-importin and released into the nucleus. The resulting drop in the level of free PE5 molecules in the cytosol displaces the RI-PE5 equilibrium toward dissociation, and then more free PE5 molecules become available for nuclear import. As a result, PE5 progressively accumulates into the nucleus.

In vitro, PE5 displays at least the same selectivity for cancer cells than onconase. In contrast to this latter protein, PE5 is mainly cytotoxic, although at low concentrations a minimal cytostatic effect can be observed (Castro et al., 2011a). Proliferation analysis using different cell lines have shown that cytotoxicity of this RNase does not require the pro-apoptotic activity of p53 (Castro et al., 2011a). It has also been described that cytotoxicity of PE5 is not prevented by a multidrug resistance (MDR) phenotype (Castro et al., 2011a), defined as the resistance of tumor cells to the cytostatic or cytotoxic effects of multiple and dissimilar drugs used in cancer chemotherapy. In fact, PE5 is highly cytotoxic against the MDR cell lines NCI/ADR-RES and NCI-H460/R (Castro et al., 2011b), which contain mutated p53 and overexpress P-glycoprotein (P-gp) and glutathione S-transferase-π (GST-π) multidrug resistance proteins.

Cytotoxic properties of PE5 have been studied using NCI/ADR-RES cells. Treatment of this cell line with PE5 generates classical hallmarks of apoptosis such as plasma membrane blebbing, apoptotic body formation, chromatin condensation, nuclear fragmentation, phosphatidylserine translocation and caspase activation, indicating that PE5 induces cell death by apoptosis (Castro et al., 2011a). This is important because a non-apoptotic mode of cell death could cause inflammatory or immune complications in patients. Regarding the apoptotic pathway, treatment with this RNase induces the activation of initiation procaspases-8 and -9, which leads to the activation of executioner procaspase-3 in NCI/ADR-RES. No changes in the proportion of Bcl-2 amounts respective to those of Bax have been detected (Castro et al., 2011a). Therefore, PE5 induces both extrinsic and intrinsic apoptotic pathways, the latter being independent of Bcl-2 and Bax. Cell death induced by PE5 is accompanied by an accumulation of cells in the S- and G_2/M-phases in NCI/ADR-RES cell line (Castro et al., 2011a). The overexpression of the cell cycle protein regulators cyclin E and $p21^{WAF1/CIP1}$ observed in PE5-treated cells explains the

alteration on the cell cycle phase distribution (Castro et al., 2011a). $p21^{WAF1/CIP1}$ also acts as a non-enzymatic inhibitor of the stress activated protein kinase JNK (Shim et al., 1996), which has an anti-apoptotic function in p53-deficient tumor cells (Liu and Lin, 2005). Accordingly, p46 form of JNK is under-phosphorylated in cells treated with PE5 (Castro et al., 2011a). These results suggest that the inhibition of JNK mediated by $p21^{WAF1/CIP1}$ in PE5-treated cells promotes the apoptosis of NCI/ADR-RES cells.

Interestingly, PE5 reduces the expression level of P-gp in the MDR cell lines NCI/ADR-RES and NCI-H460/R (Castro et al., 2011b). P-gp is a plasma membrane ATP-binding cassette (ABC) transporter protein overexpressed in most of the MDR cancers. Since the overexpression of this protein plays a critical role in the development of the MDR, inhibition of P-gp represents an approach to overcome the MDR phenotype. The reduction of P-gp caused by PE5 seems to be specific, because no changes in the accumulation of the overexpressed GST-π multidrug resistance protein have been observed (Castro et al., 2011b), and it has been suggested that it occurs through the inhibition of JNK. It has also been reported that the combination of PE5 and doxorubicin is synergistic in NCI/ADR-RES (Castro et al., 2011b). As treatment with PE5 produces an increase of doxorubicin accumulation in NCI-H460/R but not in NCI/ADR-RES (Castro et al., 2011b), the synergistic effect could be mediated by the suppression of the survival mechanisms induced by P-gp.

GENE AND MIRNA EXPRESSION PROFILES OF CELLS TREATED WITH RNASES

Microarray technology is a powerful tool for evaluating gene expression profiles of cells. It enables researchers to simultaneously analyze changes in thousands of genes and identify significant altered patterns, and therefore it is a useful approach to study cellular responses upon exposure to drugs. This technique has been used to compare gene expression profiles of onconase-treated and untreated human malignant mesothelioma cells. A total of 155 genes were found to be regulated by onconase. The activating transcription factor 3 (ATF3) and the pro-apoptotic factor interleukin

24 (IL24) were the highest upregulated genes in the two most responsive cell lines. Interleukin 6 (IL6), a cytokine that regulates immunological reactions, was also one of the most overexpressed genes. In addition, gene ontology analysis indicated that onconase affects mitogen-activated protein kinase (MAPK), cytokine-cytokine receptor interactions, and janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling (Altomare et al., 2010).

In another study, microarray technology was used to analyze miRNAs expression profiles of malignant pleural mesothelioma cells exposed to onconase. Treatment with this RNase caused a significant alteration in the expression level of 20 miRNAs. Among them, hsa-miR-17* was increased and hsa-miR-30c was decreased in all cell lines. Moreover, it was found that regulation of these miRNAs by onconase results in downregulation of the pro-survival NFKB1 gene and reduces the malignant behavior of tumor cells (Goparaju et al., 2011).

Finally, it is worth mentioning that RNase A therapy caused the boost of 116 miRNAs in tumor tissue and a significant drop of 137 miRNAs in the bloodstream of mice bearing Lewis lung carcinoma. The study was performed by high-throughput Sequencing by Oligonucleotide Ligation and Detection (SOLiD™) sequencing technology. The results suggest that a RNase belonging to the same family than PE5 and onconase promote a significant alteration of miRNA signature that leads to the attenuation of tumor malignancy (Mironova et al., 2013).

Objectives

In the recent years, several natural and engineered cytotoxic RNases have been described to be selective against cancer cells, representing an alternative approach to conventional DNA-damaging chemotherapy. Our group developed a strategy to endow HP-RNase with cytotoxic properties consisting in driving the protein into the nucleus by the inclusion of an NLS on its sequence. Formerly, we reported a cytotoxic variant of HP-RNase, named PE5, which is routed into the nucleus. Although previous studies have shown that the cytotoxic mechanism of nuclear-directed PE5 is different from that of cytosolic-directed onconase, we were interested in studying deeply this issue. Microarray technology enables researchers to simultaneously analyse changes in thousands of genes and identify significant altered patterns upon exposure to drugs, and therefore it could be a useful approach to study the molecular mechanism of PE5-induced cytotoxicity. In addition, we were aware that PE5 had to be improved to become an antitumor drug candidate. When attempting to develop a recombinant protein as a drug, two of the main desirable attributes are minimum immunogenicity and maximum potency and different strategies can be envisaged to reach them.

Taken into account these aspects, the main objectives of the present work can be summarized as follows:

1. To study the molecular effects of PE5-induced cytotoxicity and compare them with those of onconase using global gene expression and global miRNA microarray analysis.

2. To produce new nuclear-directed RNases that could be potentially less immunogenic than PE5 by back-mutating to the wild type HP-RNase sequence those residues not important for the NLS function, and to characterize the cytotoxic properties of the improved variants.

3. 3 To produce new nuclear-directed RNases by introducing an additional NLS on different sites of PE5 in order to increase PE5 cytotoxicity, and to characterize the cytotoxic properties of the improved variants.

Methods

CONSTRUCTION OF RNASE VARIANTS

Construction of plasmids expressing onconase (pONC) and PE5 (pE5) has been previously described (Leland et al., 1998; Bosch et al., 2004). These plasmids are derivative of pET22b(+) and pET17b, respectively. PE5 was constructed from PM5 by replacing Gly89 and Ser90 by Arg. PM5 is an HP-RNase variant carrying five substitutions at the N-terminus: Arg4Ala, Lys6Ala, Gln9Glu, Asp16Gly and Ser17Asn (Canals et al., 1999).

PE9 is a PE5 variant in which the five N-terminal residues mutated in PE5 (Arg4Ala, Lys6Ala, Gln9Glu, Asp16Gly and Ser17Asn) were reverted to those of the wild type enzyme (Figure 1). It was constructed from PM9 (wild type HP-RNase (Canals et al., 1999)) by exchanging Gly89 and Ser90 by Arg. For this, pE5 (Bosch et al., 2004) was digested with *Sac*I and *Sal*I and a fragment of 320bp (corresponding to the gene segment between codons 21 and 128) was ligated to pM9, in which this *Sac*I-*Sal*I segment was removed using the corresponding restriction enzymes, and transformed into *Escherichia coli* DH5α cells. The construction was checked by DNA sequencing.

PE10 is a PE5 variant in which three N-terminal residues mutated in PE5 (Gln9Glu, Asp16Gly and Ser17Asn) were reverted to those of the wild type enzyme (Figure 1). This variant was constructed from PE9 by replacing Arg4 and Lys6 by Ala and it was created using the QuickChange site-directed mutagenesis kit (Stratagene, USA) following the manufacturer's instructions. Oligonucleotides R4AK6A3 (5' CCATgTgTTgTCTTTggAACTTTgCAgCTgCAgATTCTTTCATATgTATATCTCC 3') and R4AK6A5 (5' ggAgATATACATATgAAAgAATCTgCAgCTgCAAAgTTCC AAAgACAACACATgg 3') were used. The construction was checked by DNA sequencing.

Variants NLSPE5 and scNLSPE5 incorporate the NLS of SV40 large T-antigen (PKKKRKVE) or a scrambled form (KPKERVKK), respectively, at the N-terminus of PE5. In both cases, the basic stretches are linked to the N-terminus of the RNase by a two-residue linker (AS) (Figure 1). Plasmids expressing NLSPE5 (pNLSPE5) and scNLSPE5 (pscNLSPE5) were constructed in three steps. Firstly, the HP-RNase gene of pM5 (Canals et al., 1999) was amplified by PCR using the oligonucleotides

T7TERM (5' TATgCTAgTTATTgCTCAg 3') and PLINKME_1 (5' gAgATAAAgC TTCCAAAgAATCTgCTgCTgCT 3'), which introduces a *Hind*III restriction site at 5' of the gene. The PCR product was digested with *Hind*III and *Sal*I, ligated to pET17b previously digested with *Hind*III and *Xho*I and transformed into *Escherichia coli* DH5α cells to obtain pLINKM5. Secondly, sequences coding for the NLS or scrambled NLS were introduced at the 5' of the HP-RNase gene of pLINKM5 to create pNLSPM5 and pscNLSPM5, respectively. For this, overlapping oligonucleotides NTNLS_1 (5' TATgCCgAAAAAgAAgCgTAAAgTTgA 3') and NTNLS_2 (5' AgCTTCAACTTTACgCTTCTTTTTCggCA 3') or overlapping oligonucleotides NTscNLS_1 (5' TATgAAgCCgAAAgAACgTgTTAAg AA 3') and NTscNLS_2 (5' AgCTTTCTTAACACgTTCTTTCggCTTCA 3') were mixed equimolarly, heated to 90°C and allowed to cool down to 25°C for 30 min. The assembled oligonucleotides were gel purified, ligated to pLINKM5 previously digested with *Hind*III and *Nde*I and transformed into *Escherichia coli* DH5α cells to obtain pNLSPM5 and pscNLSPM5. Thirdly, pNLSPE5 and pscNLSPE5 were obtained from pNLSPM5 and pscNLSPM5, respectively, by introducing the substitutions Gly89 and Ser90 by Arg. This step was performed using the QuickChange site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's instructions. Oligonucleotides PE5A (5' gATTgTAgACTgACTAATCgTCgTAgATACCCTAATTgTgC 3') and PE5B (5' gCACAATTAgggTATCTACgACgATTAgTCAgTCTACAATC 3') were used. All the different constructions were checked by DNA sequencing.

Variants PE5NLS and PE5scNLS carry the NLS of SV40 large T-antigen or the scrambled NLS, respectively, at the C-terminus of the protein. In variants PE5spNLS and PE5spscNLS, the NLS or scrambled NLS is, respectively, linked to the C-terminus of the protein by a five-residue linker (SVGGS) (Figure 1). PE5NLS, PE5scNLS, PE5spNLS and PE5spscNLS were obtained from PM5NLS, PM5scNLS, PM5spNLS and PM5spscNLS (Tubert, 2012), respectively, by introducing the substitutions Gly89 and Ser90 by Arg. These variants were created using the QuickChange site-directed mutagenesis kit (Stratagene, USA) following the manufacturer's instructions. Oligonucleotides PE5A and PE5B were used. All the different constructions were checked by DNA sequencing.

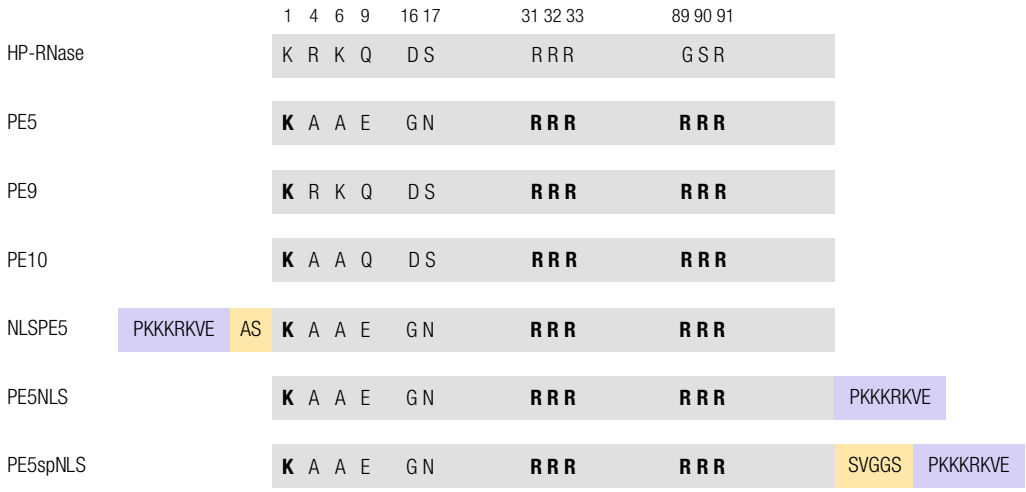

Figure 1. Schematic representation of the main HP-RNase variants used in this work. PE5 is an HP-RNase variant that carries a conformational bipartite NLS constituted by the residues shown in bold. PE5 differs in seven residues from HP-RNase (positions 4, 6, 9, 16, 17, 89 and 90), whereas PE9 differs in two residues (positions 89 and 90) and PE10 in four residues (positions 4, 6, 89 and 90). NLSPE5 carries the NLS of SV40 large T-antigen at the N-terminus of PE5, separated by a spacer. PE5NLS and PE5spNLS incorporate the same NLS at the C-terminus of PE5, without or with a spacer between PE5 and the NLS, respectively. The additional NLS of these PE5 variants is shown in violet and the spacer in yellow.

Variant NLSPE5H119A was constructed from NLSPE5 by exchanging His119 by Ala and it was created using the QuickChange site-directed mutagenesis kit (Stratagene, USA) following the manufacturer's instructions. Oligonucleotides H119A_1 (5' CCTTACgTTCCTgTTgCgTTTgATgCTAgCgTTg 3') and H119A_2 (5' CAACgCTAgCATCAAACgCAACAggAACgTAAgg 3') were used. The construction was checked by DNA sequencing.

RNASE EXPRESSION AND PURIFICATION

Recombinant onconase and HP-RNase variants were produced and purified from *Escherichia coli* BL21(DE3) cells (Studier and Moffat, 1986) transformed with the corresponding vector essentially as described previously (Ribó et al., 2001; Ribó et al., 2004). Briefly, cells containing the pONC plasmid were grown in Terrific Broth medium supplemented with 100 μg/ml ampicillin until an absorbance at 600 nm near 2 was reached. Cells containing the pE5 plasmid were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an absorbance at 550 nm near 1.5 was reached. Protein expression was induced in both cases by addition of isopropyl thiogalactoside (Fermentas, EUA) to 1 mM. After 3-4 h, cells were harvested by centrifugation at 7,500 xg for 7 min. Pellets from 2 L of induced culture were resuspended in 30 ml of 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-acetate (pH 8.0). Cells were lysed using a French press and inclusion bodies were harvested by centrifugation at 12,000 xg for 45 min at 4ºC. Pellets were then resuspended in 10 ml of 6 M guanidinium chloride, 2 mM EDTA, 100 mM Tris-acetate (pH 8.5). Reduced glutathione was added to a final concentration of 0.1 M, the pH was adjusted to 8.5 with solid Tris, and the samples were incubated at room temperature for 2 h under nitrogen atmosphere to assist protein solubilization. Insoluble material was removed by centrifugation at 14,500 xg for 30 min at 4ºC and solubilized protein was diluted dropwise into 0.5 M L-arginine, 1 mM oxidized glutathione, 2 mM EDTA, 100 mM Tris-acetate (pH 8.5) and incubated at 4°C for at least 48 h. Then proteins were concentrated by ultrafiltration using a Prep/Scale TFF cartridge (Millipore, USA). In the case of onconase, cyclization of the N-terminal Gln residue to pyroglutamic acid is essential for its full catalytic activity and its cytotoxic properties (Mosimann et al., 1994; Boix et al., 1996; Notomista et al., 2001). This is accomplished at this step by dialysis against 10 mM EDTA, 200 mM potassium phosphate (pH 7.2) at room temperature for a period of 72 h (Ribó et al., 2004). Onconase was afterwards dialyzed against 10 mM EDTA, 20 mM potassium phosphate (pH 7.2), centrifuged at 15,000 xg for 30 min at 4ºC to remove insoluble material, loaded onto a Mono-S HR 5/5 FPLC column (Amersham Biosciences, USA) equilibrated with 10 mM EDTA, 20 mM potassium phosphate (pH 7.2), and eluted with a linear gradient from 0 to 1 M NaCl. HP-RNase variants were dialyzed against 50 mM sodium acetate (pH 5.0), centrifuged at 15,000 xg for 30 min at 4ºC to

remove insoluble material, loaded onto a Mono-S HR 5/5 FPLC column (Amersham Biosciences, USA) equilibrated with 50 mM sodium acetate (pH 5.0), and eluted with a linear gradient from 0 to 1 M NaCl. Fractions containing pure RNases were dialyzed against ultra-pure water, lyophilized, and stored at -20 °C. A yield of 15–25 mg of protein per 1 L of culture was obtained. The molecular mass of each variant was confirmed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in the *Unitat cientificotècnica de suport* of the *Institut de Recerca* of the *Hospital Universitari Vall d'Hebron* (Barcelona, Spain). The protein concentration of each variant was determined by ultraviolet spectroscopy using an extinction coefficient at 280 nm of 10470 M^{-1} cm^{-1} for onconase and 7950 M^{-1} cm^{-1} for HP-RNase variants, calculated as reported previously (Pace et al., 1995).

DETERMINATION OF THERMAL STABILITY

Temperature-unfolding studies of HP-RNase variants were carried out essentially as described previously (Font et al., 2006). Depending on the experiment, proteins were dissolved to a concentration of 0.8 mg/mL in 50 mM sodium acetate buffer at pH 5.0 or 100 mM sodium acetate buffer at pH 4.0. The decrease in absorbance at 287 nm (1 nm bandpath) was recorded as a function of temperature using a Lambda Bio20 (Perkin-Elmer, USA) absorption spectrometer equipped with a thermostated cell holder. The temperature was increased from 20º to 66ºC in 2ºC steps. Before each measurement samples were equilibrated for 5 min. Temperature-unfolding transitions curves were fitted to a two-state thermodynamic model combined with sloping linear functions for the native and denatured states, and the thermal denaturation midpoint (Tm) was calculated as reported previously (Torrent et al., 1999). Assuming two-state protein folding, Tm is defined as that temperature at which both the folded and unfolded states are equally populated at equilibrium. All data are expressed as mean ± standard error (SE) of three independent determinations.

DETERMINATION OF STEADY-STATE KINETIC PARAMETERS

Spectrophotometric assays were used to determine the kinetic parameters for the hydrolysis of cytidine 2',3'-cyclic monophosphate (C>p) by the HP-RNase variants as described previously (Boix et al., 1994). The concentration of enzyme was 0.1 μM, the initial concentration of C>p ranged from 0.15 to 4.5 mM, and the activity was measured by recording the increase in absorbance at 296 nm. All assays were carried out at 25°C in 0.2 M sodium acetate buffer (pH 5.5) using 1 cm path-length quartz cells. Steady-state kinetic parameters were obtained by non-linear regression analysis using the program ENZFITTER (Elsevier Biosoft, UK). All data are expressed as mean ± SE of three independent determinations.

RNASE INHIBITOR BINDING ASSAY

RNases were tested for ribonucleolytic activity in the presence of RI (Promega, USA) using an agarose gel-based assay as described previously (Bosch et al., 2004). 15 ng of each protein in 20 μl of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 125 mM NaCl, 2.5 mM 1,4-dithio-DL-threitol, 1 mM EDTA (pH 7.0) were incubated for 10 min at 25ºC with 0 or 40 units of RI (where 1 unit is the amount of RI required to inhibit the activity of 5 ng of RNase A by 50%). Afterwards, 4 μg of 16S and 23S rRNA (Roche, Switzerland) were added and samples incubated further for 10 min at 25ºC. Reactions were stopped by the addition of 3 μl of loading buffer (40% (w/v) sucrose, 0.2% (v/v) diethyl pyrocarbonate, 0.25% (w/v) bromophenol blue) and the mixtures were subjected to electrophoresis in an agarose gel (1.2% (w/v)) containing ethidium bromide.

CELL LINES AND CULTURE CONDITIONS

NCI/ADR-RES human ovarian cancer MDR cell line (formerly MCF-7/Adr) (Liscovitch and Ravid, 2007) was a generous gift from Dr. Ramon Colomer of the *Institut Català d'Oncologia de Girona, Hospital Universitari de Girona Dr. Josep Trueta*

(Girona, Spain); Jurkat human T cell lymphoblast-like cell line and HeLa human cervical cancer cell line were obtained from Eucellbank, *Universitat de Barcelona* (Barcelona, Spain); NCI-H460/R human lung cancer MDR cell line (Pesic et al., 2006) was a generous gift from Dr. Sabera Ruzdijić of the S. Stanković Institute for Biological Research (Belgrade, Serbia); NCI-H460 human lung cancer line and OVCAR-8 human ovarian cancer cell line were obtained from the National Cancer Institute -Frederick DCTD tumor cell line repository. NCI/ADR-RES and HeLa cells were routinely grown at 37ºC in a humidified atmosphere of 5% CO_2 in DMEM (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco, Germany), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco, Germany). The other cell lines were grown at 37ºC in a humidified atmosphere of 5% CO_2 in RPMI (Gibco, Germany) supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. NCI/ADR-RES and NCI-H460/R cells were maintained in media containing 1.84 μM and 0.1 μM doxorubicin (Tedec-Meijic Farma, Spain), respectively. Cells remained free of *Mycoplasma* and were propagated according to established protocols.

CELL PROLIFERATION ASSAYS

Cells were seeded into 96-well plates at the appropriate density: 10,000 for NCI/ADR-RES, 1,500 for OVCAR-8, 3,000 for NCI-H460/R, 1,900 for NCI-H460, 1,100 for HeLa, and 6,000 for Jurkat. After 24 h incubation, cells were treated for 24, 36, 48, or 72 h with various concentrations of RNase (0.001-10 μM for onconase and NLSPE5 or 0.1-30 μM for the other RNases). RNase sensitivity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method essentially as described by the manufacturer's instructions (Sigma, USA). Data were collected by measuring the absorbance at 570 nm with a Synergy 4 multi-well plate reader (Biotek Instruments, USA). The IC_{50} value represents the concentration of the assayed enzyme required to inhibit cell proliferation by 50%, and in each case was calculated by linear interpolation from the obtained growth curves. All data are expressed as mean ± SE of at least three independent experiments with three replica in each.

CYTOSOLIC AND NUCLEAR RNA DEGRADATION ASSAY

3 x 10^6 HeLa cells were seeded into T75-flasks and then treated with 0.3 μM NLSPE5 or 1 μM PE5 or PE10 for 24 h. These concentrations caused in all cases a decrease of 10% in cell proliferation. After treatment, cells were harvested at 400 xg for 5 min at 4ºC and washed twice with cold phosphate buffered saline (PBS). Nuclear and cytosolic RNA was extracted using the PARIS kit (Applied Biosystems/Ambion, USA) according to the manufacturer's instructions and stored at -80ºC. RNA degradation of each sample was quantified in the Scientific Services of the *Centre de Regulació Genòmica* (Barcelona, Spain) using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Values of RNA integrity number (RIN) were obtained from the analysis of the electropherograms. The RIN algorithm allows calculation of RNA integrity using a trained artificial neural network based on the determination of features that can be extracted from the electrophoretic traces. The selected features which collectively catch the maximum information about the integrity levels include the ratio of area of ribosomal bands to total area of the electropherogram, the height of the 18S peak, the ratio of the area in the fast region to the total area of the electropherogram, and the height of the lower marker. The output RIN is a decimal number in the range of 1–10. A RIN of 1 is returned for a completely degraded RNA samples whereas a RIN of 10 is achieved for intact RNA sample.

PHOSPHATIDYLSERINE EXPOSURE ASSAY

Quantitative analysis of apoptotic cell death caused by NLSPE5, PE5, and PE10 treatment was performed by flow cytometry using the Alexa Fluor 488 annexin V/ propidium iodide (PI) Vybrant Apoptosis Assay Kit (Molecular Probes, USA) following the manufacturer's instructions. Briefly, NCI/ADR-RES cells (2.2 x 10^5 per well) were seeded into 6-well plates and then treated with 4.85 μM NLSPE5 or 44.5 μM PE5 or PE10 for 24, 48, and 72 h in serum-starved medium. These RNase concentrations corresponded to five times the IC_{50} after 72 h of incubation. Afterwards, attached and floating cells were harvested at 460 xg for 10 min at 4ºC, washed twice in cold PBS, and subjected to Alexa Fluor 488 annexin V and PI

staining in binding buffer at room temperature for 15 min in the dark. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, USA) using CellQuest Pro software. A minimum of 10,000 cells within the gated region were analyzed. All data are expressed as mean ± SE of at least three independent experiments.

PROCASPASE ACTIVATION ASSAY

Caspase-3, -8, and -9 catalytic activities were measured using the APOPCYTO Caspase-3, -8, and -9 colorimetric assay kits (MBL, Japan) following the manufacturer's instructions. The assay is based on cleavage of the chromogenic substrates, DEVD-pNA, IETD-pNA and LEHD-pNA, by caspases-3, -8, and -9, respectively. Briefly, NCI/ADR-RES cells (1.1 x 10^6 cells/100-mm dish) were incubated with 4.85 μM NLSPE5 or 44.5 μM PE5 or PE10 for 24, 48, and 72 h in serum-starved medium. These RNase concentrations corresponded to five times the IC_{50} after 72 h of incubation. Attached and floating cells were harvested at 460 xg for 10 min at 4ºC, washed twice in cold PBS, and then cells were lysed and centrifuged at 10,000 xg for 5 min at 4ºC. The supernatant was recovered, and the protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, USA) (Bradford, 1976). Afterwards, 10 μl of the cell lysate corresponding to 20 μg of total protein, 10 μl of 2X reaction buffer containing 10 mM 1,4-dithio-DL-threitol, and 1 μl of the 10 mM DEVD-*p*NA, IETD-*p*NA or LEHD-*p*NA substrates were mixed. Then samples were incubated at 37°C for 4 h and the reaction was measured by changes in absorbance at 405 nm. All data are expressed as mean ± SE of at least three independent experiments.

WESTERN BLOT ANALYSIS

NCI/ADR-RES cells (9 x 10^5 cells/100-mm dish) were incubated with 0.97 μM NLSPE5 or 8.9 μM PE5 or PE10 for 72 h. Treatments caused in all cases a decrease of 50% in cell proliferation (IC_{50}). Afterwards, cells were harvested at 400 xg for 5 min at 4ºC, washed twice with cold PBS, lysed in lysis buffer (Cell Signaling

Technology, USA), and the protein concentration was determined using the Bradford protein assay. Protein samples (25μg for GAPDH quantification, 50μg for Cyclin D1 and Cyclin E quantification, or 75μg for $p21^{WAF1/CIP1}$ quantification) were separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride membranes (Millipore, USA). Membranes were incubated for 1 h at room temperature in blocking buffer (3% powdered-skim milk in TBS-T (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% Tween-20)) and incubated overnight at 4ºC with monoclonal antibodies diluted in blocking buffer. Antibodies against $p21^{WAF1/CIP1}$ (# sc-6246; 1:50 dilution), Cyclin D1 (# sc-20044; 1:1,000 dilution), and Cyclin E (# sc-247; 1:1,000 dilution) were from Santa Cruz Biotechnology (Santa Cruz, USA). Antibody against GAPDH (mAB374; 1:12,000,000 dilution; Chemicon/Millipore, USA) was also used. Afterwards, membranes were incubated for 1 h at room temperature with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:30,000 dilution; Calbiochem, USA). Blots were developed with immobilon chemiluminescent HRP substrate (Millipore, USA) and images were captured by a FluorChem SP system (Alpha Innotech, USA). Quantity analysis is based on the intensity of the band using the Quantity One software (Bio-Rad Laboratories, USA). The linearity of the assay was preliminarily checked for each monoclonal antibody by submitting different amounts of untreated cell extracts to western blotting. All data are expressed as mean ± SE of at least three independent experiments.

DOXORUBICIN ACCUMULATION ASSAY

Intracellular doxorubicin levels were determined by flow cytometry. Briefly, NCI-H460/R cells (10^5 per well) were seeded into 6-well plates and then treated with NLSPE5 (0.15, 0.35 and 0.50 μM) or PE5 or PE10 (1.0, 2.6, and 4.3 μM). These concentrations caused a decrease of cell proliferation of 20%, 40%, and 50%, respectively. After 72 h of treatment, cells were incubated with 10 μM doxorubicin for 1 h at 37°C in 5% CO_2. As control experiments 10 μM verapamil (Sigma-Aldrich, USA), which is a competitive inhibitor of P-gp, was added in combination with the doxorubicin. Cells were harvested at 400xg for 5 min at 4ºC, washed twice in cold PBS, and resuspended in this same buffer. Cellular uptake was measured on a

FACSCalibur flow cytometer (Becton Dickinson, USA) using CellQuest Pro software. A minimum of 10,000 events were assayed for each sample and data are expressed as mean ± SE of at least three independent experiments.

RNA ISOLATION FOR MICROARRAY ANALYSIS

NCI/ADR-RES cells (2 x 10^5 per well) and OVCAR8 cells (5 x 10^4 per well) were seeded into 6-well plates and then treated for 36 h with concentrations of onconase or PE5 that caused a 10% decrease of cell proliferation (0.5 μM onconase or 12 μM PE5 for NCI/ADR-RES cells and 0.06 μM onconase or 0.45 μM PE5 for OVCAR8 cells). After treatment, cells were harvested at 400 xg for 5 min at 4ºC and washed twice with cold PBS. Total RNA (including miRNA) was extracted using the mirVana miRNA isolation kit (Applied Biosystems/Ambion, USA) according to the manufacturer's instructions and stored at -80ºC. Four independent preparations were performed for each cell line. RNA degradation and absorbance 260/280 nm ratio of each sample were checked in Bioarray, S.L. (Elche, Spain) using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and a ND-1000 Spectrophotometer (NanoDrop) (Thermo Fisher Scientific, USA), respectively.

GENE EXPRESSION MICROARRAYS

Gene expression microarray experiments were performed at Bioarray, S.L. using the SurePrint G3 Human Gene Expression Microarray (Agilent Technologies, USA), a high-density oligonucleotide microarray that contains 60,000 probes corresponding to 27,958 Entrez Gene RNAs and 7,419 lncRNAs. A loop-design microarray experiment was carried out for each cell line (Figure 2). Sample preparation and microarray processing procedures were done according to the Two-Color Microarray-Based Gene Expression Analysis v. 6.5 (Agilent Technologies, USA). Briefly, 200 ng of total RNA was used to synthesize double-stranded cDNA with AffinityScript-Reverse Transcriptase and Oligo dT-Promoter Primer. cDNA was simultaneously amplified and transcribed into cyanine 3- or cyanine 5-labeled cRNA employing T7 RNA Polymerase in presence of cyanine 3-CTP or cyanine 5-CTP. Labeled cRNA

(antisense) was purified, evaluated using a ND-1000 Spectrophotometer (NanoDrop) (Thermo Fisher Scientific, USA) and hybridized to the oligonucleotide microarray at 65ºC for 17 h. Microarrays were then washed and scanned on a G2565CA Microarray Scanner updated to 2 micron resolution (Agilent Technologies, USA). Data were extracted from the resulting TIFF-images using the Feature Extraction software v. 10.7 (Agilent Technologies, USA).

Raw microarray data were statistically analyzed using the software packages Marray, pcaMethods, Limma and RankProd from Bioconductor (www.bioconductor.org), which uses the R statistical environment and programming language. After performing a quality control analysis of raw microarray data (red and green signal (RG) density plot, $\log_2$ red/green intensity ratio versus average $\log_2$ intensity (MA) plot, and $\log_2$ red/green intensity ratio (M) boxplot), the non-specific signal was removed from the total intensity using the Normexp background correction method, with an offset of 20. Then intra-slide normalization was done using the Loess method to make intensities consistent within each array, and inter-slide normalization was performed employing the Aquantiles method to achieve consistency between arrays. Following normalization, the RankProd method was applied to identify differentially expressed genes. This method is a rank-based nonparametric procedure. It has the advantage that it does not rely on estimating the measurement variance for each single gene (Breitling et al., 2004), and therefore it is particularly useful to identify moderate biologically relevant expression changes between samples with important variability, as is the case of OVCAR8 cell line (see results section). Genes were considered differentially expressed when they had a false discovery rate adjusted p-value < 0.05 and a fold change > 2 or <-2.

Differentially expressed genes were characterized functionally to find over-represented gene ontology terms in the three separate ontologies (biological process, molecular function, and cellular component) (www.geneontology.org), and were also mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.kegg.jp) to find over-represented pathways. In both cases, a p-value cutoff of 0.05 was used.

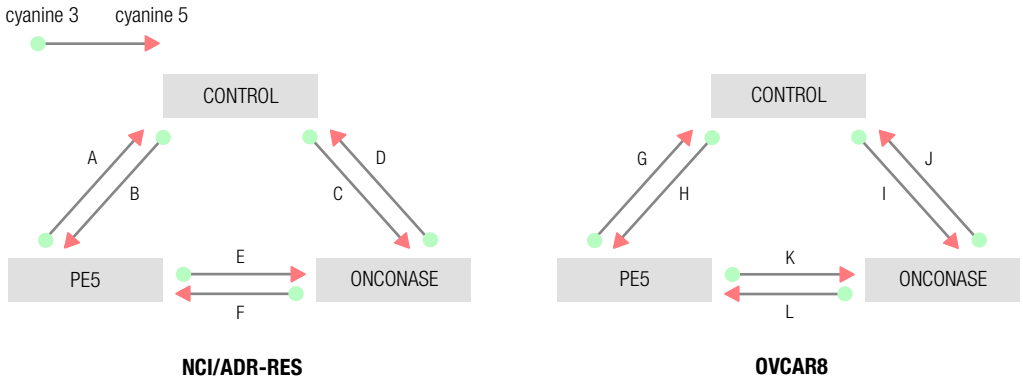

Figure 2. Experimental design of gene expression microarray experiments. Two loop-design microarray experiments were performed, one for NCI/ADR-RES cell line and one for OVCAR8 cell line. Each loop contained six hybridizations (A-F and G-L in the figure) and three experimental conditions (control, PE5 treatment, and onconase treatment). Each loop employed four independent biological replicates.

miRNA MICROARRAYS

miRNA microarray experiments were performed at Bioarray, S.L. (Elche, Spain) using the Human miRNA Microarray release 14.0 (Agilent Technologies, USA), which is based on Sanger miRBase release 14.0. It contains 15,000 probes representing 887 miRNAs. A total of 12 hybridizations were carried out for each cell line, corresponding to three experimental conditions (control, PE5 treatment, and onconase treatment) with four independent biological replicates (Table 1). Sample preparation and microarray processing procedures were done according to the miRNA Microarray System with miRNA Complete Labeling and Hyb Kit v. 2.2 (Agilent Technologies, USA). Briefly, 100 ng of total RNA were dephosphorylated with calf intestinal phosphatase at 37ºC for 30 min, denatured using 100% dimethyl sulfoxide at 100ºC for 5 min, and labeled with cyanine 3-pCp using T4 ligase by incubation at 16ºC for 2 h. In this step a molecule of cyanine 3-pCp is incorporated to the 3'-end of RNA molecules. Labeled RNA was then hybridized to the miRNA

microarray at 55ºC for 20 h. Microarrays were washed and scanned on a G2565CA Microarray Scanner updated to 2 micron resolution (Agilent Technologies, USA). Data were extracted from the resulting TIFF-images using the Feature Extraction software v. 10.7 (Agilent Technologies, USA).

Raw microarray data were statistically analyzed using the software packages AgiMicroRna, Marray and Limma from Bioconductor (www.bioconductor.org), which uses the R statistical environment and programming language. After performing a quality control analysis of raw microarray data (MA plot, density plot, relative $\log_2$ expression (RLE) plot, and expression boxplot), miRNA expression values were obtained using the robust multi-array average (RMA) approach. RMA consisted in three steps: Raw intensity values were first background corrected employing the Normexp method, they were secondly transformed to base two logarithm, and finally, they were quantile normalized to make all data comparable. Following the RMA approach, data were filtered to remove control probes and those probes not expressed in any condition, and a then a differential expression analysis was performed (principal component analysis (PCA) plot and heat map). Finally, a linear model was fitted to the normalized data, and differentially expressed miRNAs were selected using the t-test (separate analysis) from the Limma package. A false discovery rate adjusted p-value < 0.1 was considered significant.

Table 1. Nomenclature of miRNA microarray experiments.

Microarray [a]	Cell line	Experimental condition
N 01, N 04, N 07, N 10	NCI/ADR-RES	Control
N 02, N 05, N 08, N 11	NCI/ADR-RES	Onconase treatment
N 03, N 06, N 09, N 12	NCI/ADR-RES	PE5 treatment
O 01, O 04, O 07, N 10	OVCAR8	Control
O 02, O 05, O 08, O 11	OVCAR8	Onconase treatment
O 03, O 06, O 09, O 12	OVCAR8	PE5 treatment

[a] Four hybridizations were carried out for each cell line and experimental condition, corresponding to four independent biological replicates.

Results

STUDY OF THE MOLECULAR MECHANISM OF PE5- AND ONCONASE-INDUCED CYTOTOXICITY

Antitumor RNases are enzymes that cause pleiotropic effects on the cells, and therefore it is difficult to predict the mechanism by which these RNases induce the cellular death. For this reason, in the present work we have used the microarray technology to simultaneously analyze changes in thousands of genes upon exposure to PE5 and to identify which pathways are therefore altered. We have compared these results with those obtained for cells exposed to onconase. We have carried out the microarray experiments with the MDR cell line NCI/ADR-RES and its parental cell line OVCAR8.

It has been previously described that treatment with onconase alters gene expression (Altomare et al., 2010), but also leads to changes at miRNA level (Goparaju et al., 2011). Therefore, we have performed two different types of microarray analysis. First, we have used SurePrint G3 Human Gene Expression Microarrays (Agilent Technologies, USA) to identify the differentially expressed genes upon RNase treatment, and second, we have used Human miRNA Microarray release 14.0 (Agilent Technologies, USA) to detect changes in miRNA expression.

Selection of treatment conditions

In order to carry out the microarray experiments we previously selected which treatment conditions, particularly the concentration of RNase and incubation time, caused a minimal cytotoxic effect but not a considerable RNA degradation. This was important for two reasons. First, an extensive RNA degradation would prevent the possibility of performing the microarray experiments, and second, the induction of apoptosis increases the RNA turnover (Mondino and Jenkins, 1995), so an excessive cytotoxic effect would generate changes in the RNA levels due to the induction of cellular apoptosis rather than the direct action of RNase. We therefore investigated the cytotoxic effect of PE5 and onconase in NCI/ADR-RES and OVCAR8 cells at different RNase concentrations and incubation times (Figure 3). As previously described (Castro et al., 2011a), the effect of PE5 was lower than that of onconase in

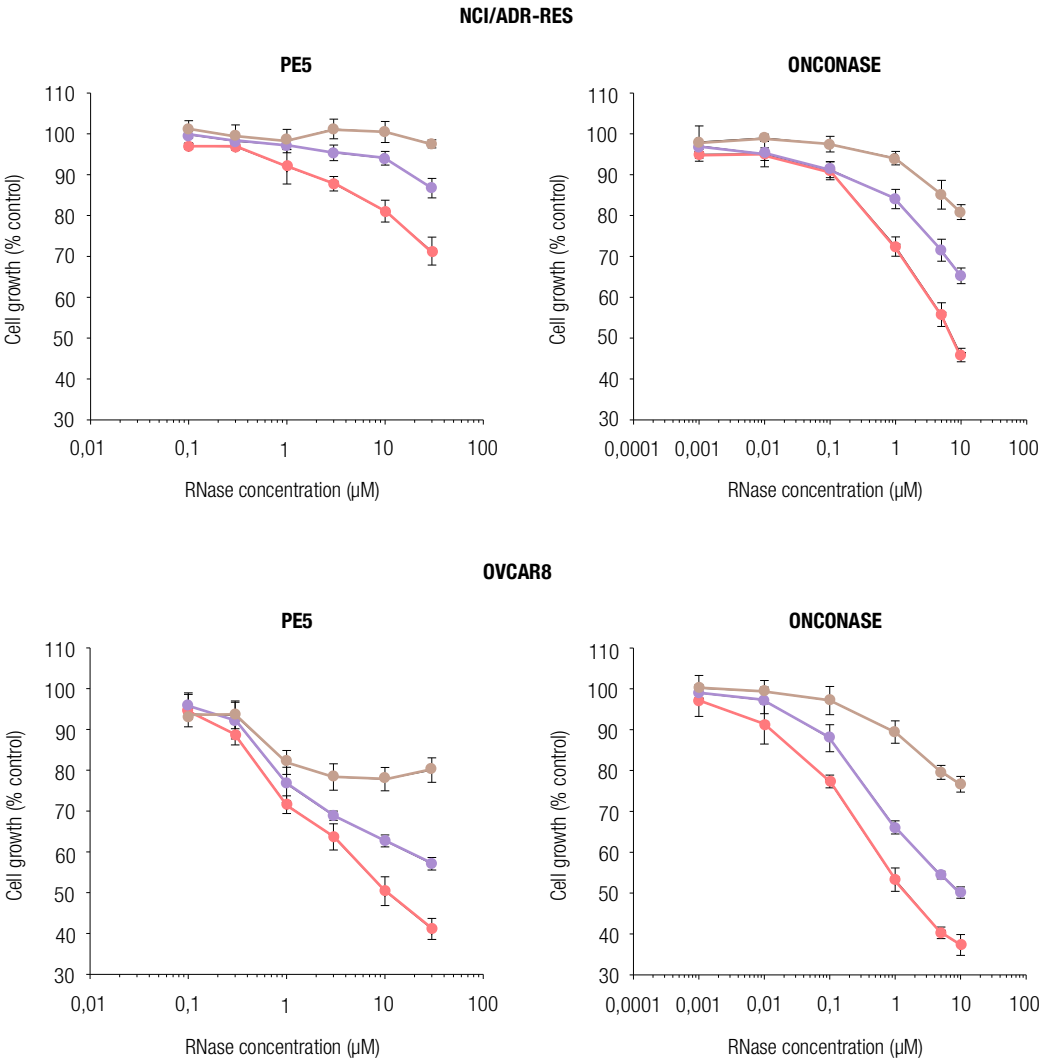

Figure 3. Cytotoxic effects of PE5 and onconase in NCI/ADR-RES and OVCAR8 cell lines. Control and RNase-treated cells were maintained for 24 (brown), 36 (violet), or 48 h (red) and metabolic activity was determined by the MTT assay. Cell growth is expressed as the percentage of activity respective to control cells using the absorbance values. The curves in the figure are from one representative experiment made in triplicates. Data are presented as mean ± SE. Equivalent results were found in at least three independent experiments.

both cell lines. A dose-dependent cytotoxic effect was apparent after 36 h of incubation with PE5 or after 24 h of incubation with onconase, and increased with incubation time. The MDR cell line NCI/ADR-RES was less sensitive to RNases than its parental cell line OVCAR8.

We then analyzed the RNA degradation caused by the different treatments. We incubated NCI/ADR-RES and OVCAR8 cells with those concentrations of PE5 or onconase that induced a decrease of cell proliferation of 5%, 10%, and 15% (IC_5, IC_{10}, and IC_{15}) after 36 h of incubation. We did not analyze higher RNase concentrations since we had previously observed in an agarose gel experiment that an extensive cleavage of nuclear RNA was produced in HeLa cells incubated with PE5 at a concentration equivalent to IC_{20} (Tubert et al., 2011). The RNA degradation of treated and untreated control cells was quantified using a bioanalyzer (Table 2). For NCI/ADR-RES cell line, RNA of untreated and onconase-treated cells was not degraded at all, while in the case of PE5 a slight RNA degradation was detected at IC_{10} and IC_{15} (RIN of 6.90 and 6.60, respectively). This difference in the RIN value between both RNases was expected since it has been described that PE5 degrades rRNA (Tubert et al., 2011) whereas onconase leaves rRNA apparently undamaged (Iordanov et al., 2000a; Saxena et al., 2002), and two of the most important features that determine the RIN value are the ratio of ribosomal bands area to total area of the electropherogram and the height of the 18S peak. Technically, RNA samples with RIN values above 8.0 have enough quality to be analyzed in a microarray experiment. The RIN value obtained for PE5 at IC_{10} was around one unit under 8.0. However, we considered that this low value was due to the rRNA degradation caused by PE5 and therefore it would not prevent the interpretation of the microarray experiments, which are performed with mRNA and microRNA. For OVCAR8 cell line, RNA samples were much less degraded (Table 2). According to these results, we decided to carry out the microarray experiments at those concentrations of PE5 and onconase that provoked a decrease of 10% in cell proliferation after 36 h of treatment (0.5 μM onconase or 12 μM PE5 for NCI/ADR-RES cells and 0.06 μM onconase or 0.45 μM PE5 for OVCAR8 cells). We considered that at IC_5 the cytotoxic effect of the RNases was too low and that at IC_{15} the RNA degradation of PE5-treated NCI/ADR-RES cells was too high.

Table 2. RIN values obtained for NCI/ADR-RES and OVCAR8 cells treated with PE5 or onconase for 36 h.

Treatment	NCI/ADR-RES		OVCAR8	
	PE5	ONCONASE	PE5	ONCONASE
Untreated	9.50	9.50	9.60	9.60
IC_5	8.80	9.60	- [a]	- [a]
IC_{10}	6.90	9.40	9.20	9.50
IC_{15}	6.60	9.60	8.90	9.30

[a] Not done.

Generation of gene expression profiles

We treated NCI/ADR-RES and OVCAR8 cells with a concentration of PE5 or onconase corresponding to IC_{10} for 36 h, and after treatment we purified the total RNA. The extracted RNA was checked in the bioanalyzer, obtaining RIN values equal to the previous experiments, and then it was used to synthesize cyanine 3- or cyanine 5- labeled cRNA. This labeled cRNA was hybridized to the human gene expression microarray. After extracting the data from the resulting images, we performed a quality control analysis to check the quality of the arrays and to determine whether normalization was needed (Figure 4). The global RG density plot, representing red (R) and green (G) signal distributions of all the microarrays, indicated that none of the arrays had a weird distribution although a light deviation between arrays and channels was observed. The deviation between channels was also observed in the individual RG density plot of each microarray. On the other hand, the individual MA plots obtained for the different microarrays, representing the $\log_2$ red/green intensity ratio (M) plotted by the average $\log_2$ intensity (A), were acceptable but the majority of points in the y-axis were not located around 0. In addition, in the global M boxplot comparing the M values of all the microarrays, the boxes were similar but not completely homogeneous and not around zero. Globally, these results indicated that all the microarrays had high quality, although a normalization process was needed.

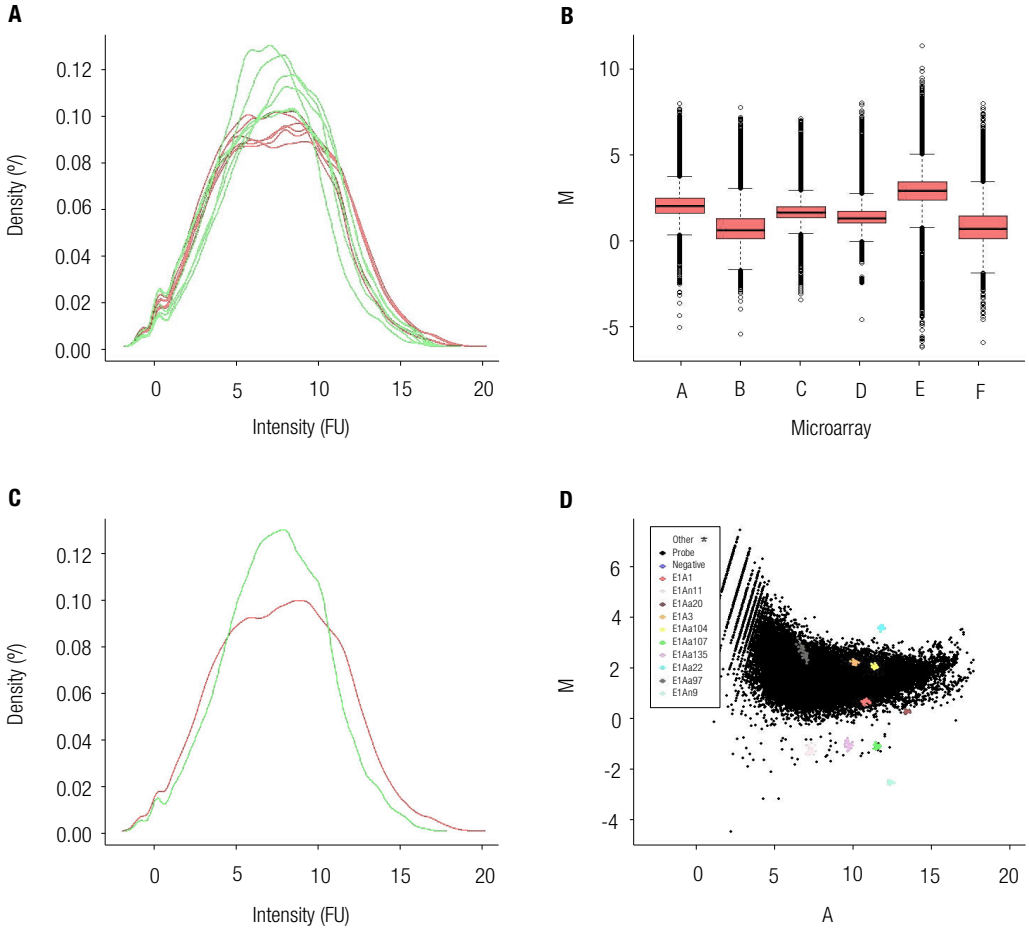

Figure 4. Quality control analysis of raw gene expression microarray data. A) Global RG density plot and B) Global M boxplot of NCI/ADR-RES cell line microarrays. Equivalent plots were obtained for OVCAR8 cell line. C) Individual RG density plot and D) Individual MA plot of a representative NCI/ADR-RES cell line microarray. Equivalent plots were obtained for all the microarrays.

Before applying the normalization, we corrected raw intensity values employing the Normexp background correction method with an offset of 20, and we performed another quality control analysis (Figure 5). Plots did not vary substantially respective those obtained before background correction, indicating that hybridization and clean processes were correct and that background was homogeneous in all the microarrays.

Following background correction, we applied intra-slide normalization using the Loess method. The quality control analysis of intra-slide normalized data indicated that intensities were consistent within each array (Figure 6). Indeed, the global RG density plot revealed that the homogeneity between samples was higher than before intra-slide normalization, and the individual RG density plots obtained for the different microarrays demonstrate and absence of deviation between channels. In addition, in the individual MA plots the majority of points in the y-axis were located around 0, and the boxes of the global M boxplot were more homogeneous and around zero.

Then we performed inter-slide normalization employing the Aquantiles method. In this case, the quality control analysis of normalized data showed that intensities were consistent between arrays (Figure 7). The global RG density plot indicated that all the arrays and channels had the same distribution, and the global M boxplot demonstrate that all the arrays were perfectly comparable.

After this process, we analyzed normalized data with the RankProd method in an attempt to identify differentially expressed genes. This method is a rank-based nonparametric procedure. It has the advantage that it does not rely on estimating the measurement variance for each single gene (Breitling et al., 2004), and therefore it is particularly useful to identify moderate biologically relevant expression changes between samples with important variability, as is the case of OVCAR8 cell line. Genes were considered differentially expressed when they had a false discovery rate adjusted p-value < 0.05 and a fold change > 2 or <-2.

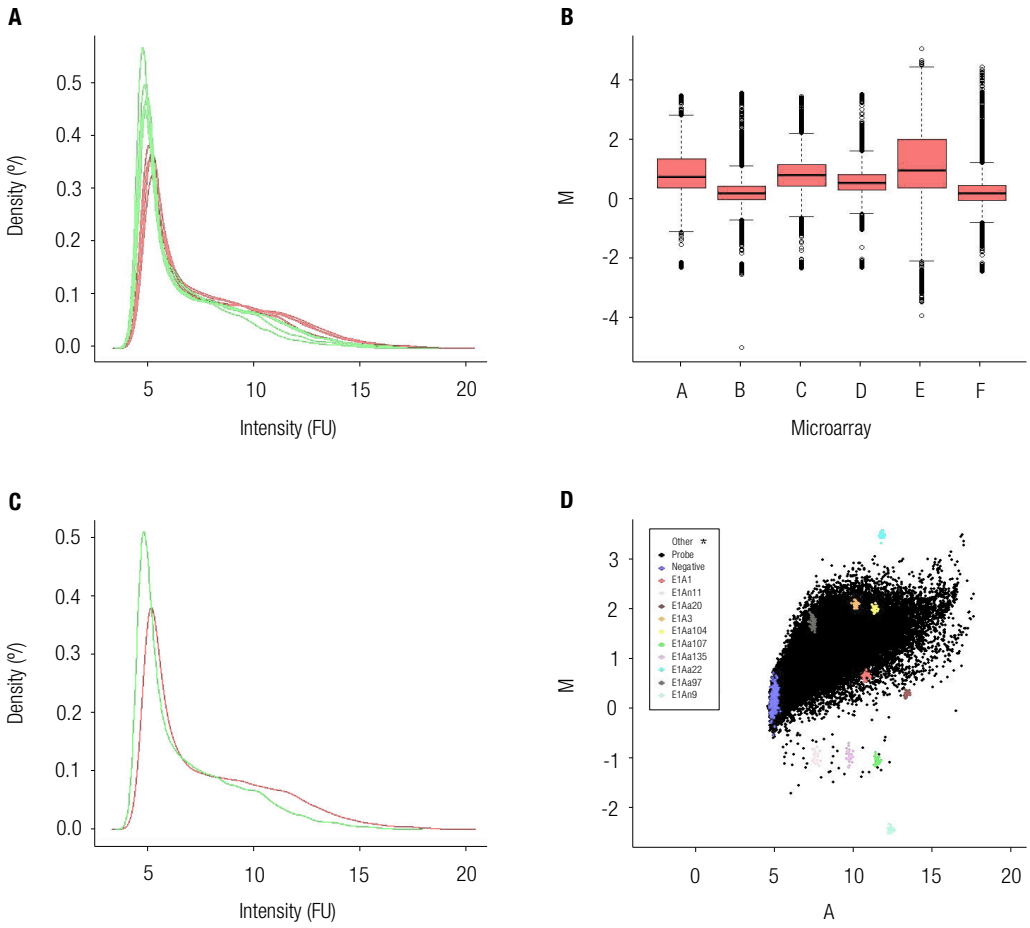

Figure 5. Quality control analysis of background corrected gene expression microarray data. Raw intensity values were background corrected employing the Normexp method with an offset of 20. A) Global RG density plot and B) Global M boxplot of NCI/ADR-RES cell line microarrays. Equivalent plots were obtained for OVCAR8 cell line. C) Individual RG density plot and D) Individual MA plot of a representative NCI/ADR-RES cell line microarray. Equivalent plots were obtained for all the microarrays.

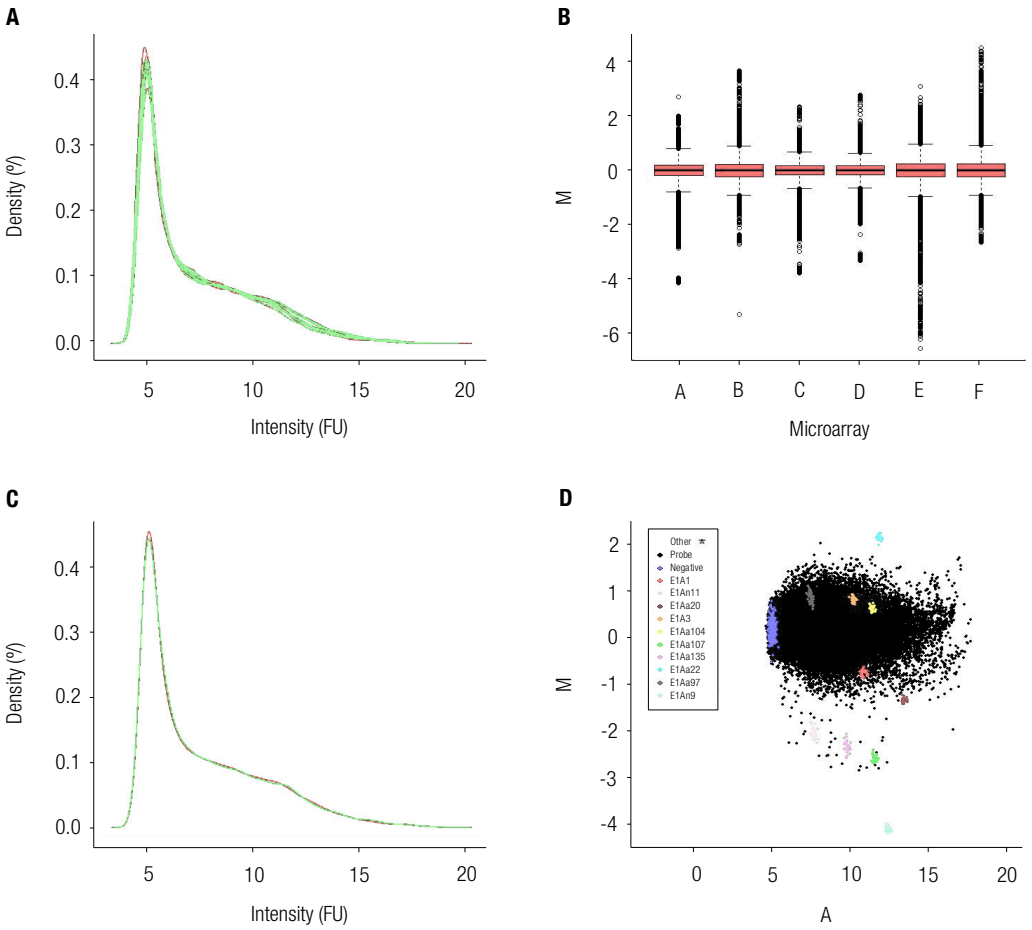

Figure 6. Quality control analysis of intra-slide normalized gene expression microarray data. Background corrected data were intra-slide normalized using the Loess method. A) Global RG density plot and B) Global M boxplot of NCI/ADR-RES cell line microarrays. Equivalent plots were obtained for OVCAR8 cell line. C) Individual RG density plot and D) Individual MA plot of a representative NCI/ADR-RES cell line microarray. Equivalent plots were obtained for all the microarrays.

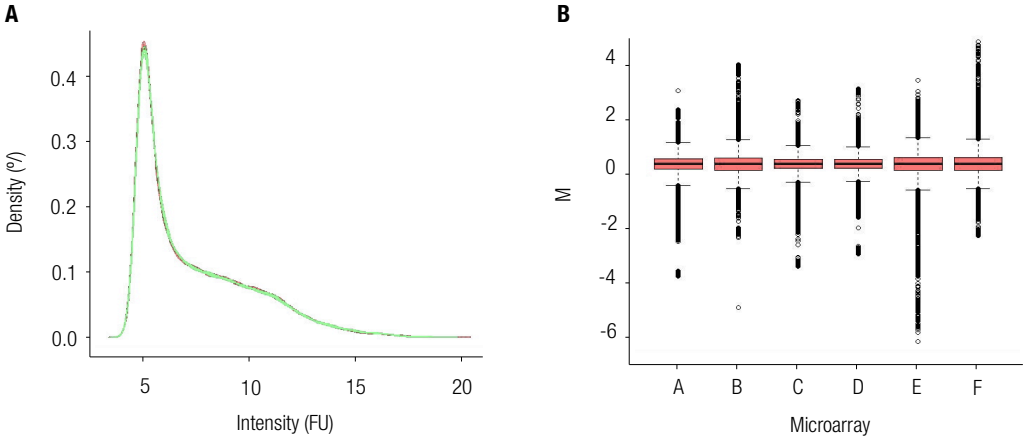

Figure 7. Quality control analysis of inter-slide normalized gene expression microarray data. Intra-slide normalized data were inter-slide normalized using the Aquantiles method. A) Global RG density plot and B) Global M boxplot of NCI/ADR-RES cell line microarrays. Equivalent plots were obtained for OVCAR8 cell line.

Identification of PE5 and onconase differentially expressed genes

A comparison of the gene expression profiles of PE5-treated and untreated NCI/ADR-RES cells revealed 647 differentially expressed genes. 53% of them were upregulated in PE5-treated cells, with an increase from untreated cells ranging from 2- to 106-fold, while 47% were downregulated, with a decrease compared to untreated cells ranging from 2- to 4-fold. This result indicates that the primary effect of PE5 is both to increase and to decrease gene expression. Table 3 shows the top 20 PE5 upregulated and downregulated genes in NCI/ADR-RES cell line. PE5 upregulated genes participate in a variety of biological processes, including transcription regulation (HMBOX1, SPEN, TEF, MXD1), cell cycle (RPA4, WTAP, CCNE2, CCNK), apoptosis (BCL2L11, RHOB, BNIP3L) and mRNA processing (WDR33, SRRM2, TRA2B, CCNL1), among others. Interestingly, some of them

function as tumor suppressors (MXD1, BNIP3L, DMTF1). On the other hand, PE5 downregulated genes are related to different biological processes such as cell adhesion and migration (GPC6, EFEMP1, ANTXR1, TGFBI), amino acid metabolism (PYCR1, BCAT1, PHGDH, ASNS), lipid metabolism (HADHA, DHCR24, ACACA, SPTLC3) and glucose metabolism (PGM1, PGAM1, LDHA, ENO1), among others. In particular, some of them are known to be upregulated in tumor cells and enhance the aggressive nature of tumors (EFEMP1), promote migration and invasion of cancer cells (GPC6) or act as proto-oncogenes (MET).

Table 3. PE5 differentially expressed genes in NCI/ADR-RES cell line: top 20 PE5 upregulated and downregulated genes.

Gene Symbol	Gene Name	Fold Change	Main Functions
HMBOX1	Homeobox containing 1	106.0	Transcription regulation
GPR179	G protein-coupled receptor 179	65.9	Sensory transduction
LRRC2	Leucine rich repeat containing 2	62.7	Unknown
GPR182	G protein-coupled receptor 182	56.3	Signaling pathway
PPP6R1	Protein phosphatase 6, regulatory subunit 1	51.5	Signal transduction
LINC00340	Long intergenic non-protein coding RNA 340	45.7	Transcription regulation
CD86	CD86 molecule	45.3	Immune response
SPEN	Spen homolog, transcriptional regulator (*Drosophila*)	41.8	Transcription regulation
KRT79	Keratin 79	40.1	Cell structural integrity
RPA4	Replication protein A4, 30kDa	39.6	Cell cycle DNA repair
BICC1	Bicaudal C homolog 1 (*Drosophila*)	26.3	Embryonic development
VPS18	Vacuolar protein sorting 18 homolog (*S. cerevisiae*)	24.8	Vesicle trafficking
PROM2	Prominin 2	19.7	Membrane organization
REP15	RAB15 effector protein	16.5	Iron metabolism
RAP1GAP	RAP1 GTPase activating protein	13.4	Signal transduction
TEF	Thyrotrophic embryonic factor	11.9	Transcription regulation
CISH	Cytokine inducible SH2-containing protein	11.7	Signal transduction

CCDC66	Coiled-coil domain containing 66	9.5	Embryonic development
DHRS4L1	Dehydrogenase/reductase (SDR family) member 4 like 1	8.9	Unknown
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5	7.0	Signal transduction
CCDC80	Coiled-coil domain containing 80	-2.7	Cell adhesion
PHGDH	Phosphoglycerate dehydrogenase	-2.7	Amino acid metabolism
BCAT1	Branched chain amino acid transaminase 1, cytosolic	-2.8	Amino acid metabolism Cell cycle
IMPA2	Inositol(myo)-1(or 4)-monophosphatase 2	-2.8	Signal transduction
SHROOM3	Shroom family member 3	-2.8	Cell shape regulation
TGFBI	Transforming growth factor, beta-induced, 68kDa	-2.8	Cell adhesion Cell proliferation
LAMA1	Laminin, alpha 1	-2.8	Embryonic development Cell adhesion Cell migration
FRAS1	Fraser syndrome 1	-2.8	Embryonic development
DKK3	Dickkopf 3 homolog (*Xenopus laevis*)	-2.8	Embryonic development Transcription regulation
UTRN	Utrophin	-2.9	Neuromuscular synapse
DPYD	Dihydropyrimidine dehydrogenase	-2.9	Pyrimidine metabolism
ANTXR1	Anthrax toxin receptor 1	-2.9	Cell adhesion Cell migration Angiogenesis
PYCR1	Pyrroline-5-carboxylate reductase 1	-3.0	Amino acid metabolism Stress response
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	-3.0	Gluconeogenesis
LRP10	Low density lipoprotein receptor-related protein 10	-3.1	Lipid metabolism
ABHD2	Abhydrolase domain containing 2	-3.1	Cell migration
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	-3.1	Cell adhesion Cell migration Transcription regulation Signal transduction
MAGED2	Melanoma antigen family D, 2	-3.1	Apoptosis Cell cycle
PSAP	Prosaposin	-3.2	Sphingolipid metabolism
GPC6	Glypican 6	-4.1	Cell migration

Gene information was taken from the UniProt database (European Bioinformatics, UK, Swiss Institute of Bioinformatics, Switzerland, Protein Information Resource, USA) (http://www.uniprot.org) and from the Entrez Gene database (National Center for Biotechnology Information, USA) (http://www.ncbi.nlm.nih. gov/gene).

In the case of onconase, we obtained 56 differentially expressed genes when the expression profiles of treated and untreated NCI/ADR-RES cells were compared. 89% of them were upregulated in onconase-treated cells, with an increase respective to untreated cells ranging from 2- to 6.5-fold, whereas 11% were downregulated, with a decrease from untreated cells ranging from 2- to 2.5-fold. This indicates that the primary effect of onconase in NCI/ADR-RES cells is activation of gene expression, as previously described for human mesothelioma cells (Altomare et al., 2010). Table 4 shows the top 20 onconase upregulated genes and the 6 downregulated genes in NCI/ADR-RES cell line. Onconase upregulated genes are involved in a large variety of biological processes, including transcription regulation (ATF3, CREB5, EGR1), cell cycle (GADD45A), apoptosis (PPP1R15A, HRK), immune response (IL6, IL1RL1, IL23A) and stress response (TXNIP, DHRS2), among others. Interestingly, some of the upregulated genes act as suppressors of tumor cell growth (TXNIP, EGR1, PPP1R15A). Onconase downregulated genes are associated with transcription regulation (RDBP, XBP1), amino acid metabolism (PHGDH, ASNS) and protein folding (HSPA1A, HSPA8).

Table 4. Onconase differentially expressed genes in NCI/ADR-RES cell line: top 20 onconase upregulated genes and the 6 downregulated genes.

Gene Symbol	Gene Name	Fold Change	Main Functions
TXNIP	Thioredoxin interacting protein	6.5	Transcription regulation Cell cycle Cell proliferation Stress response Apoptosis
ATF3	Activating transcription factor 3	6.5	Transcription regulation
CREB5	cAMP responsive element binding protein 5	6.2	Transcription regulation
HIST1H2AC	Histone cluster 1, H2ac	4.0	Nucleosome assembly
ARRDC4	Arrestin domain containing 4	3.9	Signal transduction
NEURL3	Neuralized homolog 3 (*Drosophila*) pseudogene	3.6	Transcription regulation
GADD45A	Growth arrest and DNA-damage-inducible, alpha	3.2	Cell cycle DNA repair Apoptosis

IL6	Interleukin 6 (interferon, beta 2)	3.2	Immune response Cell differentiation Cell proliferation Apoptosis Transcription regulation
AREG	Amphiregulin	3.2	Cell proliferation
SPINK6	Serine peptidase inhibitor, Kazal type 6	3.0	Epidermis development
EGR1	Early growth response 1	3.0	Transcription regulation
PPP1R15A	Protein phosphatase 1, regulatory subunit 15A	2.7	Apoptosis Stress response Translation regulation Cell cycle
ARID5B	AT rich interactive domain 5B (MRF1-like)	2.5	Transcription regulation
ANO2	Anoctamin 2	2.5	Ion transport
BHLHE41	Basic helix-loop-helix family, member e41	2.5	Transcription regulation
DHRS2	Dehydrogenase/reductase (SDR family) member 2	2.5	Stress response
MEF2C	Myocyte enhancer factor 2C	2.5	Transcription regulation Cell differentiation Immune response
IL11	Interleukin 11	2.4	Cell differentiation Cell proliferation Transcription regulation
HMBOX1	Homeobox containing 1	2.4	Transcription regulation
IL1RL1	Interleukin 1 receptor-like 1	2.4	Immune response
HSPA8	Heat shock 70kDa protein 8	-2.0	Protein folding Unfolded protein response
XBP1	X-box binding protein 1	-2.2	Transcription regulation Unfolded protein response
HSPA1A	Heat shock 70kDa protein 1A	-2.3	Protein folding Unfolded protein response
ASNS	Asparagine synthetase (glutamine-hydrolyzing)	-2.3	Amino acid metabolism Unfolded protein response
PHGDH	Phosphoglycerate dehydrogenase	-2.5	Amino acid metabolism
RDBP	RD RNA binding protein	-2.5	Transcription regulation

Gene information was taken from the UniProt database (European Bioinformatics, UK, Swiss Institute of Bioinformatics, Switzerland, Protein Information Resource, USA) (http://www.uniprot.org) and from the Entrez Gene database (National Center for Biotechnology Information, USA) (http://www.ncbi.nlm.nih. gov/gene).

Among the PE5 and onconase differentially expressed genes in NCI/ADR-RES cell line, we identified only 10 genes that were common for both RNase treatments (either 1.5% or 18% of the PE5 and onconase differentially expressed genes, respectively). This result indicates that the molecular mechanism of PE5-induced cytotoxicity is clearly different to that induced by treatment with onconase. Table 5 shows these common genes. Eight of them were upregulated in RNase-treated cells respective to untreated cells, whereas the other two genes where downregulated. Common upregulated genes are related to transcription regulation (HMBOX1, LINC00340, NEXN-AS1) and immune response (CD86, IL6). Common downregulated genes participate in amino acid metabolism (PHGDH, ASNS).

Table 5. Common PE5 and onconase differentially expressed genes in NCI/ADR-RES cell line.

Gene Symbol	Gene Name	Fold Change		Main Functions
		PE5	Onconase	
HMBOX1	Homeobox containing 1	106.0	2.4	Transcription regulation
LRRC2	Leucine rich repeat containing 2	62.7	2.2	Unknown
LINC00340	Long intergenic non-protein coding RNA 340	45.7	2.4	Transcription regulation
CD86	CD86 molecule	45.3	2.3	Immune response
IL6	Interleukin 6 (interferon, beta 2)	2.5	3.2	Immune response Cell differentiation Cell proliferation Apoptosis Transcription regulation
NEXN-AS1	NEXN antisense RNA 1 (non-protein coding)	2.2	2.1	Transcription regulation
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	2.1	2.3	Antiviral defense Apoptosis
SPINK6	Serine peptidase inhibitor, Kazal type 6	2.0	3.0	Epidermis development
ASNS	Asparagine synthetase (glutamine-hydrolyzing)	-2.3	-2.3	Amino acid metabolism Unfolded protein response
PHGDH	Phosphoglycerate dehydrogenase	-2.7	-2.5	Amino acid metabolism

Gene information was taken from the UniProt database (European Bioinformatics, UK, Swiss Institute of Bioinformatics, Switzerland, Protein Information Resource, USA) (http://www.uniprot.org) and from the Entrez Gene database (National Center for Biotechnology Information, USA) (http://www.ncbi.nlm.nih. gov/gene).

For OVCAR8 cell line we obtained a considerable lower number of differentially expressed genes than for NCI/ADR-RES. We identified only one differentially expressed gene when expression profiles of PE5-treated and untreated cells were compared. This gene (ZNF750) was upregulated in treated cells, with an increase of 2.3-fold respective to untreated cells, and it is involved in transcription regulation.

In the case of onconase, the comparison of the expression profiles of treated and untreated OVCAR8 cells revealed seven differentially expressed genes. All of them were upregulated in treated cells, with an increase compared to untreated cells ranging from 2- to 4-fold. This indicates that the primary effect of onconase in OVCAR8 cells is activation of gene expression, as observed for NCI/ADR-RES cells. Table 6 shows the onconase upregulated genes in OVCAR8 cell line. Some of them are related to transcription regulation (ATF3, NEURL3, ZNF750) and stress response (TXNIP, DHRS2). It should be noted that the PE5 upregulated gene in OVCAR8 cell line (ZNF750) was also upregulated by onconase.

Table 6. Onconase differentially expressed genes in OVCAR8 cell line.

Gene Symbol	Gene Name	Fold Change	Main Functions
ATF3	Activating transcription factor 3	4.0	Transcription regulation
TXNIP	Thioredoxin interacting protein	3.2	Transcription regulation Cell cycle Cell proliferation Stress response Apoptosis
NEURL3	Neuralized homolog 3 (*Drosophila*) pseudogene	2.9	Transcription regulation
ZNF750	Zinc finger protein 750	2.3	Transcription regulation
HIST1H2AC	Histone cluster 1, H2ac	2.3	Nucleosome assembly
DHRS2	Dehydrogenase/reductase (SDR family) member 2	2.2	Stress response
NPPB	Natriuretic peptide B	2.1	Cardiovascular homeostasis

Gene information was taken from the UniProt database (European Bioinformatics, UK, Swiss Institute of Bioinformatics, Switzerland, Protein Information Resource, USA) (http://www.uniprot.org) and from the Entrez Gene database (National Center for Biotechnology Information, USA) (http://www.ncbi.nlm.nih. gov/gene).

Table 7. Common onconase differentially expressed genes in NCI/ADR-RES and OVCAR8 cell lines.

Gene Symbol	Gene Name	Fold Change		Main Functions
		NCI/ADR-RES	OVCAR8	
TXNIP	Thioredoxin interacting protein	6.5	3.2	Transcription regulation Cell cycle Cell proliferation Stress response Apoptosis
ATF3	Activating transcription factor 3	6.5	4.0	Transcription regulation
HIST1H2AC	Histone cluster 1, H2ac	4.0	2.3	Nucleosome assembly
NEURL3	Neuralized homolog 3 (*Drosophila*) pseudogene	3.6	2.9	Transcription regulation
DHRS2	Dehydrogenase/reductase (SDR family) member 2	2.5	2.2	Stress response
NPPB	Natriuretic peptide B	2.0	2.1	Cardiovascular homeostasis

Gene information was taken from the UniProt database (European Bioinformatics, UK, Swiss Institute of Bioinformatics, Switzerland, Protein Information Resource, USA) (http://www.uniprot.org) and from the Entrez Gene database (National Center for Biotechnology Information, USA) (http://www.ncbi.nlm.nih. gov/gene).

Finally, when the results for OVCAR8 and NCI/ADR-RES cell lines were compared, we obtained that the only PE5 upregulated gene in OVCAR8 cell line was not a differentially expressed gene in NCI/ADR-RES cell line. However, six out of the seven onconase differentially expressed genes in OVCAR8 cell line were also differentially expressed in NCI/ADR-RES cell line, indicating that the initial action of onconase is similar for both cell lines. Table 7 shows these common genes. All of them were upregulated compared to untreated cells, and they are associated with transcription regulation (ATF3, NEURL3) and stress response (TXNIP, DHRS2).

Gene ontology analysis and KEGG pathway annotation of PE5 and onconase differentially expressed genes

To better understand the functional relevance of the genes regulated by PE5 and onconase in NCI/ADR-RES cells, we performed a gene ontology analysis and KEGG pathway annotation. PE5 and onconase differentially expressed genes were

used to find over-represented gene ontology terms in the three main ontologies: "biological process", consisting in operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units (cells, tissues, organs, and organisms), "molecular function", comprising the elemental activities of gene products at the molecular level, such as binding or catalysis, and "cellular component", describing the parts of a cell or its extracellular environment where the gene products are biologically relevant. PE5 and onconase differentially expressed genes were also mapped to the KEGG to find over-represented pathways. In all cases, a p-value < 0.05 was considered statistically significant.

Gene ontology analysis showed that PE5 differentially expressed genes in NCI/ADR-RES cells are related to a number of cellular events (Table 8). The most significantly affected biological process terms were lipid metabolic process, response to stress, and angiogenesis. Interestingly, cell proliferation and carbohydrate metabolic process were also affected. Regarding molecular function, the most affected terms were nucleotide binding, peptidase activity, and pyrophosphatase activity, whereas concerning cellular component, the most affected were lysosome, plasma membrane, and anchoring junction.

Onconase differentially expressed genes in NCI/ADR-RES cells are also involved in a wide diversity of cellular events (Table 9). Gene ontology analysis revealed that the most significantly affected biological process terms were cell proliferation, response to stress, and signal transduction. Other remarkable affected terms were apoptotic process and angiogenesis. On the other hand, the most affected molecular function terms were cytokine activity, nucleic acid binding transcription factor activity, and growth factor activity, and for cellular component the only affected terms were extracellular region, cell surface, and nucleus.

Analysis of the over-represented pathways collected in the KEGG database showed that 19 and 9 pathways were affected after PE5 (Table 10) and onconase (Table 11) treatments, respectively. Most of the pathways affected by PE5 were involved in cell metabolism, being very interesting those of pyruvate and glucose metabolism for their relevance in cancer cells. For onconase, some of the affected pathways were involved in growth and development process, like JAK-STAT signaling, which affects

Table 8. Gene ontology analysis of PE5 differentially expressed genes in NCI/ADR-RES cell line.

Gene ontology	Gene ontology term	Gene count [a]	P-value
Biological Process	Lipid metabolic process	64	8.11E-05
	Response to stress	131	4.70E-04
	Angiogenesis	25	6.83E-04
	Developmental process	181	4.07E-03
	Cell adhesion	47	8.26E-03
	Cell proliferation	69	1.33E-02
	Cell migration	41	1.61E-02
	Carbohydrate metabolic process	36	4.34E-02
Molecular Function	Nucleotide binding	115	3.52E-05
	Peptidase activity	34	1.34E-03
	Pyrophosphatase activity	42	2.58E-03
	Oxidoreductase activity	38	3.30E-03
	Monosaccharide binding	7	4.58E-03
	Cytoskeletal protein binding	34	4.95E-03
	Glycoprotein binding	6	1.28E-02
	Coenzyme binding	12	1.77E-02
Cellular Component	Lysosome	31	2.31E-07
	Plasma membrane	177	1.27E-04
	Anchoring junction	16	1.67E-03
	Endoplasmic reticulum	61	2.25E-03
	Cell surface	28	2.41E-03
	Extracellular region	92	3.05E-03
	Endosome	29	7.86E-03
	Golgi apparatus	53	8.01E-03
	Actin cytoskeleton	19	2.87E-02

[a] Number of differentially expressed genes that belong to these terms.

Table 9. Gene ontology analysis of onconase differentially expressed genes in NCI/ADR-RES cell line.

Gene ontology	Gene ontology term	Gene count [a]	P-value
Biological Process	Cell proliferation	15	2.45E-05
	Response to stress	21	3.18E-05
	Signal transduction	24	2.03E-04
	Cell differentiation	18	4.34E-04
	Apoptotic process	13	4.81E-04
	Developmental process	24	7.50E-04
	Immune response	10	9.98E-04
	Transcription, DNA-dependent	18	3.44E-03
	Phosphorylation	10	3.95E-03
	Cell-cell signaling	9	4.46E-03
	Chromatin assembly	3	5.46E-03
	Protein metabolic process	20	5.80E-03
	Intracellular protein kinase cascade	7	1.45E-02
	Gene expression	20	2.20E-02
	Angiogenesis	4	2.24E-02
	Growth	6	2.66E-02
	RNA metabolic process	18	2.84E-02
	Cell migration	6	3.94E-02
	DNA metabolic process	6	4.52E-02
Molecular Function	Cytokine activity	4	3.66E-03
	Nucleic acid binding transcription factor activity	8	1.10E-02
	Growth factor activity	3	1.38E-02
	Hormone activity	2	4.48E-02
Cellular Component	Extracellular region	15	6.05E-04
	Cell surface	6	2.35E-03
	Nucleus	24	2.30E-02

[a] Number of differentially expressed genes that belong to these terms.

basic cellular functions such as cell growth, differentiation, and death (Reviewed in Aaronson and Horvath, 2002), or MAPK signaling pathway, that can also regulate a wide variety of cellular functions, including cell proliferation, differentiation, and stress responses (Reviewed in Tanoue and Nishida, 2002). Many pathways related to infection or immunological diseases were also affected. This is the case of rheumatoid arthritis, systemic lupus erythematosus, or graft-versus-host disease.

Table 10. KEGG pathway annotation of PE5 differentially expressed genes in NCI/ADR-RES cell line.

KEGG term	Gene count [a]	P-value
Lysosome	14	8.84E-05
Metabolic pathways	59	8.73E-04
Pyruvate metabolism	6	2.64E-03
Steroid biosynthesis	4	3.97E-03
Propanoate metabolism	5	5.01E-03
Glycolysis / Gluconeogenesis	7	7.91E-03
Nicotinate and nicotinamide metabolism	4	9.47E-03
Gastric acid secretion	7	1.56E-02
Homologous recombination	4	1.63E-02
Vitamin B6 metabolism	2	1.72E-02
Selenocompound metabolism	3	2.09E-02
Other glycan degradation	3	2.09E-02
Aminoacyl-tRNA biosynthesis	6	2.38E-02
Focal adhesion	13	2.55E-02
Glycosaminoglycan degradation	3	2.83E-02
ECM-receptor interaction	7	3.10E-02
Viral myocarditis	6	3.75E-02
Salivary secretion	7	3.85E-02
Bile secretion	6	3.98E-02

[a] Number of differentially expressed genes that belong to these terms.

Table 11. KEGG pathway annotation of onconase differentially expressed genes in NCI/ADR-RES cell line.

KEGG term	Gene count [a]	P-value
Prion diseases	3	4.59E-04
Rheumatoid arthritis	4	6.22E-04
MAPK signaling pathway	6	9.09E-04
Systemic lupus erythematosus	4	2.77E-03
Protein processing in endoplasmic reticulum	4	5.55E-03
Graft-versus-host disease	2	1.39E-02
Intestinal immune network for IgA production	2	1.88E-02
JAK-STAT signaling pathway	3	3.01E-02
Antigen processing and presentation	2	4.40E-02

[a] Number of differentially expressed genes that belong to these terms.

Globally, these results indicate that PE5 and onconase participate in a large variety of cell phenomena and functions but in a different way, since most of the over-represented gene ontology terms and KEGG pathways were different. Lipid metabolic process, cell adhesion, and carbohydrate metabolic process were gene ontology biological process terms affected by PE5 but not for onconase, while signal transduction, cell differentiation, apoptotic process, immune response, transcription DNA-dependent, phosphorylation, cell-cell signaling, chromatin assembly, protein metabolic process, intracellular protein kinase cascade, gene expression, growth, RNA metabolic process, and DNA metabolic process were only affected by onconase. There were only five gene ontology biological terms common between PE5 and onconase treated cells, specifically response to stress, angiogenesis, developmental process, cell proliferation, and cell migration. Gene ontology molecular function and cellular component terms were also clearly distinct. Indeed, all the affected molecular function terms were different between both RNases, and for cellular component the unique common terms were cell surface and extracellular region. Similarity, KEGG pathways affected by PE5 and onconase were completely different.

Generation of miRNA expression profiles

We treated NCI/ADR-RES and OVCAR8 cells for 36 h with a concentration of PE5 or onconase corresponding to an IC_{10} effect, and we then purified the total RNA. This RNA was labeled with cyanine 3-pCp and hybridized to the miRNA microarray. After extracting the data from the resulting images, we performed a quality control analysis (Figure 8). The individual MA plots obtained for the different microarrays, representing the $\log_2$ transformation of fold-change (M) plotted by the average $\log_2$ intensity (A), were acceptable but the fitting line was not straight on the zero line. In the global density plot, showing the intensity distributions of all the microarrays, the distributions were similar but not identical. A slight deviation between arrays was observed, although none of them had a weird distribution. In the RLE plot, computed by calculating for each probe-set and each array the ratio between the expression of a probe-set and the median expression of this probe-set across all the arrays, the boxplots were not completely homogeneous but were centered near zero and had similar spread. Finally, the expression boxplot comparing the expression levels of the different microarrays revealed that all of them had similar expression levels. Collectively, these results indicated that all the microarrays had high quality but a normalization process was needed.

We obtained the miRNA expression values using the RMA approach, which consisted in three steps. First, raw intensity values were background corrected employing the Normexp method, second, they were $\log_2$ transformed, and third, they were quantile normalized. After this process, we performed another quality control analysis (Figure 9). In the individual MA plots obtained for the different microarrays, the fitting line was straight on the zero line. The global density plot revealed that all the microarrays had the same distribution, and the boxplots of the RLE plot were more homogeneous than before the RMA process. In addition, the expression boxplot indicated that all the microarrays had similar expression levels. Therefore, the quality control analysis demonstrated that all the arrays were perfectly comparable.

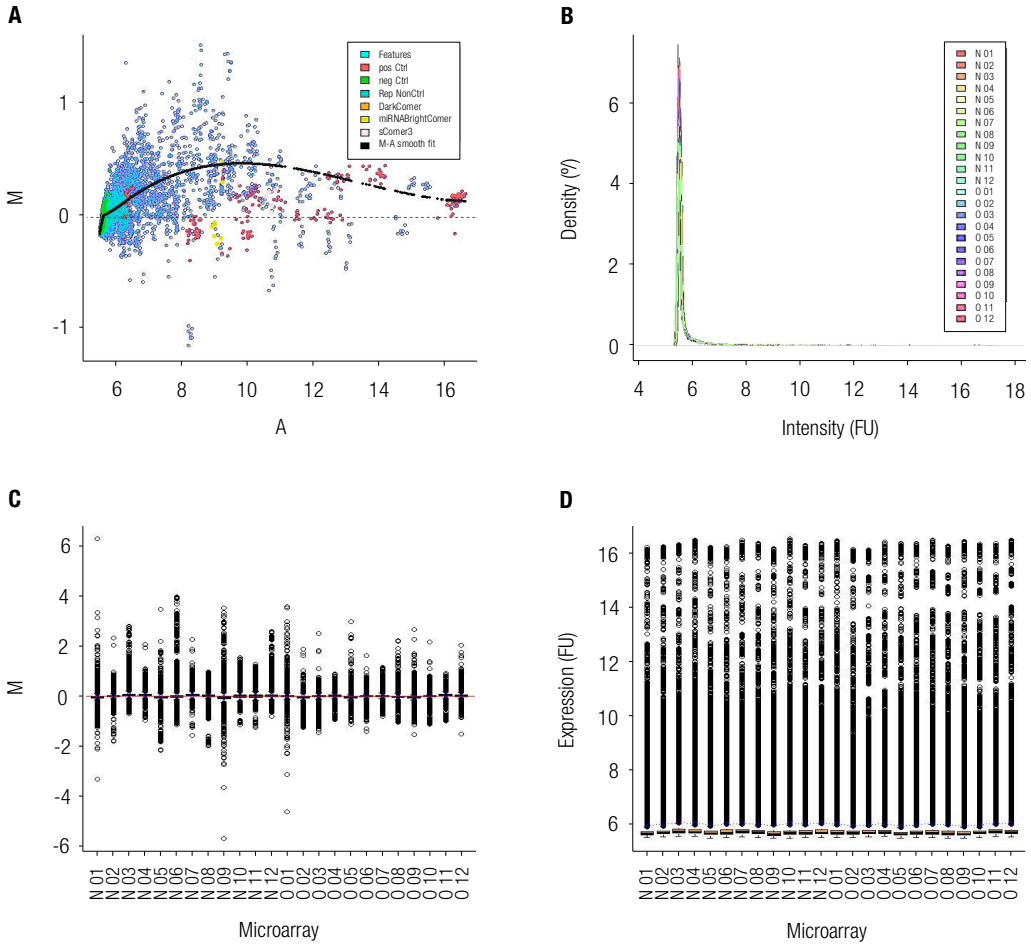

Figure 8. Quality control analysis of raw miRNA microarray data. A) Individual MA plot of a representative NCI/ADR-RES cell line microarray. Equivalent plots were obtained for all the microarrays. B) Global density plot, C) RLE plot, and D) Expression boxplot of all the microarrays.

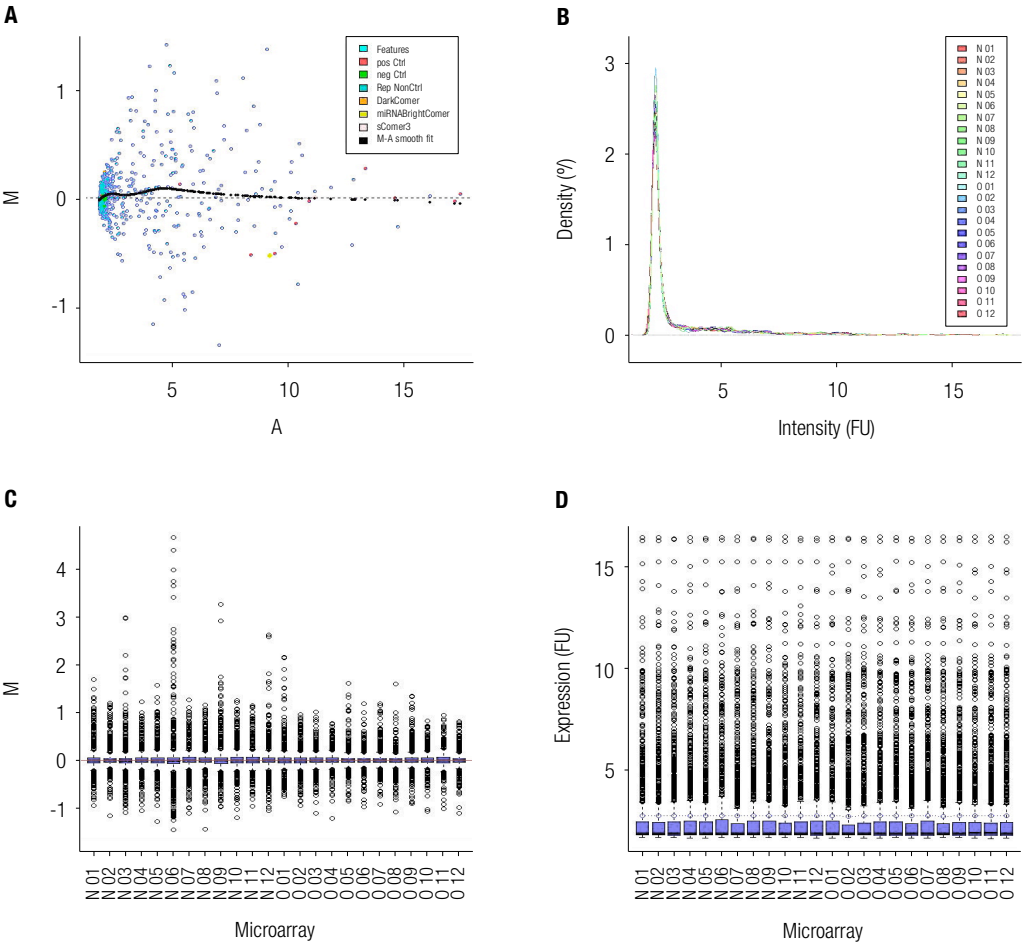

Figure 9. Quality control analysis of RMA processed miRNA microarray data. Raw intensity values were background corrected using the Normexp method, log2 transformed, and then quantile normalized. A) Individual MA plot of a representative NCI/ADR-RES cell line microarray. Equivalent plots were obtained for all the microarrays. B) Global density plot, C) RLE plot, and D) Expression boxplot of all the microarrays.

Following the RMA approach, we filtered the data to remove control probes and those probes not expressed in any condition, and then we performed a differential expression analysis to check the correlations of expression between arrays (Figure 10). The PCA plot, in which the data are projected on PC1 versus PC2, showed two groups of microarrays, one for each cell line used. For NCI/ADR-RES cell line, PE5 arrays were clearly separated from the rest, and only a slight differentiation was observed between onconase and control arrays. For OVCAR8 cell line, all the arrays were grouped together. The heat map, representing the expression level of the 50 high variance miRNAs across the different microarrays, confirmed this clustering.

Finally, a linear model was fitted to the normalized data, and differentially expressed miRNAs were selected using the t-test (separate analysis) from the Limma package. A false discovery rate adjusted p-value < 0.1 was considered significant.

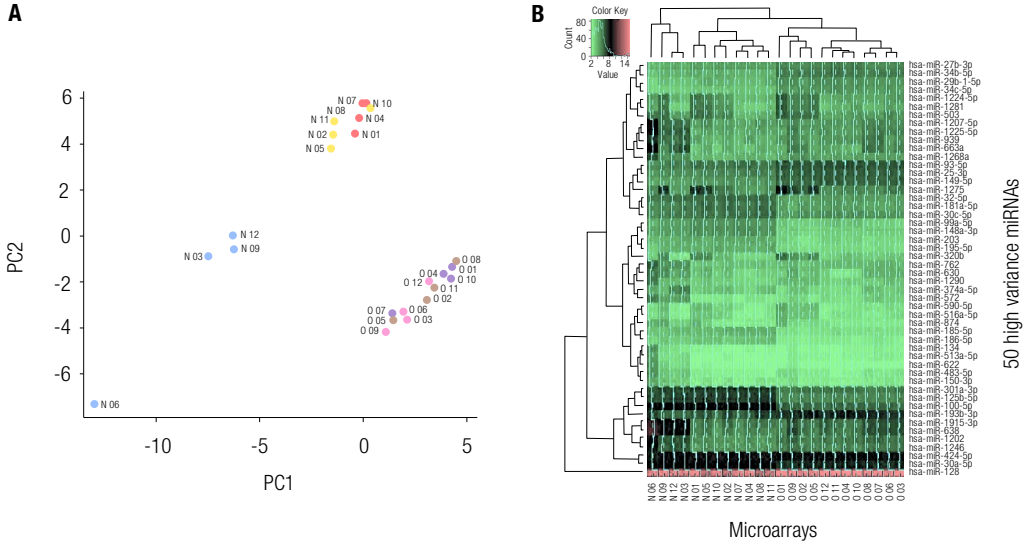

Figure 10. Differential expression analysis of RMA processed and filtered miRNA microarray data. A) PCA plot and B) Heat-map of all the microarrays.

Identification of PE5 and onconase differentially expressed miRNAs

miRNA expression profiles of RNase-treated and untreated NCI/ADR-RES cells were compared. For PE5, we obtained 181 differentially expressed miRNAs. 24% of them were upregulated in PE5-treated cells, with an increase respective to untreated cells ranging from 1.2- to 15.8-fold, while 76% were downregulated, with a decrease from untreated cells ranging from 1.1- to 2.2-fold. This result indicates that the primary effect of PE5 is to decrease miRNA expression. Table 12 shows the top 20 PE5 upregulated and downregulated miRNA in NCI/ADR-RES cell line.

In the case of onconase, we identified 13 miRNA that were differentially expressed between treated and untreated NCI/ADR-RES cells. 23% of them were upregulated in onconase-treated cells, with an increase from untreated cells ranging from 1.2- to 1.3-fold, whereas 77% were downregulated, with a decrease compared to untreated cells ranging from 1.1- to 1.7-fold. This indicates that the initial effect of onconase in NCI/ADR-RES cells is to decrease miRNA expression, as obtained for PE5. Table 13 shows the onconase differentially expressed miRNAs in NCI/ADR-RES cell line.

Table 12. PE5 differentially expressed miRNAs in NCI/ADR-RES cell line: top 20 PE5 upregulated and downregulated miRNAs.

Upregulated miRNA	Fold Change	Downregulated miRNA	Fold Change
hsa-miR-638	15.8	hsa-miR-503	-2.2
hsa-miR-1915-3p	12.6	hsa-miR-424-5p	-1.6
hsa-miR-663a	7.8	hsa-miR-99a-5p	-1.5
hsa-miR-1225-5p	6.8	hsa-let-7c	-1.5
hsa-miR-1202	4.7	hsa-miR-193b-3p	-1.5
hsa-miR-1207-5p	4.3	hsa-miR-522-3p	-1.4
hsa-miR-572	4.3	hsa-miR-18a-5p	-1.4
hsa-miR-939	3.9	hsa-miR-21-3p	-1.4
hsa-miR-1246	3.8	hsa-miR-331-3p	-1.4

hsa-miR-630	3.4	hsa-let-7d-5p	-1.4
hsa-miR-762	3.2	hsa-miR-130b-3p	-1.4
hsa-miR-1268a	2.8	hsa-miR-324-5p	-1.4
hsa-miR-622	2.8	hsa-miR-98	-1.4
hsa-miR-483-5p	2.7	hsa-miR-27b-3p	-1.4
hsa-miR-1973	2.7	hsa-miR-148a-3p	-1.4
hsa-miR-150-3p	2.5	hsa-miR-374a-5p	-1.4
hsa-miR-513a-5p	2.4	hsa-miR-361-5p	-1.4
hsa-miR-134	2.3	hsa-miR-196a-5p	-1.4
hsa-miR-1290	2.3	hsa-miR-30e-5p	-1.4
hsa-miR-548q	2.0	hsa-miR-30a-3p	-1.4

Table 13. Onconase differentially expressed miRNAs in NCI/ADR-RES cell line.

Upregulated miRNA	Fold Change	Downregulated miRNA	Fold Change
hsa-miR-20a-3p	1.3	hsa-miR-503	-1.7
hsa-miR-181a-3p	1.2	hsa-miR-1260a	-1.7
hsa-miR-424-3p	1.2	hsa-miR-744-5p	-1.4
		hsa-miR-149-5p	-1.3
		hsa-miR-24-3p	-1.2
		hsa-miR-99b-5p	-1.2
		hsa-miR-324-5p	-1.2
		hsa-miR-18a-5p	-1.2
		hsa-miR-138-5p	-1.2
		hsa-miR-769-5p	-1.1

When PE5 and onconase differentially expressed miRNAs in NCI/ADR-RES cell line were compared, we detected nine miRNAs common for both RNase treatments. This number represents 69% of onconase differentially expressed miRNAs, but only 5% in the case of PE5. As it is shown in Table 14, all of them were downregulated.

Table 14. Common PE5 and onconase differentially expressed miRNAs in NCI/ADR-RES cell line.

miRNA	Fold Change	
	PE5	Onconase
hsa-miR-769-5p	-1.1	-1.1
hsa-miR-138-5p	-1.2	-1.2
hsa-miR-1260a	-1.3	-1.7
hsa-miR-744-5p	-1.3	-1.4
hsa-miR-99b-5p	-1.3	-1.2
hsa-miR-149-5p	-1.4	-1.3
hsa-miR-324-5p	-1.4	-1.2
hsa-miR-18a-5p	-1.4	-1.2
hsa-miR-503	-2.2	-1.7

For OVCAR8 cell line we obtained a considerable lower number of differentially expressed miRNAs, equally than for gene expression microarrays. We did not detect any differentially expressed miRNA when expression profiles of PE5-treated and untreated OVCAR8 cells were compared, and only two (hsa-miR-138-5p and hsa-miR-769-5p) for onconase. They were downregulated in onconase-treated cells, with a decrease of 1.2-fold respective to untreated cells, suggesting that the primary effect of this RNase in OVCAR8 cell line is to decrease miRNA expression. These two downregulated miRNAs in OVCAR8 cells were also downregulated in NCI/ADR-RES cells treated with onconase (compare with Table 13), indicating that the initial action of onconase in miRNA expression is similar for both cell lines.

GENERATION OF NEW CYTOTOXIC HP-RNASE VARIANTS DIRECTED TO THE NUCLEUS

PE5 is an antitumor HP-RNase variant that carries a non-contiguous extended bipartite NLS. This NLS drives the protein into the nucleus, where it cleaves nuclear RNA inducing the apoptosis of cancer cells. In the present work, we have designed strategies to improve the properties of PE5 as an antitumor drug candidate. When attempting to develop a recombinant protein as a drug, two of the main desirable attributes are minimum immunogenicity and maximum potency. Therefore the improvements of PE5 have been designed to obtain both goals. First, in order to reduce the potential immunogenicity of the protein, we have studied which residues mutated in PE5 can be reverted to those of the wild type HP-RNase without decreasing its cytotoxicity and second, in an effort to obtain a more cytotoxic enzyme, we have investigated the effect of introducing an additional NLS at different sites of PE5.

Half of the residues mutated in PE5 can be back-mutated to HP-RNase without decreasing its cytotoxicity

In an attempt to reduce the potential immunogenicity of PE5, we studied which PE5 residues different from those of the wild type HP-RNase enzyme could be back-mutated to the human sequence without decreasing its cytotoxicity. Firstly, we constructed PE9 variant, in which the five N-terminal residues mutated in PE5 (positions 4, 6, 9, 16, and 17), that are not important for the NLS, were reverted to those of the wild type enzyme. This variant still carries the replacements involved in the NLS of PE5 (positions 89 and 90) (Table 15), and it was expected that it exhibited the same cytotoxicity. Surprisingly, PE9 was clearly less cytotoxic than PE5 in NCI/ADR-RES cells (Table 15). The five changes that incorporate PE9 respective to PE5 did not vary the catalytic efficiency of the enzyme but decreased its T_m by about 4ºC (Table 15). This latter result was expected since we had previously described that these five changes present in PE5 increased the thermal stability of the HP-RNase by 4ºC (Benito et al., 2002). Among them, substitutions of Arg4 and Lys6 by Ala mainly contributed to the increase in stability.

Table 15. Biochemical characterization of PE5 variants back-mutated to HP-RNase.

RNase	Replacements [a]	T_m (ºC) [b]	k_{cat}/K_m (%) [c]	IC_{50} (μM) [d]
PE5	R4A, K6A, Q9E, D16G, S17N, **G89R**, **S90R**	45.4 ± 0.1	100.0 ± 0.0	8.9 ± 0.5
PE9	**G89R**, **S90R**	41.1 ± 0.1	126.6 ± 0,6	> 30 [e]
PE10	R4A, K6A, **G89R**, **S90R**	45.5 ± 0.1	128.9 ± 6.2	8.9 ± 1.1

[a] Replacements in relation to HP-RNase. Replacements Gly89Arg and Ser90Arg (bold) belong to the NLS of the RNases.
[b] Measured at pH=5.0 (mean ± SE).
[c] Catalytic efficiency of hydrolysis of C>p relative to that of PE5 (mean ± SE).
[d] Measured in NCI/ADR-RES cell line (mean ± SE).
[e] The IC_{50} was not reached under the tested conditions.

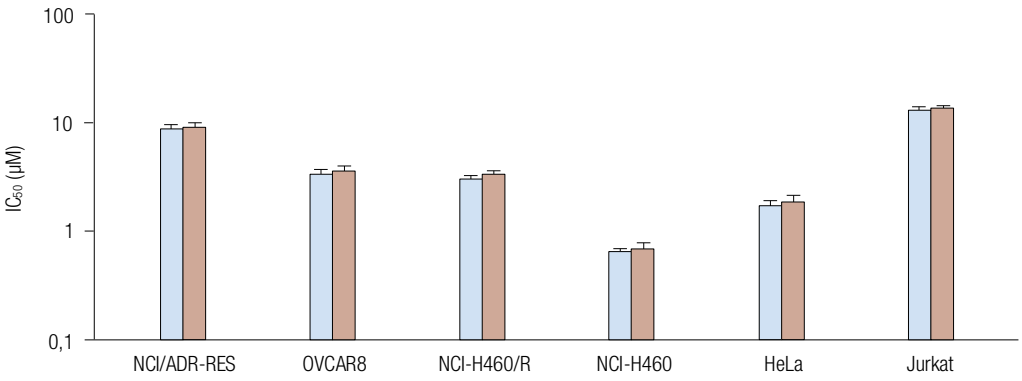

Figure 11. Cytotoxicity of PE5 and PE10 for a panel of human tumor cell lines. Control and RNase-treated cells were maintained for 72 h and metabolic activity was determined by the MTT assay. IC_{50} values of PE5 (blue bars) and PE10 (brown bars) are indicated for a variety of cell lines. Data are presented as mean ± SE of at least three independent experiments made in triplicates.

We also constructed PE10 variant, in which residues Arg4 and Lys6 of PE9 were replaced by Ala. This new variant differs from PE5 in three residues (positions 9, 16, and 17) that are not critical for the NLS functionality, and carries the other four substitutions of PE5 (positions 4, 6, 89, and 90) (Table 15). The proliferation assay showed that the cytotoxicity of PE10 in NCI/ADR-RES cells was equal to that of PE5 (Table 15). This cytotoxicity was not restricted to a single cell line, since PE10 displayed the same cytotoxicity than PE5 in a panel of diverse cell lines representative of different human cancers (Figure 11). Moreover, the T_m and the catalytic efficiency of PE10 were similar to those of PE5 (Table 15). We therefore obtained a new cytotoxic PE5 variant in which half of the residues mutated in PE5 are back-mutated to wild type HP-RNase.

The introduction of an additional NLS may increase the cytotoxicity of PE5 depending on the insertion site

In order to increase the ability of PE5 to reach the nucleus and obtain a more cytotoxic enzyme, we designed the variants NLSPE5 and PE5NLS, which incorporate the NLS of SV40 large T-antigen at the N- or C-terminus of PE5, respectively. This sequence had been previously used to direct green fluorescent protein inside the nucleus (Fanara et al., 2000). In an effort to exclude charge-dependent effects, we also created the control variants scNLSPE5 and PE5scNLS, carrying a scrambled NLS at the N- or C-terminus of PE5, respectively. A scrambled NLS is a non-NLS sequence with the same amino acid composition than the NLS. We checked that this scrambled sequence was not an NLS using the protein analysis tool PredictNLS (http://cubic. bioc. columbia.edu/predictNLS/).

We measured the thermal stability of these variants (Table 16). For some of them we could not calculate the T_m at pH 5.0 because they presented a non-reversible denaturation process that precluded the determination of the T_m. Different pHs were checked and it was observed that at pH 4.0 the unfolding was fully reversible, so we measured the thermal stability of all the variants at this pH. Stability of pancreatic RNases decreases at acidic pHs (Pace et al., 1990), and T_m values obtained at pH4.0 are therefore underestimating the stability at neutral pH. Accordingly, the T_m of PE5

at pH 5.0, and also the T_m of those variants whose unfolding was reversible at this pH, increased about 8.5 ºC respective to the value obtained at pH 4.0 (compare Tables 15 and 16). Assuming this approach, we obtained that the thermal stability of NLSPE5 and scNLSPE5 is similar to that of PE5, while that of PE5NLS and PE5scNLS is clearly lower (Table 16). We also checked the catalytic efficiency of these variants using C>p as a substrate. The catalytic efficiency of NLSPE5 and scNLSPE5 was equivalent to that of PE5 but we could not detect the catalytic activity of PE5NLS and PE5scNLS even at the higher substrate concentration used (Table 16). We postulated that the NLS basic sequence at the C-terminus of the protein was interfering somewhat with its structure, and we decided not to carry out further analysis with PE5NLS and PE5scNLS. Alternatively, we constructed PE5spNLS and PE5spscNLS variants that carry an additional spacer (SVGGS) between PE5 and the NLS or scrambled NLS, respectively. The T_m and the catalytic efficiency of these two new variants were nearly equal to that of PE5 (Table 16).

Table 16. Biochemical characterization of PE5 variants carrying an additional NLS or a scrambled NLS.

RNase	T_m (ºC) [a]	k_{cat}/K_m (%) [b]	IC_{50} (μM) [c]
PE5	36.6 ± 0.1	100.0 ± 0.0	8.9 ± 0.5
NLSPE5	35.8 ± 0.1	84.4 ± 2.5	1.0 ± 0.1
scNLSPE5	35.9 ± 0.1	83.7 ± 2.2	2.3 ± 0.1
PE5NLS	23.5 ± 0.1	__ [d]	ND [e]
PE5scNLS	23.8 ± 0.1	__ [d]	ND [e]
PE5spNLS	35.1 ± 0.1	93.2 ± 3.0	10.6 ± 0.9
PE5spscNLS	35.1 ± 0.3	110.5 ± 0.6	7.7 ± 0.2

[a] Measured at pH=4.0 (mean ± SE).
[b] Catalytic efficiency of hydrolysis of C>p relative to that of PE5 (mean ± SE).
[c] Measured in NCI/ADR-RES cell line (mean ± SE).
[d] The catalytic activity was undetectable at the higher concentration of substrate used.
[e] Not done.

We assayed the cytotoxicity of the RNase variants in NCI/ADR-RES cells (Table 16). NLSPE5 had an IC_{50} clearly lower than its control scNLSPE5 and than PE5, whereas PE5spNLS and PE5spscNLS were as cytotoxic as the parental PE5. The higher cytotoxicity of NLSPE5 was not restricted to this cell line, since NLSPE5 exhibited a degree of cytotoxicity similar to that of onconase and 6-14 times higher than that of PE5 in a large panel of human tumor cell lines (Figure 12). The IC_{50} values for the MDR cell lines NCI/ADR-RES and NCI-H460/R were higher than for their respective parental cell lines OVCAR-8 and NCI-H460. Therefore, we obtained a much more cytotoxic PE5 variant, NLSPE5, by inserting an additional NLS at the N-terminus of the protein.

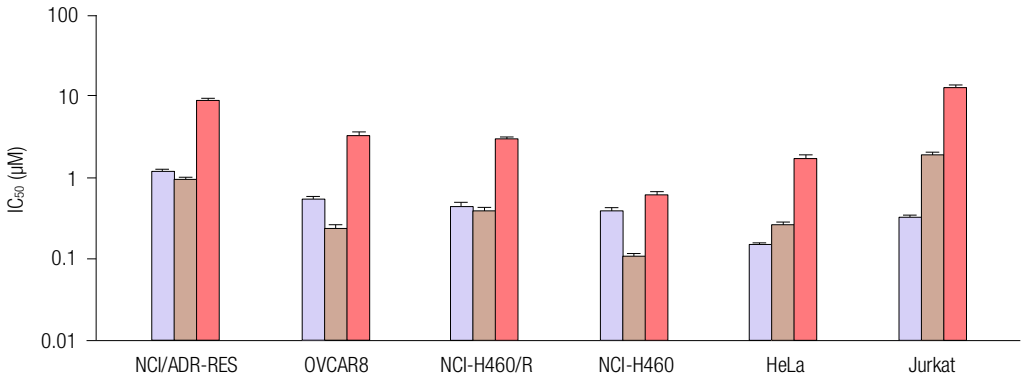

Figure 12. Cytotoxicity of onconase, NLSPE5, and PE5 for a panel of human tumor cell lines. Control and RNase-treated cells were maintained for 72 h and metabolic activity was determined by the MTT assay. IC_{50} values of onconase (violet bars), NLSPE5 (brown bars), and PE5 (red bars) are indicated for a variety of cell lines. Data are presented as mean ± SE of at least three independent experiments made in triplicates.

The cytotoxicity of NLSPE5 and PE10 is dependent on the cleavage of nuclear RNA

We were interested in characterizing NLSPE5 and PE10, the two improved PE5 variants. First, we investigated whether the ribonucleolytic activity was essential for the cytotoxicity of NLSPE5. Consequently, we abolished the ribonucleolytic activity of NLSPE5 by creating a new variant in which the catalytic residue His119 was substituted by Ala. This variant did not show catalytic activity using C>p as a substrate and was not cytotoxic in NCI/ADR-RES cell line, indicating that the NLS does not confer a cytotoxic activity *per se* and that the ribonucleolytic activity is essential for the cytotoxicity of NLSPE5.

Another factor that could be critical for the cytotoxicity of RNases is their ability to evade the RI. Therefore, RI inhibition of the different variants was qualitatively analyzed using an agarose gel-based assay (Figure 13). All the variants were fully inhibited by the RI, but NLSPE5 showed a slight RNA degradation. This result could indicate that the increase in cytotoxicity of NLSPE5 would be caused by the acquisition of the ability to evade the RI and cleave cytosolic RNA. To check this possibility, we investigated the integrity of the nuclear and cytosolic RNA of HeLa cells treated with the RNases, as previously done for PE5 (Tubert et al., 2011).

We incubated HeLa cells with 0.3 μM NLSPE5 or 1 μM PE5 or PE10 for 24 h. The conditions were chosen because they correspond to the minimal incubation time and concentrations necessary to observe a cytotoxic effect (inhibition of 10% of cell growth), and therefore it can be considered that the RNA degradation is due to the direct action of the RNases but not to the induction of cellular apoptosis (Mondino and Jenkins, 1995). RNA degradation of treated and untreated control cells was quantified using a bioanalyzer (Figure 14). Cytosolic RNA was not degraded at all in the different samples, while nuclear RNA degradation was evident for NLSPE5-, PE5- and PE10-treated cells but not for untreated cells. This result indicate that the cytotoxicity of NLSPE5 and PE10 is dependent on the cleavage of nuclear RNA, as previously observed for PE5 (Tubert et al., 2011), and it is not caused by the acquisition of the ability to cleave cytosolic RNA.

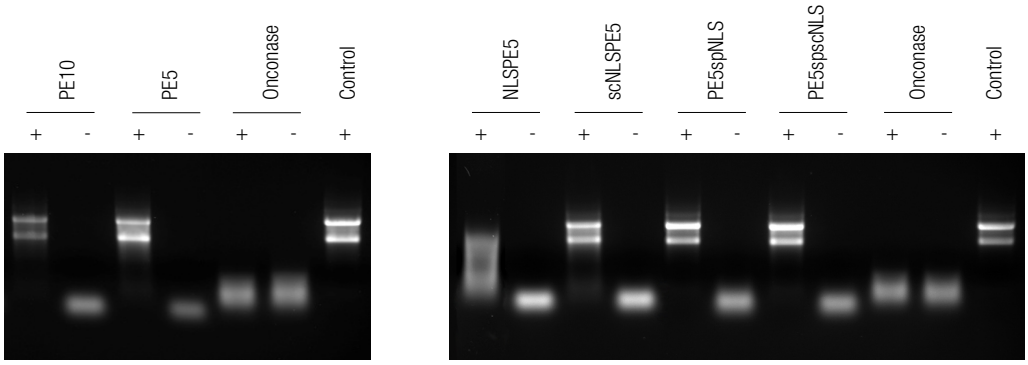

Figure 13. Inhibition of ribonucleolytic activity by the RI. RNase catalyzed degradation of 16S and 23S rRNA in presence (+) and absence (-) of 6-fold excess of RI was visualized in an agarose gel. The intensity and the position of the rRNA bands correlate with the degree of ribonucleolytic activity. If it decreases, both band intensity and apparent molecular mass increase.

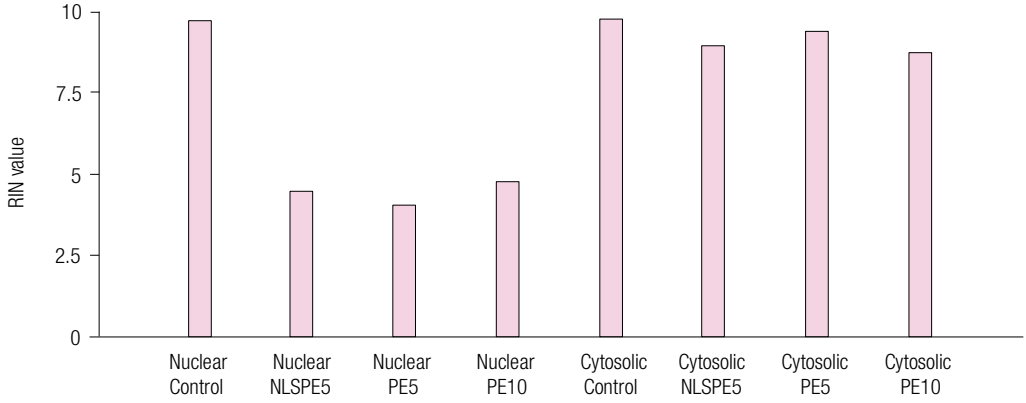

Figure 14. Nuclear and cytosolic RNA degradation induced by NLSPE5, PE5, and PE10 in HeLa cell line. Cells were treated with 0.3 μM NLSPE5 or 1 μM PE5 or PE10 for 24 h, nuclear and cytosolic RNA was extracted, and RNA degradation was quantified using a bioanalyzer.

The effects induced by NLSPE5 and PE10 on cancer cells are analogous to those of PE5

We investigated whether the cytotoxic properties of NLSPE5 and PE10 differed from those of PE5. First, we analyzed the induction of apoptosis by the translocation of phosphatidylserine to the external hemi-membrane, as previously described for PE5 (Castro et al., 2011a). We quantified by flow cytometry the percentage of NCI/ADR-RES cells in early apoptosis, late apoptosis, and necrosis after 24, 48, and 72 h of incubation with 4.85 μM NLSPE5 or 44.5 μM PE5 or PE10 (Figure 15). In all cases early apoptosis was evident after 24 h of treatment whereas an important fraction of cells were in late apoptosis after 48 h of treatment. The different variants did not induce necrosis of the cells even after 72 h of incubation. The percentage of cells in early and late apoptosis was equivalent for the treatments assayed.

We also studied the mechanism of apoptosis induced by the RNase variants. We analyzed the activation of procaspases-3, -8, and -9 in NCI/ADR-RES cells after treatment with 4.85 μM NLSPE5 or 44.5 μM PE5 or PE10 (Figure 16). NLSPE5 and PE10 induced the activation of initiator procaspases-8 and -9 and executioner procaspase-3, as previously described for PE5 (Castro et al., 2011a). The pattern of procaspase activation did not differ between the different variants. Procaspase activation was evident at 48 h and increased at 72 h of incubation with the RNases.

We had previously shown that PE5 arrests the cell cycle of NCI/ADR-RES cells at S- and G_2/M-phases and this is accompanied by a two-fold accumulation of cyclin E and $p21^{WAF1/CIP1}$ but unchanged levels of cyclin D1 (Castro et al., 2011a). Our results indicated that the cytotoxicity of PE5 was mediated by the increase of the expression of $p21^{WAF1/CIP1}$, which could explain why PE5 reduces the level of P-gp in MDR cell lines (Castro et al., 2011a; Castro et al., 2011b). We were therefore interested in investigating whether the cytotoxic mechanisms of NLSPE5 and PE10 were similar to that of PE5. We studied by western blot the effect caused by 0.97 μM NLSPE5 and 8.9 μM PE5 or PE10 on the accumulation of cyclin D1, cyclin E, and $p21^{WAF1/CIP1}$ in NCI/ADR-RES cells after 72 h of incubation (Figure 17). Treatment with these RNases induced a two-fold accumulation of cyclin E and $p21^{WAF1/CIP1}$ but did not affect the levels of cyclin D1.

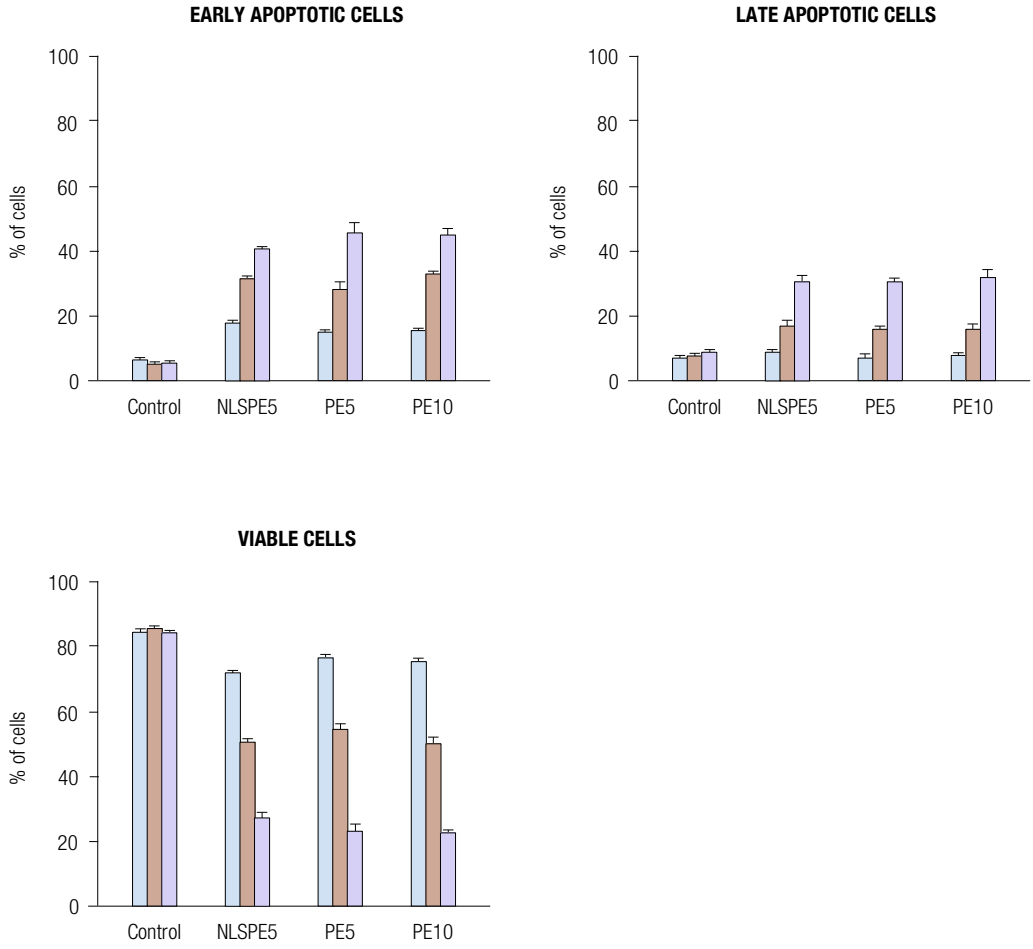

Figure 15. Apoptosis induced by NLSPE5, PE5, and PE10 in NCI/ADR-RES cells measured by Alexa Fluor 488 annexin V/PI staining. Cells were treated with 4.85 μM NLSPE5 or 44.5 μM PE5 or PE10 for 24 (blue bars), 48 (brown bars), or 72 h (violet bars), stained with Alexa 488 annexin V and PI, and analyzed by flow cytometry. Cells undergoing early apoptosis were positive for annexin V and negative for PI (annexin V+/PI-), late apoptotic cells were annexin V+/PI+, and necrotic cells were annexin V-/PI+. Values obtained for necrotic cells were between 0.2 and 1.4 % in all the cases (results not shown). Values were analyzed from 10,000 total events for each experimental sample. Data are presented as mean ± SE of at least three independent experiments.

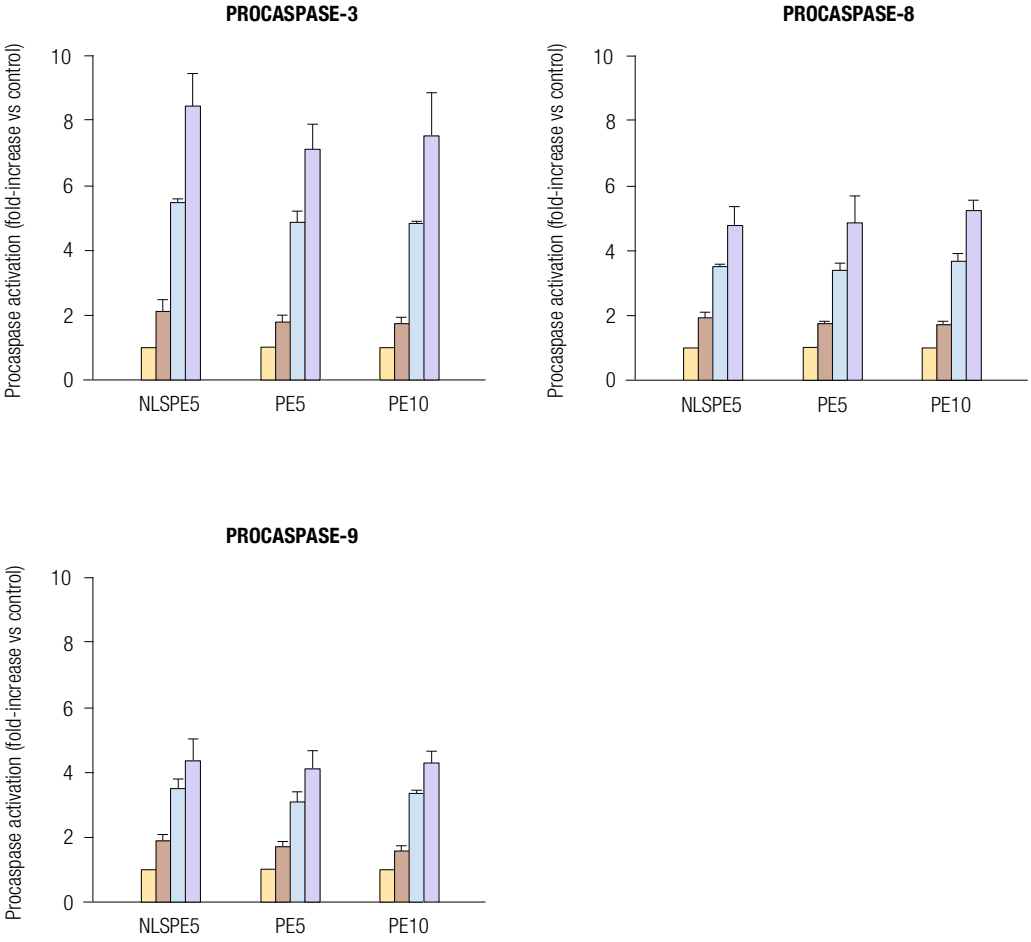

Figure 16. Procaspase-3, -8, and -9 activation in NCI/ADR-RES cells treated with NLSPE5, PE5, or PE10. Cells were incubated with 4.85 μM NLSPE5 or 44.5 μM PE5 or PE10 for 24 (brown bars), 48 (blue bars), or 72 h (violet bars) and caspase-3, -8, and -9 catalytic activities were measured in whole cell lysates using a quantitative colorimetric assay. Procaspase activation is expressed as fold-increase respective to control cells (yellow bars). Data are presented as mean ± SE of at least three independent experiments.

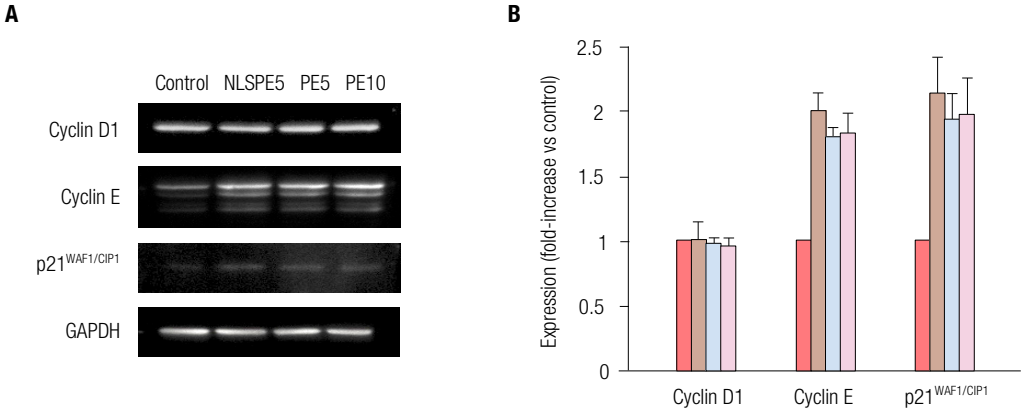

Figure 17. Effects of NLSPE5, PE5, and PE10 on the expression of cyclin D1, cyclin E, and $p21^{WAF1/CIP1}$ in NCI/ADR-RES cell line. Cells were treated with 0.97 μM NLSPE5 or 8.9 μM PE5 or PE10 for 72 h, lysed, and analyzed by Western blot. A) Western blot of a representative experiment. B) Densitometric analysis of the GAPDH-normalized immunoblots of NLSPE5- (brown bars), PE5- (blue bars), and PE10- (pink bars) treated cells. Cyclin D1, Cyclin E, and $p21^{WAF1/CIP1}$ expression is shown as fold-increase respective to control cells (red bars). Data are presented as mean ± SE of at least three independent experiments.

We had also previously shown that the decrease of the expression level of P-gp in MDR cell lines induced by PE5 could be functionally confirmed by measuring the accumulation of doxorubicin in MDR cells treated with the RNase (Castro et al., 2011b). Therefore, we investigated by flow cytometry the doxorubicin accumulation in NCI-H460/R cells treated with different concentrations of NLSPE5 (0.15, 0.35, and 0.50 μM) or PE5 or PE10 (1.0, 2.6, and 4.3 μM) for 72 h (Figure 18). As expected, after treatment with NLSPE5 and PE10, doxorubicin accumulation increased in a dose-dependent manner up to 40% with respective to untreated control cells at the highest RNase concentration assayed. The increase in doxorubicin accumulation was analogous in the different treatments assayed.

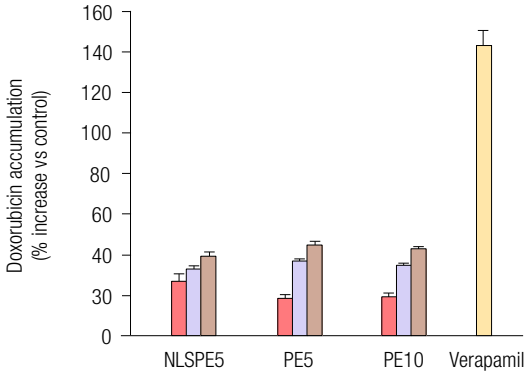

Figure 18. Effect of NLSPE5, PE5, and PE10 on doxorubicin accumulation in NCI-H460/R cell line. Cells were treated with RNase concentrations that caused a decrease of cell proliferation of 20% (red bars), 40% (violet bars), and 50% (brown bars) for 72 h and then exposed to 10 μM doxorubicin for 1 h. 10 μM verapamil (yellow bar) was used as a positive control. Intracellular fluorescence of doxorubicin was measured by flow cytometry. Values were analyzed from 10,000 total events for each experimental sample. Doxorubicin accumulation is expressed as % increase respective to control cells. Data are presented as mean ± SE of at least three independent experiments.

Discussion

RNases are promising agents to be used in anticancer therapy. In contrast to most chemotherapeutic drugs, which interfere with DNA synthesis and cell division, antitumor RNases have the advantage of being non-mutagenic agents that exert their effects by interfering with RNA functions such as protein synthesis or gene regulation. Another interesting feature of antitumor RNases is that they cause pleiotropic effects on the cells, implicated in a large variety of pathways and biological processes. This quality can be useful to combat the multifactorial complexity of the cancer cell phenotype. In addition, it may difficult the acquisition of tumor resistance to RNases and it could explain the synergisms encountered between them and other antitumor agents. However, because of these pleiotropic effects it is difficult to study and predict the mechanism by which antitumor RNases induce the cellular death.

Microarray technology is a powerful tool for evaluating gene expression profiles of cells, and it has been widely used to investigate cellular responses upon exposure to drugs (Daigeler et al., 2008; Che et al., 2013). In particular, large-scale microarray analysis of gene expression enables researchers to simultaneously analyze changes in thousands of genes and identify significant altered patterns. In the present work, we have used global gene expression and global miRNA microarrays to study the pleiotropic effects caused by PE5 and onconase, and therefore elucidate the molecular mechanism of the cytotoxicity induced by these antitumor RNases. We have analyzed and compared the expression patterns of the human ovarian carcinoma cell lines NCI/ADR-RES and OVCAR8. The first displays a MDR phenotype and it is representative of poor-prognosis ovarian cancer. It was selected because it is very sensitive to the action of PE5 and onconase (Castro et al., 2011a). It is worth mentioning that NCI/ADR-RES is especially resistant to doxorubicin (IC_{50} of 76.4 μM), compared to other MDR cell lines as for example NCI-H460/R (IC_{50} of 1.16 μM) (Castro et al., 2011 b). In this cell line, both PE5 and onconase induce cell death through apoptosis, but the cytotoxic mechanisms are different (Castro et al., 2011a). PE5 increases the number of cells in S and G_2/M cell cycle phases, which is accompanied by the increased expression of cyclin E and $p21^{WAF1/CIP1}$ together with the under-phosphorylation of p46 forms of JNK. In contrast, the treatment of NCI/ADR-RES cells with onconase does not alter the cell cycle phase distribution and it is accompanied by a decreased expression of XIAP (Castro et al., 2011a).

OVCAR8 cell line was chosen because it is the parental cell line of NCI/ADR-RES cell line (Liscovitch and Ravid, 2007).

We carried out the microarrays experiments at those concentrations of PE5 and onconase that provoked a decrease of 10% in cell proliferation after 36 h of treatment, since in a preliminary experiment we determined that these concentrations caused a minimal cytotoxic effect but not a considerable RNA degradation (Table 2). This was important for two reasons. First, an extensive RNA degradation would prevent the possibility of performing the microarray experiments, and second, the induction of apoptosis increases the RNA turnover (Mondino and Jenkins, 1995), so an excessive cytotoxic effect would generate changes in the RNA levels due to the induction of cellular apoptosis rather than the direct action of RNase. It is word mentioning that at these treatment conditions onconase causes a highly cytostatic effect, but PE5 exerts both cytostatic and cytotoxic effects (Castro et al., 2011a).

Gene expression microarrays of NCI/ADR-RES cells revealed 647 PE5 differentially expressed genes. These genes are associated with a variety of biological processes, including transcription regulation, cell cycle, apoptosis, mRNA processing, cell adhesion and migration, and amino acid, lipid, and glucose metabolism (Table 3). In the case of onconase, we identified 56 differentially expressed genes. They are also involved in many biological processes, including transcription regulation, cell cycle, apoptosis, immune response, stress response, amino acid metabolism, and protein folding (Table 4). All these results confirm that PE5 and onconase cause pleiotropic effects on the cells.

Gene expression microarray experiments also showed that the effects caused by PE5 and onconase are clearly different in NCI/ADR-RES cells, which is consistent with the fact that PE5 is directed into the nucleus (Bosch et al., 2004) where it cleaves nuclear RNA (Tubert et al., 2011), while onconase is driven into the cytoplasm and targets t-RNAs (Saxena et al., 2002) and microRNAs (Zhao et al., 2008). First, we obtained that PE5 regulates the expression of numerous genes, whereas in the case of onconase the number of differentially expressed genes was considerable lower (11.5-fold less). Second, the percentage of upregulated and downregulated genes was different between the two RNases. 53% of PE5 differentially expressed genes were

upregulated and 47% were downregulated, indicating that the primary action of PE5 is both to increase and to decrease gene expression, while for onconase 89% of genes were upregulated and 11% were downregulated, indicating that the initial action of this RNase is activation of gene expression. Third, among the affected genes, only 10 were altered by both RNase treatments (Table 5), and none of them appear to be significant for the antitumor activity of either PE5 or onconase. Finally, the affected gene ontology terms and KEGG pathways are distinct (Tables 8, 9, 10 and 11), showing that the mechanisms by which PE5 and onconase exert its cytotoxic activity toward tumor cells are completely different, as will be discussed below.

For OVCAR8 cell line, we obtained a considerable lower number of differentially expressed genes than for NCI/ADR-RES cells. Indeed, we identified only one differentially expressed gene for PE5 treatment and seven for onconase (Table 6). We hypothesize that the difference could be due to the fact that OVCAR8 cell line is not as resistant to stress conditions as NCI/ADR-RES, and therefore in this cell line fewer changes in gene expression profiles are required for induction of cytotoxicity. Supporting this, we found that in order to cause a 10% decrease in cell proliferation, 30-fold less PE5 or 9-fold less onconase were needed in OVCAR8 cells respective to NCI/ADR-RES cells. Nevertheless, we cannot discard the possibility that RNase concentrations used for OVCAR8 treatment actually provoked less than a 10% decrease in cell proliferation, especially in the case of PE5. Cytotoxic curves have a low gradient at low percentages of reduction of cell proliferation, and therefore it exists an important error in the determination of the concentrations that cause a very low decrease of cell proliferation, such as 10%. Diverse observations are in agreement with this hypothesis. First, we only identified one PE5 differentially expressed gene in OVCAR8 cell line but 647 in NCI/ADR-RES, which represents an excessive difference. Second, this gene does not appear among those changed in NCI/ADR-RES cells. Third, RNA samples of OVCAR8 cells treated with PE5 were much less affected than those of NCI/ADR-RES cells (Table 2). Therefore, at least for PE5, the second hypothesis is more likely.

In the following sections we will discuss the effects caused by PE5 and onconase on gene expression, as well as the molecular mechanism of the cytotoxicity induced by these RNases. The biological processes and the genes presented in the following

sections have been chosen based on over-represented gene ontology and KEGG terms, on differentially expressed genes belonging to these terms, and on the main functions of the top 60 upregulated and downregulated differentially expressed genes. Because of the reasons described above, for PE5 we will focus on NCI/ADR-RES cells, while for onconase the effects on both cell lines will be discussed.

PE5 causes pleiotropic effects on cells

PE5 displays multiple effects on NCI/ADR-RES cells and changes the expression level of a high number of genes. Gene ontology analysis revealed that PE5 differentially expressed genes are involved in interesting biological processes such as carbohydrate metabolism, lipid metabolism, cell proliferation, and response to stress (Table 8). In addition, we have observed that this RNase also acts on other processes like amino acid and reactive oxygen species (ROS) metabolism, alters the expression of different oncogenes and tumor suppressors, and regulates MDR-associated genes, among others. Therefore, PE5 inhibits the growth and development of tumor cells through a diversity of mechanisms.

PE5 reduces the expression of numerous genes involved in metabolic pathways

Cancer cells have altered metabolism compared to normal cells, at least in part to support the biosynthetic demands of rapid proliferation. Both glucose and glutamine, major nutrients for energy production and precursors for *de novo* synthesis of macromolecules, are known to have a crucial role in the reprogrammed metabolic state of many cancer cells. The potential contributions of other nutrients, including fatty acids and other amino acids, have also been appreciated. (Reviewed in Galluzzi et al., 2013). Here, we found that PE5 reduces the expression of multiple genes implicated in glucose, lipid, and amino acid metabolism. The changes in gene expression observed in our experiments were generally low although very significant. This is not surprising since the chosen PE5 treatment only provoked a decrease of 10% in cell proliferation. We believe that treatment at higher concentrations would cause more important variations of the effects described here.

Glucose metabolism

It is known that cells can obtain energy by glucose breakdown through the oxygen-dependent pathway of oxidative phosphorylation (OXPHOS) and through the oxygen-independent pathway of glycolysis. Since OXPHOS is more efficient in generating ATP than glycolysis, it is recognized that the presence of oxygen results in the activation of OXPHOS and the inhibition of glycolysis (Pasteur effect). However, cancer cells and non-malignant proliferating cells are able to maintain high rates of glycolysis even under the presence of adequate oxygen levels, a process known as aerobic glycolysis or Warburg effect (Warburg, 1956). Aerobic glycolysis can provide different advantages to cancer cells (Reviewed in Gillies et al., 2008). First, it produces lactate that, when excreted, acidifies tumor surroundings. This acidification helps tumor invasion and inhibits the immune system. Second, glycolysis furnishes the cell with important substrates for pentose phosphate pathway (PPP), which generates NADPH necessary for the biosynthesis of new molecules and for the quenching of ROS. Third, tumor cells use glycolytic intermediates for anabolic biosynthesis of carbohydrates, proteins, lipids, and nucleic acids. These building blocks are required for cell proliferation. Finally, glycolysis reduces the dependence for oxygen of the growing tumor. Accordingly, accumulating evidences suggest that maintaining a high level of glycolysis plays a crucial role in cancer development and it is indispensable for survival and growth of cancer cells. In this sense, inhibition of the increased glycolytic capacity of cancer cells leads to a significant inhibition of cell growth and induction of cell death, and therefore it represents a key anticancer strategy (Reviewed in Pelicano et al., 2006).

Interestingly, microarray experiments showed that PE5 decreases the expression of some genes that codify for proteins related to glucose metabolism, in particular phosphoglucomutase 1 (PGM1), glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate mutase 1 (brain) (PGAM1), enolase 1 (alpha) (ENO1), lactate dehydrogenase A (LDHA), phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2) and malic enzyme isoform 3 (ME3) (Figure 19). It is worth mentioning that none of these enzymes are regulated allosterically. Activation states of allosteric enzymes are of course not detectable in a microarray experiment. The main roles of these proteins and their relationship with cancer are briefly described below.

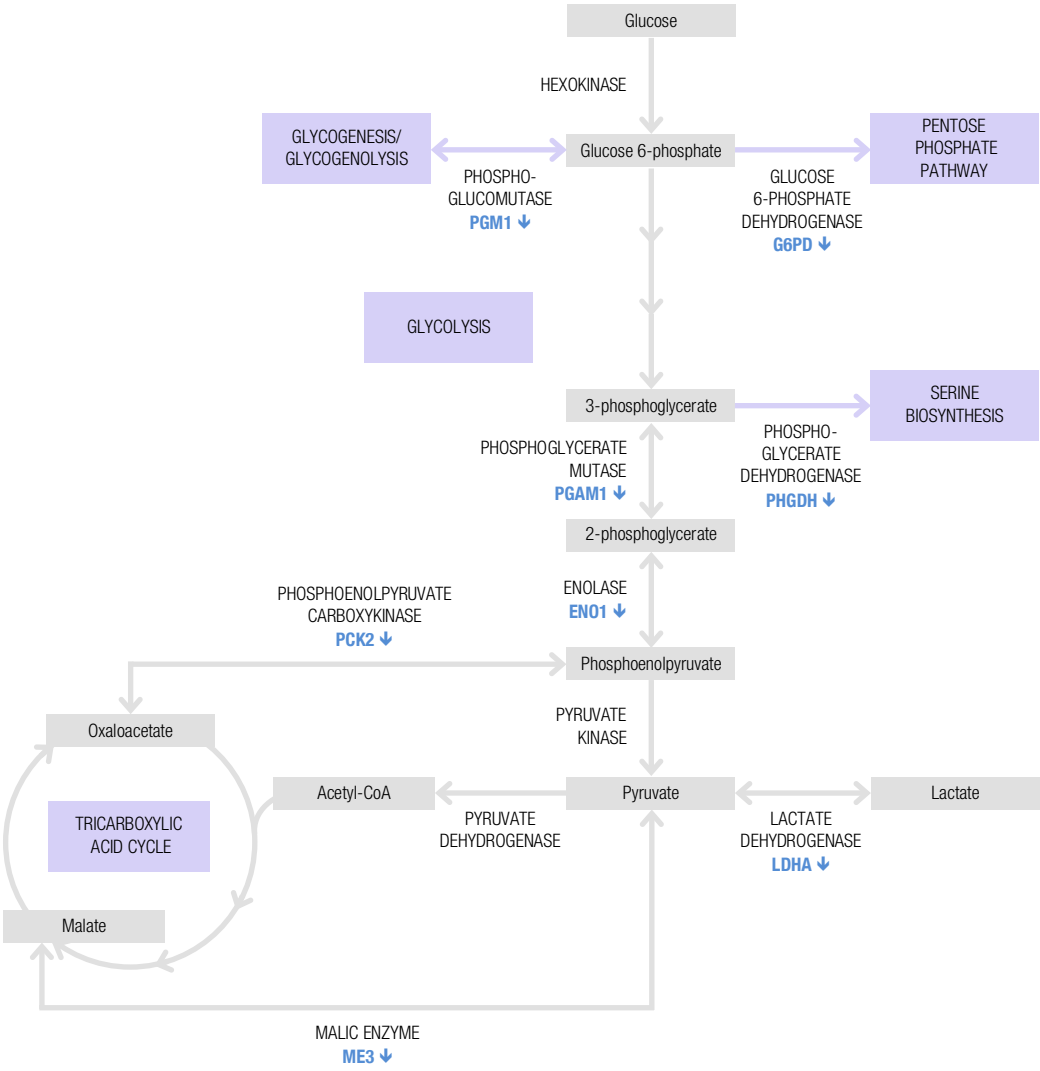

Figure 19. Effects of PE5 on glucose metabolism. The figure shows a general schema of glycolysis and its connections with glycogenesis/glycogenolysis, PPP, serine biosynthesis and TCA cycle. Three arrows indicate more than one step. PE5 differentially expressed genes in NCI/ADR-RES cell line are shown in blue. All of them are downregulated (↓).

PGM1 catalyzes the inter-conversion of glucose 1-phospate and glucose 6-phospate, and it participates in both the breakdown and synthesis of glycogen (Figure 19). Glucose 6-phosphate can move down the glycolytic pathway to generate energy or, alternatively, can enter into the PPP, where it undergoes a series of reactions to yield NADPH and/or ribose supporting *de novo* nucleotide biosynthesis.

G6PD catalyzes the first and rate-limiting reaction in the PPP (Figure 19). The expression or activity of this enzyme is elevated in multiple cancers and in some cases is associated with poor prognosis (Van Driel et al., 1999; Langbein et al., 2008; Wang et al., 2012). It has also been reported that G6PD promotes tumor cell proliferation and survival (Hu et al., 2013).

PGAM1 catalyzes the reversible reaction of 3-phosphoglycerate to 2-phosphoglycerate in the glycolytic pathway (Figure 19). This enzyme is overexpressed in many human cancers enabling increased glycolytic flux (Fang et al., 2004; Li et al., 2006; Turhani et al., 2006) and it is correlated with decreased survival of patients (Ren et al., 2010; Gao et al., 2013). It is believed that increased expression of PGAM1 in cancer cells is due to the loss of p53 because p53 negatively regulates PGAM1 gene expression (Corcoran et al., 2006). It has been reported that overexpression of PGAM1 can immortalize mouse embryonic fibroblasts and promote cell proliferation, suggesting its potential oncogenic property (Kondoh et al., 2005). Furthermore, different studies have shown that repression of PGAM1 reduces cell proliferation and tumor growth, and this effect is accompanied by a significant decrease of glycolysis (Ren et al., 2010; Hitosugi et al., 2012).

In addition to its involvement in glycolysis, PGAM1 modulates two biosynthetic pathways derived from glycolysis: the oxidative branch of the PPP and the serine biosynthesis pathway. Silencing the expression of PGAM1 causes an increase in the intracellular level of 3-phosphoglycerate and a decrease in 2-phosphoglycerate level (substrate and product of PGAM1, respectively) (Hitosugi et al., 2012). The increase in 3-phosphoglycerate directly inactivates the PPP enzyme 6-phosphogluconate dehydrogenase (6PGD), whereas the decrease of 2-phosphoglycerate levels is accompanied by a reduction in phosphoglycerate dehydrogenase (PHGDH) activity, which carries out the first and rate-limiting step in the serine biosynthesis pathway (Figure 19) (Hitosugi et al., 2012). As we will discus later, this latter effect may be increased in NCI/ADR-RES cells treated with PE5, since this RNase reduces the expression of PHGDH.

ENO1 is one of the three enolase enzymes that catalyze the inter-conversion between 2-phosphoglycerate and phosphoenolpyruvate in the glycolytic and gluconeogenic pathways (Figure 19). ENO1 increases Warburg effect and it is frequently overexpressed in various types of cancer (Altenberg and Greulich, 2004; Li et al., 2006). Overexpression of ENO1 has been described to be associated with aggressive phenotype and poor survival outcomes in different cancers (Chang et al., 2006; Hamaguchi et al., 2008; Tsai et al., 2010; Yonglitthipagon et al., 2012), suggesting an oncogenic role for this enzyme. Moreover, ENO1 is highly expressed in metastatic cancer cells compared to primary cancer cells (Tsai et al., 2010; Linge et al., 2012; Yoshida et al., 2013) indicating that it may contribute to tumor invasion.

LDHA catalyzes the conversion of pyruvate to lactate (Figure 19). This enzyme has been reported to be involved in tumor progression, and its expression is frequently increased in human cancers where it is an indicator of poor prognosis (Rong et al., 2013; Yao et al., 2013; Fujiwara et al., 2013). Forced expression of LDHA promotes growth of different kinds of cancer cells, while knocking down its expression or inhibiting its activity reduces cell

growth, cell migration, tumorigenicity and induces apoptosis (Rong et al., 2013; Yao et al., 2013; Fujiwara et al., 2013).

PCK2 and **ME3** are mitochondrial enzymes that catalyze the reversible conversion of oxaloacetate to phosphoenolpyruvate and malate to pyruvate, respectively (Figure 19). Although these reactions are reversible, the formation of phosphoenolpyruvate and pyruvate appear to be favored under physiological conditions. Therefore, PCK2 and ME3 can be considered cataplerotic enzymes. Their major function is to remove tricarboxylic acid (TCA) cycle intermediates that are formed by the entry of the carbon skeletons of amino acids into the cycle. In this process, carbon skeletons of amino acids, such as glutamine and glutamate, entry into the TCA cycle where they are converted to malate, which in turn can be oxidized to oxaloacetate by NAD:malate dehydrogenase. The reduction of PCK2 and ME3 indirectly reduces the glycolytic pathway since less intermediate metabolites can enter into it. Interestingly, PCK2 has been associated with cell proliferation in cancer (Liu et al., 2012) and it has been found overexpressed in metastatic lesions (Chaika et al., 2012).

The effects on glucose metabolism described here indicate that PE5 may arrest critical metabolic pathways in NCI/ADR-RES cells, which reduce important energy sources for cancer cells and also diminishes anabolic processes required for cell growth and proliferation. We have found that PE5 decreases the expression of different enzymes and this may lead to an inhibition of glycolysis and cataplerosis, together with a reduction of intermediate metabolites of glycolysis for the glycogenesis, PPP or serine biosynthesis. Also the conversion of pyruvate to lactate may be reduced, decreasing the acidosis of the surrounding tumor environment.

Lipid metabolism

In the last decade, the altered lipid metabolism has been recognized as a common property of tumor cells, and like glucose metabolism, it is believed to be important for the initiation and progression of tumors (Reviewed in Zhang and Du, 2012). Indeed, cancer cells are highly dependent on *de novo* lipid biosynthesis, since lipids are involved in membrane synthesis to support cell growth and proliferation, in membrane saturation that can be important for oxidative stress resistance, and also in the synthesis of cholesterol and lipid hormones that mediate signal transduction relevant for cell proliferation, survival, and invasion. Moreover, in response to

glucose limitation, lipids can be consumed through β-oxidation to provide key substitute energy for cancer cell survival.

We found that PE5 decreases the expression of several genes implicated in lipid metabolism. These genes could be grouped in different categories based on their roles in lipid biosynthesis, lipid catabolism, and lipid transport. Among the genes related to lipid biosynthesis, we can mention acetyl-CoA carboxylase alpha (ACACA), serine palmitoyltransferase, long chain base subunit 3 (SPTLC3), N-acylsphingosine amidohydrolase (acid ceramidase) 1 (ASAH1), 24-dehydrocholesterol reductase (DHCR24), transmembrane 7 superfamily member 2 (TM7SF2), and methylsterol monooxygenase 1 (MSMO1), and among those involved in lipid catabolism we can emphasize hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit (HADHA), and propionyl CoA carboxylase, beta polypeptide (PCCB) (Figure 20). A brief description of these proteins and their involvement in cancer are presented below.

Diverse studies have shown that tumor cells reactivate *de novo* fatty acid synthesis (Reviewed in Zhang and Du, 2012), supporting that it plays an important role in cancer pathogenesis. The expression and activity of many enzymes implicated in fatty acid synthesis, like ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) are upregulated in numerous types of cancer and their inhibition leads to apoptosis in different cases (Reviewed in Zhang and Du, 2012). As example, chemical inhibition of ACC, the enzyme that carboxylates acetyl-CoA to produce malonyl-CoA, induces growth arrest and apoptosis in prostate cancer cells (Beckers et al., 2007). In this sense, it is interesting to note that PE5 treatment significantly decrease one of the two isoforms of ACC, known as **ACACA** (Figure 20).

As stated above, lipid synthesis is also critical to support cell growth and proliferation. The importance of membrane synthesis in cancer cells has been highlighted by the observation that the expression and activity of choline kinase, an enzyme required for the synthesis of phosphatidylcholine and phosphatidylethanolamine (the major phospholipids found in cellular membranes) is increased in tumors from various tissues and correlates with poor prognosis (Ramírez de Molina et al., 2002a; Ramírez de Molina et al., 2002b; Iorio et al., 2010). Different genes involved in sphingolipid synthesis have a decreased expression upon treatment with PE5. Among them, **SPTLC3** catalyzes the rate-limiting step of the *de novo* synthesis of sphingolipids, and **ASAH1** catalyzes the synthesis and degradation of ceramide into sphingosine and fatty acid (Figure 20).

Mevalonate pathway, which facilitates the synthesis of cholesterol, is another important biosynthetic process within lipid metabolism. Cholesterol is an important component of biological membranes as it modulates the

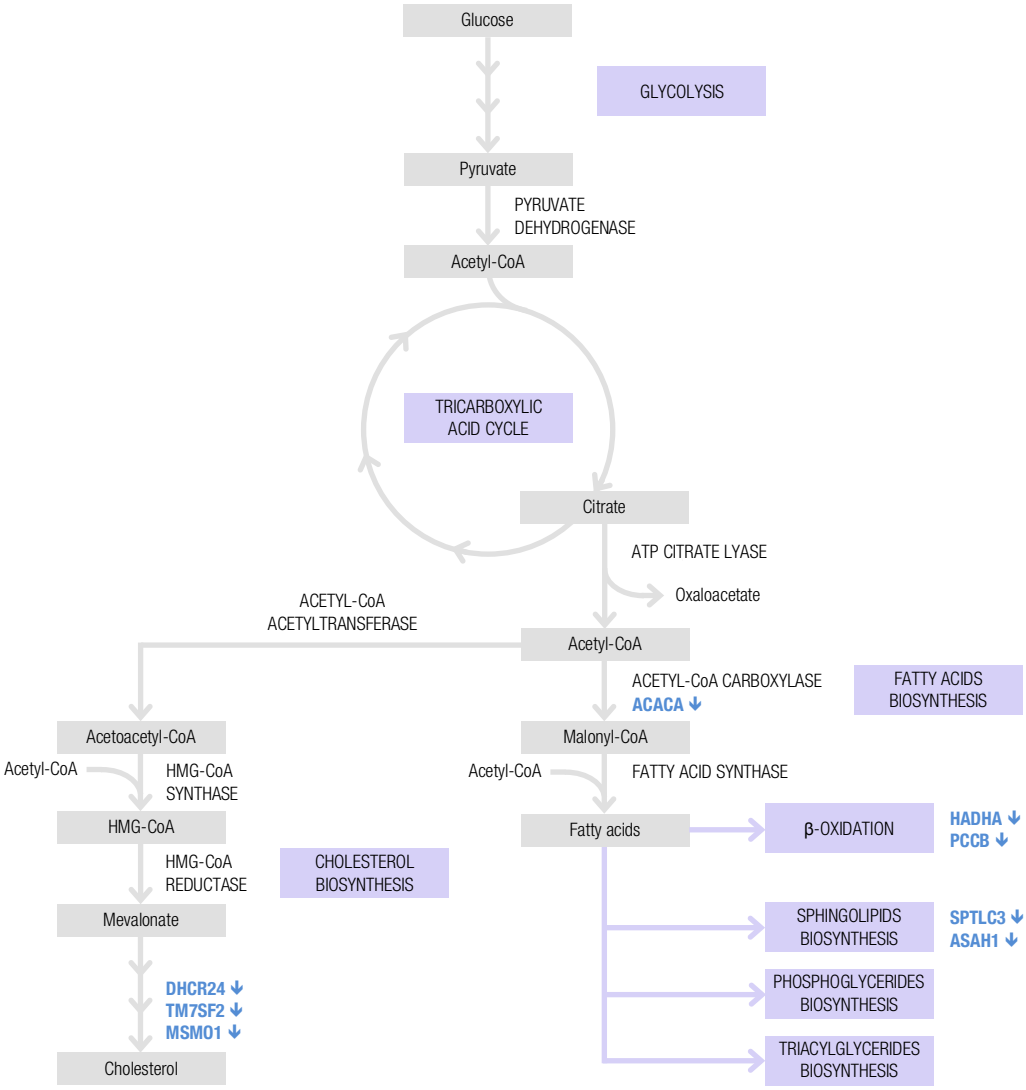

Figure 20. Effects of PE5 on lipid metabolism. The figure shows a general schema of lipid metabolism with the connections between fatty acids, sphingolipids, phosphoglycerides, triacylglycerides, and cholesterol biosynthesis, β-oxidation, glycolysis, and TCA cycle. Three arrows indicate more than one step. PE5 differentially expressed genes in NCI/ADR-RES cell line are shown in blue. All of them are downregulated (↓).

fluidity of the lipid bilayer and also forms lipid rafts that coordinate the activation of some signal transduction pathways (Reviewed in Lingwood and Simons, 2010). Accumulation of cholesterol has been reported in cancer (Reviewed in Hager et al., 2006) and deregulation of the mevalonate pathway has been associated with transformation (Clendening et al., 2010). Remarkably, statins, a class of cholesterol-lowering drugs, show antiproliferative activity in several cancer-cell lines that range from cell cycle arrest to apoptosis (Newman et al., 1997; Gray-Bablin et al., 1997; Rao et al., 1998). It is interesting to note that some key enzymes involved in cholesterol synthesis are underexpressed upon PE5 treatment. We can emphasize, as examples, **DHCR24** that catalyzes the reduction of the delta-24 double bond of sterol intermediates during cholesterol biosynthesis, **TM7SF2** that participate in the conversion of lanosterol to cholesterol, and also **MSMO1**, which has been postulated to function in cholesterol biosynthesis (Figure 20).

Regarding lipid catabolism, different studies have shown that β-oxidation is a key pathway for energy generation in cancer (Zha et al., 2005; Khasawneh et al., 2009). Furthermore, pharmacological activation of β-oxidation can rescue the glucose dependency of Akt-transformed cells (Buzzai et al., 2005), suggesting that this pathway can provide important metabolites for cancer-cell survival. Again, some enzymes involved in the oxidation of fatty acids are underexpressed upon treatment with PE5. We can mention **HADHA**, that catalyzes the last three steps of mitochondrial β-oxidation of long chain fatty acids, and **PCCB**, that participates in the catabolism of propionyl-CoA (Figure 20).

In summary, our results indicate that PE5 affects the expression of numerous genes implicated in different lipid metabolic pathways relevant for cancer progression and growth.

Amino acid metabolism

Like glucose and lipid metabolism, amino acid metabolism is linked to cancer. Tumor cells tend to switch from recycling amino acids to their *de novo* synthesis (Richardson et al., 2008; Possemato et al., 2011). Remarkably, the treatment with PE5 reduced the expression level of different genes implicated in amino acid biosynthesis that are relevant for cancer progression, including phosphoglycerate dehydrogenase (PHGDH), pyrroline-5-carboxylate reductase 1 (PYCR1), and asparagine synthetase (ASNS) (Figure 21). PE5 also reduced the catabolizing amino acid enzyme branched chain amino acid transaminase 1 (BCAT1). The main functions of these enzymes and their relationship with cancer are summarized below.

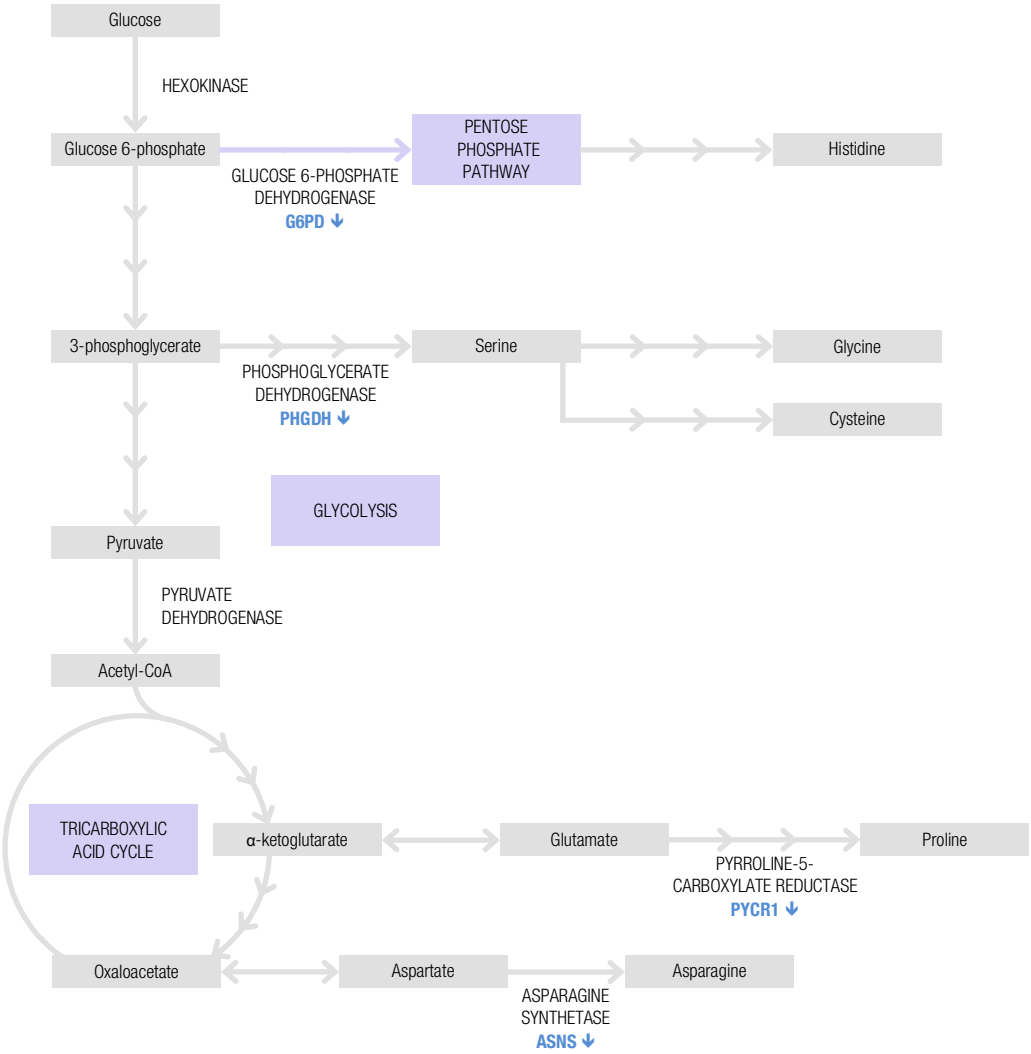

Figure 21. Effects of PE5 on amino acid metabolism. The figure shows a general schema of amino acid biosynthesis and its connections with glycolysis, PPP and TCA cycle. Three arrows indicate more than one step. PHGDH catalyzes the first step in the serine biosynthesis pathway. PYCR1 catalyzes the last step in the proline biosynthesis pathway. PE5 differentially expressed genes in NCI/ADR-RES cell line are shown in blue. All of them are downregulated (↓).

PHGDH catalyzes the first step in the serine biosynthetic pathway (oxidation of 3-phosphoglycerate) and controls flux from glycolysis into the serine biosynthesis pathway (Figure 21). Biosynthesis of serine is required for glycine and cysteine biosynthesis, and it is key to tumor growth (Possemato et al., 2011). Diverse studies have shown that PHGDH is amplified or overexpressed in different cancers, correlating with the more aggressive tumors (Possemato et al., 2011; Locasale et al., 2011; Liu, J. et al., 2013; Jing et al., 3013). Moreover, inhibition of this enzyme strongly reduces cell proliferation, invasion and tumorigenicity in cells that overexpress it (Possemato et al., 2011; Locasale et al., 2011; Liu, J. et al., 2013).

Proline biosynthesis is increased in cancer cells compared to non-cancer cells (Richardson et al., 2008). **PYCR1** catalyzes the NAD(P)H-dependent conversion of pyrroline-5-carboxylate to proline, the last step in proline biosynthesis from glutamate (Figure 21). PYCR1 was found to be overexpressed in different cancers (Ernst et al., 2002; Roth et al., 2010) and involved in tumor growth (Possemato et al., 2011).

ASNS catalyzes the conversion of aspartate and glutamine to asparagine and glutamate in an ATP-dependent reaction (Figure 21). Elevated expression of this enzyme is associated with resistance to L-asparaginase anticancer therapy in a number of cancers (Aslanian et al., 2001; Lorenzi and Weinstein, 2009). Several lines of evidence suggest that inhibiting ASNS activity represents a viable strategy for treating L-asparaginase-resistant leukemia in the clinic (Reviewed in Richards and Kilberg, 2006).

In addition to affect the synthesis of serine (and consequently glycine and cysteine), proline, and asparagine, PE5 indirectly reduces the synthesis of histidine. This is because PE5 reduces the expression of G6PD, the first enzyme of the PPP that produces ribose-5P, which is the substrate for histidine synthesis (Figure 21).

BCAT1 catalyzes the first step in the break-down of the branched-chain amino acids leucine, isoleucine and valine at the cytosol. This enzyme is highly expressed in various cancers (Ju et al., 2009; Zhou et al., 2013; Tönjes et al., 2013), and it has been found that it promotes cancer cell proliferation, migration and invasion through amino acid catabolism (Zhou et al., 2013; Tönjes et al., 2013).

Finally, PE5 may also inhibit the protein synthesis since we identified many aminoacyl tRNA synthetases as PE5 downregulated genes. Particularly, these are cysteinyl-tRNA synthetase (CARS), alanyl-tRNA synthetase (AARS), glycyl-tRNA synthetase (GARS), isoleucyl-tRNA synthetase (IARS), tyrosyl-tRNA synthetase (YARS), and glutamyl-prolyl-tRNA synthetase (EPRS). This result is in agreement with previous results that showed that treatment of different cancer cell lines with PE5 reduces cell protein synthesis compared to untreated cells (Bosch et al., 2004).

PE5 decreases the expression of various genes implicated in the quenching of ROS

We identified some genes underexpressed in PE5 treated cells that are implicated in the quenching of ROS, a diverse class of radical species continuously produced in all cells by respiring mitochondria. Cancer cells have increased ROS levels compared to normal cells because of their accelerated metabolism and rapid cell proliferation (Reviewed in Cairns et al., 2011). At high levels, ROS can cause damage to macromolecules, including DNA; induce the activation of protein kinase Cδ, triggering senescence; and/or cause permeabilization of the mitochondria, leading to the release of cytochrome c and apoptosis. In order to counteract the accumulation of ROS, cancer cells upregulate the thioredoxin and glutathione antioxidant systems, which scavenge ROS and repair ROS-induced damage.

Treatment with PE5 reduced the expression of two genes implicated in the detoxification of ROS: thioredoxin reductase 2 (TXNRD2) and glutathione peroxidase 3 (GPX3), belonging to thioredoxin and glutathione antioxidant systems, respectively. Thus, downregulation of these enzymes can induce apoptosis due to the high levels of ROS species. A brief description of these proteins is presented below.

TXNRD2 is a mitochondrial oxidoreductase that maintains thioredoxins in a reduced state, and it is particularly important for scavenging ROS in the mitochondria (Reviewed in Kowaltowski et al., 2009).
GPX3 is a plasma antioxidant enzyme that catalyzes the reduction of hydrogen peroxide and lipid peroxides by reduced glutathione. It also maintains genomic integrity by detoxifying ROS, known DNA-damaging agents and mediators of cancer chemotherapy response (Reviewed in Comhair and Erzurum, 2005).

Importantly, glutathione and thioredoxin antioxidant systems rely on the reducing power of NADPH to maintain their activities. There are three major mechanisms of NADPH production in mammalian cells: PPP, conversion of malate to pyruvate by malic enzyme, and decarboxylation of isocitrate to α-ketoglutarate catalyzed by

isocitrate dehydrogenase (IDH). Treatment of NCI/ADR-RES with PE5 not only reduced the expression of G6PD, which catalyzes the first and rate-limiting reaction of PPP, and ME3, the mitochondrial NADP(+)-dependent isoform of malic enzyme, but also reduced the expression of IDH2, the mitochondrial NADP(+)-dependent isoform of IDH.

IDH2 is the NADP(+)-dependent isocitrate dehydrogenase that converts isocitrate to α-ketoglutarate in the mitochondria. Although the physiologic function of IDH2 has not been well characterized, it is thought that this enzyme plays roles in the metabolism of glucose, fatty acids, and glutamine, in addition to contribute to the maintenance of normal cellular redox status. Interestingly, suppression of melanoma cell tumorigenesis is observed in IDH2-knock-out mice along with significant elevation of oxidative stress (Kim et al., 2014).

PE5 decreases the expression of oncogenes and increases that of tumor suppressors

Apart from the effect on different metabolic cell pathways that can alter the growth and development of cancer cells, PE5 directly decreased the expression of a variety of oncogenes and increased that of different tumor suppressors.

Among the genes with oncogenic functions downregulated by PE5, we can mention glypican 6 (GPC6), EGF containing fibulin-like extracellular matrix protein 1 (EFEMP1), met proto-oncogene (hepatocyte growth factor receptor) (MET), transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase) (TGM2), platelet-derived growth factor receptor, beta polypeptide (PDGFRB), and clusterin (CLU). The most important functions of these genes, as well as their contributions to caner development and progression are summarized below.

GPC6 is the highest PE5 downregulated gene. It is one of the six members of the glypican family of cell surface heparan sulfate proteoglycans, whose main function is to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors, and bone morphogenetic proteins (Reviewed in Filmus et al., 2008). A role in tumorigenesis has also been reported for different members of this family. However, relatively little is known concerning the expression or functional roles of GPC6 in human solid tumors and phenotypes associated with cancer progression. In this sense, it has been found that nuclear factor of activated T-cells (NFAT), a transcription factor highly expressed in aggressive breast cancer cells that mediates tumor invasion, stimulates GPC6 transcription, which in turn promotes invasive migration through upregulation of Wnt5A signaling. In addition, GPC6 induction of Wnt5A stimulates the activation of JNK and p38 MAPK (Yiu et al., 2011). Also, GPC6 was found to be upregulated in a mouse model harbouring an oncogenic mutation of the receptor tyrosine kinase Kit present in sporadic gastrointestinal stromal tumors (Gromova et al., 2009).

EFEMP1, a member of the fibulin family of extracellular matrix glycoproteins, is one of highest PE5 downregulated genes. Paradoxically, EFEMP1 can demonstrate either oncogenic or tumor-suppressive behavior depending on the cancer types. Increased expression of EFEMP1 has been reported in glioma, pancreatic, cervical and ovarian cancers, and it has been linked to tumor progression and poor prognosis (Hu et al., 2009; Seeliger et al., 2009; En-lin et al., 2010; Chen, J. et al., 2013). Particularly, it has been found that EFEMP1 promotes cell motility and invasion, increases vascular endothelial growth factor (VEGF) production stimulating angiogenesis, and plays a role in metastasis development (Seeliger et al., 2009; Song et al., 2011; Chen, J. et al., 2013). This fibulin also promotes cell survival and tumor growth through regulation of Notch pathway (Hu et al., 2012). In contrast, EFEMP1 displays a cancer-suppressing function in other types of cancer (Sadr-Nabavi et al., 2009; Hwang et al., 2010; Tong et al., 2011).

MET is a proto-oncogenic transmembrane receptor. The binding with its ligand (hepatocyte growth factor) activates several downstream signaling pathways, including phosphoinositide 3-kinase/Akt, Ras-Rac/Rho, MAPK, and phospholipase C-γ, that stimulate morphogenic, proliferative and anti-apoptotic pathways involved in cell detachment, motility, and invasiveness (Reviewed in Benvenuti and Comoglio, 2007). In accordance with its oncogenic role, enhanced MET expression has been reported in a broad spectrum of solid human cancers and correlates with poor clinical prognosis (Reviewed in Benvenuti and Comoglio, 2007). Cells which overexpress MET are tumorigenic when implanted into nude mice and become extremely metastatic (Rong et al., 1994), and transgenic mice for MET develop metastatic tumors (Takayama et al., 1997). On the contrary, cancer cells become less aggressive when MET is switched off (Matsumoto and Nakamura, 2003; Christensen et al., 2003; Taulli et al., 2006; Lutterbach et al., 2007). Moreover, overexpression of MET confers resistance to epidermal growth factor receptor (EGFR) inhibitors (Benedettini et al., 2010).

TGM2 catalyzes the formation of an amide bond between the carboxamide groups of peptide-bound glutamine residues and primary amino groups in various compounds. Expression of this enzyme is upregulated in multiple cancer cell types, particularly those associated with poor disease outcome, drug resistance, and increased incidence of metastasis (Reviewed in Mehta et al., 2010). It has been reported that expression of TGM2 enhances cancer cell survival, and also initiates a complex series of signaling networks that contribute to the development of drug resistance and invasive phenotype. For example, TGM2 activates the pro-survival NF-κB (Mann et al.,

2006), and focal adhesion kinase/Akt (Verma et al., 2008), whereas it negatively regulates the tumor suppressor phosphatase and tensin homologue (PTEN) (Verma et al., 2008).

PDGFRB is one of the two isoforms of platelet-derived growth factor receptor (PDGFR). The binding of PDGFR with its endogenous ligands (PDGF-A, -B, -C and –D) initiates an intracellular signaling that leads to stimulation of cell proliferation, migration and angiogenesis (Reviewed in Östman and Heldin, 2007). PDGF and PDGFR expression are increased in a large variety of human cancers, and their overexpression is correlated with poor survival (Reviewed in Raica and Cimpean, 2010). It has been described that PDGF and PDGFR are involved in autocrine stimulation of tumor cell growth, and additionally, PDGF signaling acts in a paracrine manner on nontumor cells, such as cells in tumor blood vessels and stromal fibroblasts, which may also be important for tumor growth and angiogenesis (Hermanson et al., 1992; Guo et al., 2003). Furthermore, PDGF and PDGFR are implicated in tumor metastasis promoting migration, invasiveness, and the spread of tumor cells via lymphatic vessels (Yi et al., 2002; Cao et al., 2004; Jechlinger et al., 2006). Otherwise, blockade of PDGFR results in growth inhibition in a number of cell lines and cancers (Reviewed in Raica and Cimpean, 2010). Inhibition of PDGFR is also associated with an improved tumor drug uptake and a concomitantly enhanced therapeutic effect of chemotherapeutical drugs (Pietras et al., 2002).

CLU is a stress-induced and secreted cytoprotective chaperone that maintains partially unfolded proteins in a state appropriate for subsequent refolding by other chaperones. The cytoplasmic form of CLU is also involved in several biological activities, including inhibition of apoptosis, protection against complement mediated cell lysis, membrane lipid recycling, and lipid transport, and it is implicated in pathological disorders such as cancer (Reviewed in Jones and Jomary, 2002). CLU exhibits a pro-survival function in cancer cells, since it activates the pro-survival Akt (Ammar and Closset, 2008) while inhibiting the pro-apoptotic Bax (Trougakos et al., 2009). A role for CLU in tumor invasiveness and metastasis has also been proposed because of its interaction with the ERK/slug pathway (Chou et al., 2009) and its role in transforming growth factor beta (TGF-β)-promoted epithelial-mesenchymal transition (Shiota et al., 2012). Moreover, it blocks therapy-induced apoptosis and confers resistance to a broad-spectrum of anti-cancer treatments (Miyake et al., 2000; Zellweger et al., 2003). Therefore, it is not strange that overexpression of CLU had been detected in many tumor types and it had been linked with more aggressive tumors and poorer outcomes (Steinberg et al., 1997; Miyake et al., 2002; Xie et al., 2005; Kevans et al., 2009).

Regarding tumor suppressor genes upregulated by PE5 treatment, we can emphasize, as examples, BCL2-like 11 (apoptosis facilitator) (BCL2L11), MAX dimerization protein 1 (MXD1), ras homolog gene family, member B (RHOB), BCL2/adenovirus E1B 19kDa interacting protein 3-like (BNIP3L), cyclin D binding myb-like transcription factor 1 (DMTF1), WW domain containing oxidoreductase (WWOX), and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

(NFKBIA). A description of these genes and their roles attenuating cancer development and progression are presented below.

BCL2L11 belongs to the Bcl-2 protein family, which are central mediators of the intrinsic pathway of apoptosis. This protein interacts with all anti-apoptotic members and act as an apoptotic activator (Chen et al., 2005), playing important roles in tumorigenesis and tumor treatment. BCL2L11 deficiency was found to facilitate epithelial tumor growth in mice (Tan et al., 2005), and loss of even one BCL2L11 allele accelerate Myc-induced development of tumors (Egle et al., 2004). Moreover, BCL2L11 is deleted or silenced by methylation in a proportion of cancers, and loss of this protein is associated with shorter patient survival (Tagawa et al., 2005; Mestre-Escorihuela et al., 2007; Sinicrope et al., 2008). Otherwise, BCL2L11 suppression is important for tumor metastasis. Indeed, BCL2L11 plays a key role in the anoikis, or apoptosis induced by detachment, of a variety of tumor cells (Reviewed in Akiyama et al., 2009). These tumor cells have to bypass or abrogate BCL2L11-mediated cell death in order to metastasize. Finally, various anticancer drugs induce BCL2L11 expression and use this protein as a mediating executioner of cell death. Consistently, its downregulation contributes to chemotherapy resistance (Reviewed in Akiyama et al., 2009).

MXD1 is a member of the MYC/MAX/MXD1 network of basic helix-loop-helix leucine zipper transcription factors, whose deregulation contributes to the genesis of many human cancers. MXD1 forms heterodimers with MAX and functions as a transcriptional repressor of Myc target genes. Thus, MXD1 is considered an antagonist of the oncogene Myc (Reviewed in Grandori et al., 2000). Importantly, the levels of MXD1 are generally low in many human cancers (Shapiro et al., 1994; Han et al., 2000; Osaki et al., 2007), and the decrease of MXD1 expression correlates with loss of cell differentiation during tumorigenesis (Hurlin et al., 1995; Lymboussaki et al., 1996). Furthermore, ectopic expression of this transcription factor inhibits cell cycle progression and proliferation of cancer cells (Roy and Reisman, 1995; Roussel et al., 1996; Ohta et al., 2002; Osaki et al., 2007).

RHOB is a small guanosine triphosphatase (GTP)-binding protein that appears to mediate apoptosis and to be a negative regulator of cancer (Couderc et al., 2008). Loss of RHOB expression has been reported in different cancers, and has been associated with more aggressive and invasive tumors (Forget et al., 2002; Mazieres et al., 2004; Volanis et al., 2011; Liu, Y. et al., 2013). Accordingly, RHOB knockout in mice is associated with increased susceptibility to tumorigenesis (Liu et al., 2001a), while its overexpression is highly effective in suppressing tumor growth (Couderc et al., 2008; Chen et al., 2000). In addition, RHOB is required for apoptosis in response to a variety of anticancer compounds, and consequently its deletion renders cells resistant to apoptosis (Liu et al., 2001b).

BNIP3L is a member of the BCL2/adenovirus E1B 19 kDa-interacting protein (BNIP) family. It interacts with anti-apoptotic proteins, such as E1B 19kDa, Bcl-2 and Bcl-XL, and induces apoptosis by altering mitochondrial membrane permeability (Matsushima et al., 1998; Imazu et al., 1999). BNIP3L was found deleted or silenced by hypermethylation in various cancers (Calvisi et al., 2007; Liu et al., 2008; Brown et al., 2011), and interestingly,

experimental induction of its expression causes significant growth suppression in different cancer cell lines (Matsushima et al., 1998).

DMTF1 is a transcription factor regulated by binding of D-cyclins. It is induced by the oncogenic Ras signaling pathway and functions as a tumor suppressor by activating the transcription of alternative reading frame (ARF), and thus the ARF-p53 pathway, to arrest cell growth or induce apoptosis (Inoue et al., 1999; Zhu et al., 2013). On the other hand, the DMTF1 promoter is repressed by overexpression of E2F transcription factors and also by physiological mitogenic signaling, which indicates that DMTF1 is a marker of cells that have exited from the cell cycle (Mallakin et al., 2006). The DMTF1 gene is frequently deleted in a number of cancers (Bodner et al., 1999; Mallakin et al., 2007; Maglic et al., 2013), and remarkably, DMTF1-null mice are prone to spontaneous tumor development, which is accelerated when the animals are neonatally treated with carcinogenesis agents (Inoue et al., 2000; Inoue et al., 2001).

WWOX participates in different cellular processes including bone and germ-cell development, transcription control, induction of apoptosis, cell growth inhibition, and tumor suppression in multiple cancers (Reviewed in Salah et al., 2010). There is a correlation between the loss of WWOX expression and cancer development, as well as an association between WWOX absence and poor prognosis and outcome in various cancer types (Reviewed in Salah et al., 2010). WWOX interacts with different oncogenic proteins, like transcription factor AP-2γ, receptor tyrosine kinase ErbB4, and c-Jun, sequestering them in the cytoplasm and thereby inhibiting their oncogenic activity (Aqeilan et al., 2004; Aqeilan et al., 2005; Gaudio et al., 2006).

NFKBIA is a member of the NF-κB inhibitor family. It inhibits the transcription factor NF-κB by masking its NLSs and keeping it sequestered in an inactive state in the cytoplasm (Jacobs and Harrison, 1998). NF-κB is a pro-survival transcription factor required for maintaining normal immune system function, however inadequate NF-κB activation can mediate inflammation and tumorigenesis. Indeed, it is constitutively activated in most tumor cells (Reviewed in Karin and Greten, 2005). Since NF-κB is repressed by NFKBIA, the latter is considered a tumor suppressor gene. NFKBIA has been found silenced or deleted in some cancers, as well as associated with unfavorable outcomes (Bredel et al., 2011; Furukawa et al., 2013). Importantly, restoration of NFKBIA expression attenuates the malignant phenotype and increases the vulnerability to chemotherapy of cancer cells (Bredel et al., 2011).

PE5 reduces the expression of diverse MDR-associated genes

We had previously demonstrated that treatment of different MDR cell lines with PE5 reduces the expression of P-gp protein and consequently causes an increase of doxorubicin accumulation (Castro et al., 2011b). This effect is dose-dependent. For NCI/ADR-RES cells, the decrease of P-gp expression was of 25%, 65%, and 74% respective to untreated cells when they were treated at IC_{20}, IC_{30}, and IC_{40} for 72 h,

respectively. It is therefore not surprising that in the microarray experiment, in which RNA comes from cells treated with PE5 at IC_{10} during 36 h, we did not observe a significant decrease of the gene encoding P-gp, named ABCB1 or MDR1 gene. Besides, we were interested in investigating whether we could detect changes in the expression of other genes related to different processes of MDR.

As stated above, PE5 treatment reduces the expression of some genes implicated in the quenching of ROS. Particularly, it seems to decrease the expression level of relevant proteins involved in the production of NADPH (G6PD, ME3 and IDH2) that is, at its turn, necessary for the glutathione reduction. Reduced glutathione has an important role in a number of drug resistance mechanisms, since glutathione-S-transferase enzymes conjugate it to a wide diversity of electrophilic anticancer drugs. We have also stated before that PE5 reduces the expression of diverse genes implicated in MDR: ASNS (which is involved in resistance to L-asparaginase), MET (whose activation is related to resistance to epidermal growth factor receptor inhibitors), TGM2 (that contributes to the development of drug resistance), PDGFRB (whose inhibition is associated with an improved tumor drug uptake) and CLU (that interferes with apoptotic signaling and confers resistance to a broad-spectrum of anti-cancer treatments). In addition, we found that PE5 downregulates the expression of aldo-keto reductase family 1 member A1 (AKR1A1) and DNA topoisomerase II, alpha isozyme (TOP2A), which also contribute to drug resistance. Both proteins are described below.

AKR1A1 is an enzyme involved in the reduction of some aromatic and aliphatic aldehydes to their corresponding alcohols. It activates several procarcinogens, such as polycyclic aromatic hydrocarbons trans-dihydrodiols, and it is also implicated in the metabolism of various xenobiotics and drugs. It is known that AKR1A1 metabolizes anthracyclines, such as daunorubicin and doxorubicin into inactive anthracycline alcohol metabolites, thereby contributing to anticancer drug resistance (Reviewed in Jin and Penning, 2007). Furthermore, AKR1A1 has been associated with acquired resistance to irradiation through the suppression of p53 activation (Kim et al., 2012).

TOP2A is a DNA topoisomerase, a key enzyme in DNA replication, cell cycle progression, and chromosome segregation. It is the molecular target of topoisomerase II inhibitors such as doxorubicin or epirubicin, and therefore mutations in this gene have been associated with development of drug resistance (Reviewed in Di Leo and Isola, 2003).

Finally, PE5 increased the expression of different genes known to be necessary for anticancer drug action: BCL2L11 (that mediates cell death of various anticancer drugs and accordingly its downregulation contributes to chemotherapy resistance), RHOB (that is required for apoptosis in response to a variety of anticancer compounds and consequently its deletion renders cells resistant to apoptosis) and NFKBIA (which increases the vulnerability to chemotherapy).

Onconase causes pleiotropic effects different from those of PE5

Gene ontology analysis of the genes differentially expressed upon treatment with onconase in NCI/ADR-RES cells showed three interesting categories that could help understand how onconase induces cytotoxicity. These categories are cell proliferation, response to stress, and apoptosis (Table 9). It is also interesting to note that seven out of the 12 most upregulated genes in NCI/ADR-RES cells treated with onconase (Table 4) correspond to transcription regulators that are functionally related among them, and that are implicated in the three biological processes stated above. These transcription regulators are early growth response 1 (EGR1), activating transcription factor 3 (ATF3), growth arrest and DNA-damage-inducible, alpha (GADD45A), thioredoxin interacting protein (TXNIP), cAMP responsive element binding protein 5 (CREB5), arrestin domain containing 4 (ARRDC4), and protein phosphatase 1, regulatory subunit 15A (PPP1R15A). The relationship between them is depicted in Figure 22.

Briefly, it is known that EGR1 increases the expression of ATF3 (Bottone et al., 2005) and GADD45A (Thyss et al., 2005). ATF3 seems to play a central role in the downstream effects caused by onconase since it upregulates the expression of GADD45A (Xu et al., 2011) and PPP1R15A (Jiang et al., 2004). Additionally, it can indirectly increase TXNIP expression, since ATF3 promotes upregulation of the transcription factor Krüppel-like factor 6 (KLF6) (Xu et al., 2011), whose overexpression increases TXNIP expression (Qi et al., 2009). Moreover, ATF3 can bind to CREB5 to form an heterodimer (Newman and Keating, 2003). Although the physiological significance of this dimer is not clear, the general view is that its formation can alter DNA binding specificity and transcriptional activity, thus

expanding the ability of these transcription factors to regulate gene expression (Reviewed in Hai, 2006). On the other hand, TXNIP and ARRDC4 are related, since transcription regulation of both proteins is controlled through the MondoA transcription factor (Stoltzman et al., 2008).

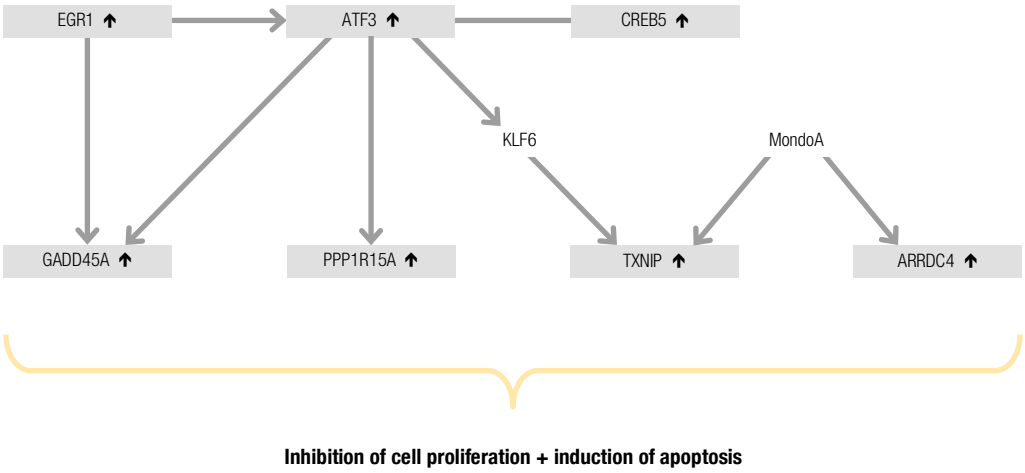

Figure 22. Relationship between the transcription regulators overexpressed (↑) in NCI/ADR-RES cells treated with onconase. The overexpression of these transcription regulators may explain the cytotoxic effect of onconase. Arrows connecting two transcription regulators indicate that one of them increases the expression of the other (pointed by the arrow). The line between ATF3 and CREB5 indicates that the binding between both proteins occurs and they might regulate different genes.

For OVCAR8 cell line, ATF3 and TXNIP were also identified as differentially expressed genes upregulated by onconase (Table 6). Furthermore, a slight but significant increase of expression of the rest of transcription regulators was observed,

with fold change values between 1.8 and 1.5. In addition, six out of the seven onconase differentially expressed genes found in OVCAR8 cell line were also differentially expressed in NCI/ADR-RES cell line (Table 7). These results indicate that the effect induced by onconase on both cell lines is similar but more intense for NCI/ADR-RES. However, it is worth mentioning that treatment with onconase increases the expression of ATF3 and GADD45A in different human mesothelioma cell lines (Altomare et al., 2010), suggesting that these genes have a role in onconase-induced cytotoxicity and that their upregulation is not cell type specific.

As it has been done for PE5, we will discuss here the most interesting biological processes in which these transcription regulators are involved. Although some of them cause diverse and sometimes contradictory effects depending on the cell line or kind of tumor used as a model, in general their overexpression is related to a reduction of cell proliferation and suppression of tumorigenesis.

EGR1 is a Cys2-His2-type zinc-finger transcription factor activated by a broad range of extracellular stimuli, comprising growth factors, cytokines, and stress signals (Cao et al., 1990; Khachigian et al., 1997; Yan et al., 1999). EGR1 is expressed at low levels in several types of cancer (Levin et al., 1995; Huang et al., 1997; Calogero et al., 2001), and it has been demonstrated that it acts as a tumor suppressor, activating apoptosis and having a lasting effect on cell survival and tumor progression. For example, it transactivates the promoter of the p53 gene and upregulates its expression to induce apoptosis of cancer cells (Nair et al., 1997). EGR1 also forms a complex with Yes-associated protein 1 (YAP1) and transactivates the promoter of the Bax gene, thereby activating procaspase-3 and -9 and inducing apoptosis in a p53-independent manner (Zagurovskaya et al., 2009). In addition, another study has demonstrated that the ability of curcumin to act as tumor suppressor by induction of $p21^{WAF1/CIP1}$ is mediated p53-independently through activation of EGR1 (Choi et al., 2008). It is interesting to note here that onconase exerts its cytotoxicity independently of p53 phenotype (Iordanov et al., 2000a). The antiproliferative effect of EGR1 is not observed in all cases since it has also been reported that this transcription factor promotes prostate tumor progression by modulating the androgen receptor-signaling pathway (Yang and Abdulkadir, 2003).

ATF3 and **CREB5** are basic-region leucine zipper (bZIP) transcription factors of cAMP responsive element-binding (CREB) protein family. An analysis of bZIP association by coil-coil array showed that binding of human ATF3 and human CREB5 occurs, although the physiological significance of this dimer is not clear (Newman and Keating, 2003). ATF3 is an adaptive response gene that participates in cellular processes to adapt to extra- and/or intra-cellular changes, where it transduces signals from various receptors to activate or repress gene

expression. ATF3 protein is expressed at low levels in normal and quiescent cells, but it can be rapidly and highly induced in different cell types in response to multiple and diverse extracellular signals, including growth factors, cytokines, physiological stresses, and genotoxic agents (Reviewed in Hai, 2006). Current literature indicates that ATF3 affects cell death and cell cycle progression, although controversy exist since it has been demonstrated to be both a negative and a positive regulator of these processes. Ectopic expression of this transcription factor induces apoptosis in a variety of cancer cells (Syed et al., 2005; Huang et al., 2008), or enhances the ability of different drugs to induce apoptosis (Mashima et al., 2001; Yan et al., 2005; O'Brien et al., 2012; Niknejad et al., 2014), but in contrast, other studies have shown that ATF3 has an anti-apoptotic role (Nobori et al. 2002; Nakagomi et al., 2003). Similarity, it has been reported that it can promote (Allan et al., 2001; Perez et al., 2001) or suppress (Fan et al., 2002; Lu et al., 2006) cell cycle progression. One potential explanation for these conflicting findings is the diverse cell types used in the studies. Other explanations include the varying levels and durations of ATF3 expression, and the differences in the approaches used in the studies; some used the gain-of-function whereas others used the loss-of-function approach.

It is interesting to note that expression of both EGR1 and ATF3 is controlled through common upstream signal transduction cascades that have been previously described to be altered by onconase. Three MAPK families, namely ERK1/2, JNK and p38 MAPK, are most commonly involved in EGR1 activation. While ERK1/2 mediates EGR1 expression in response to growth factors (Hodge et al., 1998), a combination of ERK1/2, p38 MAPK and/or JNK is required to induce EGR1 in response to stress (Lim et al., 1998; Rolli et al., 1999). In the case of ATF3, it has been reported that p38 MAPK signaling pathway, but not ERK1/2 or JNK, play a critical role on the induction of this transcription factor. Moreover, ATF3 is functionally important to mediate the pro-apoptotic effects of p38 MAPK (Lu et al., 2007). Accordingly, onconase is a potent activator of the stress-activated protein kinases JNK1, JNK2 and p38 MAPK in primary and immortalized fibroblasts (Iordanov et al., 2000b).

TXNIP is a protein with multiple functions, but specially plays an important role in redox homeostasis: it binds to the cysteine residues of the catalytic active center of thioredoxin and inhibits its antioxidative function, resulting in an increased production of ROS, oxidative stress, and finally induction of cellular apoptosis (Junn et al., 2000). Interestingly, reduced expression of this protein has been found in a wide range of human tumor tissues, and sometimes it has been associated with poor disease progression and prognosis (Ikarashi et al., 2002; Han et al., 2003; Tome et al., 2005; Nishizawa et al., 2011). The suppression of TXNIP induces tumorigenesis (Sheth et al., 2006), whereas the enforced expression inhibits cell cycle progression, cancer cell proliferation (Han et al., 2003), and metastasis (Goldberg et al., 2003), indicating that TXNIP acts as a tumor suppressor.

The mechanism by which TXNIP modulates apoptosis is related to its redox homeostasis function and involves apoptosis signal-regulating kinase 1 (ASK1), a key regulator of oxidative stress-induced apoptosis in many types of cells through activation of JNK and p38 MAPK pathways (Tobiume et al., 2001). In mammalian cells, cytosolic and mitochondrial thioredoxins are the major disulfide oxidoreductases supplying electrons to enzymes for cell proliferation and viability. The reduced/dithiol form of thioredoxin binds to ASK1 and inhibits its activity to prevent stress- and cytokine-induced apoptosis (Saitoh et al., 1998). In an oxidative stress environment, ROS induces oxidation of thioredoxin, which dissociates from ASK1 and apoptosis is stimulated (Saitoh et al., 1998). TXNIP binds and inhibits thioredoxin, thus allowing activated ASK1 to induce apoptosis (Junn et al., 2000). On the other

hand, TXNIP can act independently of its binding to thioredoxin. It is known that TXNIP interacts with a variety of molecules to regulate cell cycle, and that its overexpression inhibits cell proliferation through cell cycle arrest. For instance, TXNIP represses the transcription of cyclin A and induces that of $p16^{INK4A}$, leading to cell cycle arrest at G_0/G_1 (Han et al., 2003; Nishinaka et al., 2004). Also, TXNIP increases $p27^{KIP1}$ stability by inhibiting c-Jun activation domain-binding protein-1 (JAB1), resulting in suppression of cell proliferation (Jeon et al., 2005). Furthermore, it is known that TXNIP suppresses glucose uptake and mediates metabolic reprograming, which contributes to cell growth inhibition (Elgort et al., 2010). Accordingly, loss of TXNIP inhibits mitochondrial oxidation while promoting glycolysis, potentially favoring tumor cell growth through the Warburg effect (Hui et al., 2008; Kaadige et al., 2009). The glucose uptake function is intrinsic to the arrestin domains of TXNIP (Patwari et al., 2009). Another arrestin, **ARRDC4**, is an equally potent inhibitor of glucose uptake (Patwari et al., 2009). The metabolic function is not the unique feature common between TXNIP and ARRDC4, since transcription regulation of both proteins is controlled through the MondoA transcription factor, related to the carbohydrate response element binding protein (ChREBP) (Stoltzman et al., 2008).

GADD45A belongs to the growth arrest and DNA damage-inducible (GADD) family of genes, whose cognate proteins are key players in cellular stress responses. It is induced by a wide spectrum of DNA-damaging agents and growth arrest treatments, and it has important roles in the control of cell cycle checkpoints, DNA repair process, signaling transduction, and genome stability (Reviewed in Rosemary Siafakas and Richardson, 2009). It is known that GADD45A inhibits cell growth through G_2/M cell cycle arrest (Wang et al., 1999). Particularly, it interacts with cyclin-dependent kinase 1 (CDK1) and dissociates the CDK1/cyclin B1 complex, which suppresses the activity of this complex, thereby resulting in G_2/M cell cycle arrest (Zhan et al., 1999). It is interesting to note that onconase induces the expression of genes whose encoded proteins promote cell cycle arrest at different points (G_2/M in the case of GADD45A and G_0/G_1 in the case of TXNIP). This is in accordance with the fact that onconase arrest cell growth of Jurkat and NCI/ADR-RES cells without altering the proportion of the different cell cycle phases (Tsai et al., 2004; Castro et al., 2011a). GADD45A can also participate in the induction of apoptosis. Indeed, this protein interacts with mitogen-activated protein kinase kinase kinase 4 (MEKK4) and activates the JNK/p38 MAPK signaling pathway, which induces apoptosis (Takekawa and Saito, 1998).

Interestingly, multiple evidences show that GADD45A has a tumor suppression function. On the one hand, reduced expression of this gene has been frequently observed in several types of human cancer (Wang et al., 2005; Ramachandran et al., 2009; Na et al., 2010). On the other hand, GADD45A is regulated by tumor suppressor genes and oncogenes. For instance, GADD45A expression is upregulated by the tumor suppressors p53 and breast cancer 1, early onset (BRCA1) (Zhan et al., 1998; Harkin et al., 1999). Remarkably, the phenotype of GADD45A-null mice is similar to that found in p53-deficient mice, showing severe genomic instability and enhanced tumorigenesis following DNA damaging agents (Hollander et al., 1999; Hollander et al., 2001a). Otherwise, increased expression of the proto-oncogene c-Myc leads to decreased GADD45A expression (Amundson et al., 1998). Upregulation of transcription factor NF-κB also downregulates GADD45A, and it has been proposed that repression of GADD45A mediated by NF-κB is essential for cancer cell survival (Zerbini et al., 2004). In this sense, it is worth mentioning that the growth suppression effect of onconase in Jurkat cells is accompanied by an altered subcellular distribution and reduced expression of NF-κB (Tsai et al., 2004). It has

also been reported that the increase of hsa-miR-17* and decrease of hsa-miR-30c levels in malignant pleural mesothelioma cells treated with onconase result in downregulation of NFKB1 gene, which encodes NF-κB subunit p50 (Goparaju et al., 2011).

PPP1R15A is an endoplasmic reticulum-associated protein known to be one of the principal mediators of endoplasmic reticulum stress–induced apoptosis. In addition, PPP1R15A is induced by other conditions of cellular stress, such as DNA damage, heat shock, virus infection, and energy depletion (Fornace et al., 1989; Novoa et al., 2001; Cheng et al., 2005). Interestingly, overexpression of PPP1R15A in several cell lines promotes apoptosis or cell cycle arrest, suggesting that it is a tumor suppressor gene (Adler et al., 1999; Hollander et al., 2001b). In agreement, it has been reported that some antitumor agents inhibit cancer cell proliferation and induce apoptosis through overexpression of PPP1R15A (Fishel et al, 2006; Kato et al., 2010). The mechanism by which PPP1R15A mediates these effects is unknown, although diverse theories exist. For example, it has been proposed that PPP1R15A promotes apoptosis and growth arrest inducing p53 phosphorylation and $p21^{WAF1/CIP1}$ transcription, respectively (Yagi et al., 2003).

Together with these transcription factors, there are other genes whose expression changed in NCI/ADR-RES treated with onconase that merit a comment. Interleukin 6 (IL6) expression was highly upregulated, while asparagine synthetase (ASNS) and phosphoglycerate dehydrogenase (PHGDH) were underexpressed as a result of onconase treatment. In addition to be upregulated in NCI/ADR-RES, IL6 expression was slightly but significantly increased in OVCAR-8 cells, with a fold change of 1.6. Overexpression of IL6 has also been observed in human mesothelioma cell lines treated with onconase (Altomare et al., 2010), suggesting that overexpression of this gene is independent of the cell type used.

IL6 is a pleiotropic cytokine that regulates immunological reactions in host defense, inflammation, haematopoiesis, and oncogenesis (Reviewed in Akira et al., 1993). It is known that dependent on the cell type and the presence or absence of IL6 receptor, IL6 can either stimulate or inhibit cancer cell proliferation. For example, it stimulates proliferation of various tumor cells (Miles et al., 1990; Okuno et al., 1992; Giri et al., 2001) and high IL-6 levels are associated with increasing tumor stages and tumor size, metastasis, and decreased survival in different cancers (Nakashima et al., 2000; Knüpfer and Preiss, 2010; Lane et al., 2011). However, when tumor cells are weakly antigenic or devoid of IL-6 receptor, a tumor inhibiting effect of IL6 has been demonstrated, presumably because of its immune enhancing properties (Sun et al., 1992; Mulé et al., 1992).

Since IL6 is related to immune and inflammatory response, it could be involved in the toxicity of onconase encountered in clinical trials (Reviewed in Costanzi et al., 2005) and, as proposed previously (Altomare et al., 2010), targeting of this gene might improve treatment of patients with onconase.

ASNS and **PHGDH** are enzymes implicated in amino acid biosynthesis. As discussed before, they are relevant for cancer progression and were downregulated in NCI/ADR-RES cells treated with PE5.

Both PE5 and onconase cause significant changes in miRNA expression

miRNAs are potent post-transcriptional regulators of gene expression. Typically, mature miRNAs bind with partial complementarity to the 3' untranslated region (3' UTR) of a target mRNA, which causes translational repression and/or mRNA cleavage, reducing the final protein output. miRNAs participate in many cellular processes, including cell proliferation, differentiation, and apoptosis, and their deregulation can lead to the development of diverse human diseases, such as cancer. Indeed, miRNAs are deregulated in almost all types of human cancer (Reviewed in Ling et al., 2013). Cancer cells acquire miRNA signature patterns distinct form those of normal cells, which contribute to the development of specific phenotypes that allow them to proliferate, survive in adverse conditions, modify metabolism, evade immune recognition, elicit local angiogenesis, metastasize, and develop chemoresistance. Based on their influence on the cancer cell phenotype, miRNAs can function either as tumor suppressors or oncogenes. Remarkably, because of their roles in cancer development, miRNAs have a significant potential as therapeutic targets.

In the present work, we used microarrays to compare the miRNA expression profiles of cells treated with PE5 or onconase with those of untreated cells. We found for the first time that PE5 cause significant changes in miRNA expression and confirmed that onconase also alters miRNA levels, as previously reported (Goparaju et al., 2011). These changes in miRNA expression could explain, at least in part, how PE5 and onconase mediate cytotoxicity and regulate the expression of the differentially expressed genes obtained in the gene expression microarrays. For NCI/ADR-RES cell line, we identified a total of 181 miRNAs that were differentially expressed in

PE5-treated cells (24% upregulated and 76% downregulated), and 13 in onconase-treated cells (23% upregulated and 77% downregulated) (Tables 12 and 13). In the case of OVCAR8 cell line, we obtained a considerable lower number of differentially expressed miRNAs, similarity to gene expression microarrays. As mentioned before, this difference could be due to the fact that OVCAR8 cell line requires fewer changes in gene expression for induction of cytotoxicity or that RNase concentration used for OVCAR8 treatment actually provoked less than a 10% decrease in cell proliferation, especially in the case of PE5. Because of the lower number of miRNAs identified in OVCAR8 cell line, here we will focus on the differentially expressed miRNAs encountered in NCI/ADR-RES.

For NCI/ADR-RES cell line, when comparing the microarray experiments between both RNases, our results showed that an important number of onconase differentially expressed miRNAs (nine out of 13) are also deregulated in PE5 treated cells (Table 14). However, we consider that the effects caused by PE5 and onconase on cells through regulation of miRNAs may be different. First, nine miRNAs represent a very low proportion among the 181 PE5 differentially expressed miRNAs. Second, these nine common miRNAs are all downregulated, whereas no coincidences were obtained between upregulated miRNAs. And third, PE5 differentially expressed miRNAs are globally related to tumor development and progression, regulation of chemoresistance, and control of metabolism, while practically no relationship with drug chemoresistance or metabolism has been described for the miRNAs regulated by onconase. This is in agreement with the results of gene expression microarray experiments, where we detected important differences between the effects of both RNases. On the other hand, we observed that PE5 and onconase upregulate and downregulate the same percentage of miRNAs. For the two RNases, approximately 25% of differentially expressed miRNAs were upregulated whereas 75% were downregulated, which indicates that the primary action of PE5 and onconase is to decrease miRNA expression. The changes in the miRNA expression profiles upon RNase treatments contrast with those of mRNA in the sense that the proportion of mRNAs that are downregulated (47% for PE5, 11% for onconase) is much lower than that of miRNAs. This could suggest that miRNAs are more likely primary targets of these RNases that mRNAs.

It is important to note that each miRNA can regulate the expression of numerous target genes and conversely several miRNAs can function cooperatively to regulate the same target gene, and therefore miRNAs and mRNAs form a complex network of interactions. For this reason it is difficult to study and predict the effects caused by the deregulation of a given miRNA or a group of miRNAs. In addition, miRNA activity can be dependent on the cellular environment, and the same miRNA can have different targets in different cell types and consequently opposite effects. In agreement, contradictory information has been reported. For example, miR-221 (hsa-miR-221-5p), that is one of the PE5 downregulated miRNAs, exerts an oncogenic function in liver cancer (Pineau et al., 2010), but acts as a tumor suppressor in erythroblastic leukemia (Felli et al., 2005). Similarity, miR-27a (hsa-miR-27a-3p), which is also downregulated by PE5, increases the expression level of P-glycoprotein in ovarian cancer reducing the sensitivity to chemotherapy (Li et al., 2010), whereas the opposite effect has been observed in hepatocellular carcinoma (Chen, Z. et al., 2013). Finally, it is worth mentioning that the effects of miRNAs have not been fully characterized. In this sense, for many miRNAs, *in silico* analysis predict roughly more than ten-fold mRNA targets than the number of targets validated experimentally.

In the following sections we will discuss the effects caused by PE5 and onconase on miRNA expression. For PE5, the biological processes and the miRNAs presented here have been chosen based on the main functions and validated targets of the top 20 upregulated and top 60 downregulated differentially expressed miRNAs out of the 181. For onconase, the 13 differentially expressed miRNAs have been considered.

PE5 differentially expressed miRNAs support the general results of gene expression microarrays

Although most of the validated targets of PE5 differentially expressed miRNAs do not correspond with PE5 differentially expressed genes, the general effects are predicted to be very similar. As it will be described below, PE5 differentially expressed miRNAs in NCI/ADR-RES cells are involved in tumor development and progression, regulation of chemoresistance, and control of some metabolic pathways, which is consistent with the results of gene expression microarrays.

PE5 regulates de expression of diverse tumor suppressor and oncogenic miRNAs

miRNAs have a variety of roles in cancer development and progression, acting as tumor suppressors or oncogenes. As mentioned above, the effect of miRNAs is highly dependent on the cellular context, and for some miRNAs both oncogenic and tumor suppressor activities have been reported. Nevertheless, among the PE5 differentially expressed miRNAs implicated in tumor progression or suppression, those reported to be increased in ovarian cancer cells are decreased by the PE5 treatment and *viceversa*.

Microarray experiments showed that PE5 increases the expression of several tumor suppressor miRNAs, including miR-638 (hsa-miR-638), miR-663a (hsa-miR-663a), miR-630 (hsa-miR-630), miR-622 (hsa-miR-622), miR-134 (hsa-miR-134), miR-150* (hsa-miR-150-3p), and miR-7 (hsa-miR-7-5p). It has been reported that these miRNAs exert their function by targeting for degradation oncogenic protein-coding mRNAs, which results in inhibition of proliferation, invasion, migration, metastasis, and induction of apoptosis. A brief description of these miRNAs is presented below.

All of the above miRNAs have been stated to be significantly underexpressed in a variety of human cancers (Pan et al., 2010; Sand et al., 2012; Yang et al., 2013; Niu et al., 2013). It has also been found that they target oncogenic protein-coding miRNAs. To mention some examples, **miR-663** promotes inhibition of H-Ras (Yang et al., 2013) and eukaryotic translation elongation factor 1 alpha 1 (EEF1A2) (Vislovukh et al., 2013), **miR-630** inhibits insulin-like growth factor 1 receptor (IGF-1R), (Farhana et al., 2013), **miR-622** downregulates K-Ras (Han et al., 2012), **miR-134** inhibits forkhead box M1 (FOXM1) (Li et al., 2012), **miR-150*** inhibits c-Myb and IGF-1R (Farhana et al., 2013), and finally, **miR-7** has been described to downregulate EGFR expression (Webster et al., 2009). This is in accordance with our gene expression microarrays, since we encountered that EEF1A2 was downregulated in cells treated with PE5.

On the other hand, microarray experiments showed that PE5 also decreases the expression of various miRNAs involved in tumor development and progression. Among the most downregulated miRNAs, we identified miR-503 (hsa-miR-503),

miR-424 (hsa-miR-424-5p), miR-99a (hsa-miR-99a-5p), miR-193b (hsa-miR-193b-3p), and different members of the miR-17-92 cluster, let-7 family, and miR-200 family. Most of these miRNAs have been described to exert an oncogenic activity by targeting tumor suppressor genes and promoting proliferation, invasion and metastasis while inhibiting apoptosis. These miRNAs are described below.

miR-503, **miR-424**, **miR-99a**, and **miR-193b** are highly expressed in diverse human cancers, and in some cases they are related to poor survival and correlated with advanced clinical stage in patients (Özata et al., 2011; Wu et al., 2013; Zhang et al., 2013; Lenarduzzi et al., 2013). Furthermore, miR-424 suppresses the expression of suppressor of cytokine signaling 6 (SOCS6) (Wu et al., 2013), miR-99a inhibits the expression of CTD small phosphatase-like (CTDSPL) and tribbles pseudokinase 2 (TRIB2) (Zhang et al., 2013), and miR-193b reduces neurofibromin 1 (NF1) (Lenarduzzi et al., 2013), all o them coding for tumor suppressor proteins.

miR-18a (hsa-miR-18a-5p), miR-19a (hsa-miR-19a-3p), and miR-17 (hsa-miR-17-5p), which belong to the **miR-17-92 cluster**, were identified as PE5 downregulated miRNAs. It has been widely described that members of this cluster are oncogenic miRNAs, and they are overexpressed and associated with poor prognosis in a broad range of cancer types (Reviewed in Mendell, 2008; Ling et al., 2013). Moreover, a number of studies have shown that certain tumor suppressors are direct targets of these miRNAs. For example, the tumor suppressor PTEN has been identified as a direct target negatively regulated by miR-19a (Jia et al., 2013), whereas the pro-apoptotic and tumor suppressor gene BCL2L11 and the potent cell cycle inhibitor $p21^{WAF1/CIP1}$ have been reported to be targets of miR-17 (Yan et al., 2012; Shen et al., 2013). This is in agreement with our results of gene expression microarrays, where we detected BCL2L11 as a PE5 upregulated gene, and consistent with a previous study from our research group that showed that PE5 increases $p21^{WAF1/CIP1}$ protein levels (Castro et al., 2011a).

Among the members of **let-7 family**, we found that PE5 decreases the expression of let-7c (hsa-let-7c), let-7d (hsa-let-7d-5p), miR-98 (hsa-miR-98), let-7b (hsa-let-7b-5p), let-7e (hsa-let-7e-5p), let-7g (hsa-let-7g-5p), and let-7i (hsa-let-7i-5p). Some let-7 members are underexpressed in various cancer types, and known relevant targets of let-7 members are oncogenes, indicating a tumor suppressive function of let-7 family (Reviewed in Ling et al., 2013). However, miRNAs belonging to this family also display a potential oncogenic role. As examples, the serum levels of let-7c are significantly elevated in esophageal cancer patients (Tanaka et al., 2013), let-7d is upregulated during the transition from ductal carcinoma in situ to invasive ductal carcinoma (Volinia et al., 2012), and the expression of miR-98 is significantly upregulated in cancer (Yao et al., 2009; Sukata et al., 2011). Interestingly miR-98 was confirmed to target and reduce the expression of tumor suppressor candidate 2 (TUSC2) and p53 (Du et al., 2009; Zhang et al., 2011).

A similar situation occurs with **miR-200 family**. miRNAs belonging to this family are often reported as tumor suppressors (Reviewed in Mongroo and Rustgi, 2010), but an oncogenic function for these miRNAs has also been described. For instance, miR-141 (hsa-miR-141-3p) and miR-200c (hsa-miR-200c-3p), the two members of

miR-200 family downregulated by PE5, are among the most significantly overexpressed miRNAs in different types of cancer, and high expression of both miRNAs has been found to be correlated with poor prognosis (Nam et al., 2008; Waltering et al., 2011; Tanaka et al., 2013).

We found that PE5 also regulates the expression of miRNAs implicated in stemness. Increasing evidences suggest that the tumor growth potential depends on cancer stem cells, which represent a small subset of highly malignant cancer cells with stem cell properties. It is generally accepted that cancer stem cells possess the capacity to self-renew and the ability to differentiate and give rise to all cell types found in a particular tumor. It has been hypothesized that these cells remain in patients after conventional therapy has been completed, and therefore effective tumor eradication require obtaining agents able to target cancer stem cells (Reviewed in Clarke et al., 2006). As examples, miR-1202 (hsa-miR-1202), miR-1181 (hsa-miR-1181), miR-150* (hsa-miR-150-3p), miR-134 (hsa-miR-134), and miR-1915 (hsa-miR-1915-3p) are miRNAs upregulated by PE5 involved in the reduction of tumor stem cell like properties (Tay et al., 2008; Nam et al., 2012; Farhana et al., 2013; Sallustio et al., 2013). Moreover, PE5 decreased the expression of miR-503 (hsa-miR-503), miR-424 (hsa-miR-424-5p), miR-27b (hsa-miR-27b-3p), miR-23b (hsa-miR-23b-3p), and miR-27a (hsa-miR-27a-3p), which have been described to be overexpressed in ovarian cells showing stem cell features (Park et al., 2013).

PE5 alters the expression of various miRNAs involved in MDR

In addition to be involved in tumor development and progression, it is known that miRNAs play a key role in the development of drug resistance. Abnormal expression of miRNAs has been observed in diverse chemoresistant cancer cells, where they contribute to drug resistance through alteration of the drug target, alteration of the drug, modification of the cell cycle and apoptosis, increased DNA damaged repair, failure of the drug to enter the cell through loss of cell surface receptors or transporters, or ejection of the drug from the cell by reducing drug efflux pumps (Reviewed in Allen and Weiss, 2010).

We found that PE5 changes the expression of different miRNAs involved in the regulation of P-glycoprotein (P-gp; from MDR1 or ABCB1 gene), multidrug resistance associated protein 1 (MRP1; from ABCC1 gene) and breast cancer resistant protein (BCRP; from ABCG2 gene), which are ABC transporters overexpressed in several types of cancer. They allow the elimination of a wide range of therapeutic agents from cancer cells, and therefore are involved in the development of MDR (Reviewed in Liu, 2009). Among the PE5 upregulated miRNAs, we identified miR-134 (hsa-miR-134) and miR-7 (hsa-miR-7-5p). Concerning miRNAs downregulated by PE5, we can mention miR-27 (hsa-miR-27a-3p), miR-19a (hsa-miR-19a-3p), and miR-130a (hsa-miR-130a-3p). Their contributions to MDR are summarized below.

Both **miR-134** and **miR-7**, which are PE5 upregulated miRNAs, have been found underexpressed in MDR cells. It has been reported that they target and negatively regulate MRP1 expression and protein levels (Guo et al., 2010; Pogribny et al., 2010). In agreement, ectopic expression of miR-134 greatly increases the sensitivity of MDR cells to cisplatin, etoposide, and doxorubicin (Guo et al., 2010), whereas miR-7 increases the sensitivity of resistant cells to cisplatin (Pogribny et al., 2010).

miR-27, **miR-19a**, and **miR-130a**, which are PE5 downregulated miRNAs, are overexpressed in various MDR cells (Zhu et al., 2008; Liang et al., 2011; Yang et al., 2012; Wang et al., 2013). Moreover, it has been described that miR-27 upregulates MDR1 expression by targeting homeodomain interacting protein kinase 2 (HIPK2), a serine/threonine kinase involved in transcription regulation (Li et al., 2010). Accordingly, inhibition of miR-27a decreases the levels of MDR1 mRNA and protein, increases HIPK2 expression, increases paclitaxel-induced apoptosis and the sensitivity to paclitaxel in resistant cells (Li et al., 2010), and enhances the sensitivity to vinblastine as well as intracellular accumulation of doxorubicin in MDR cells (Zhu et al., 2008). This is consistent with our results of gene expression microarrays, since HIPK2 was overexpressed in NCI/ADR-RES cells treated with PE5. On the other hand, it has been reported that miR-19a, which is a member of the miR-17-92 cluster, directly targets and inhibits the tumor suppressor PTEN in cancer cells to modulate the expression of different MDR-related transporters. In agreement, downregulation of this miRNA increases PTEN mRNA and protein expression levels, decreases the transporters MDR1, MRP1, and BCRP mRNA and the corresponding proteins, and restores the sensitivity of MDR cells to the chemotherapeutic agents taxol, VP-16, mitoxantrone, cisplatin, 5-fluorouracil, and adriamycin (Liang et al., 2011; Wang et al., 2013). Finally, miR-130a is also associated with MDR1-mediated drug resistance. Inhibition of this miRNA remarkably decreases the expression of MDR1 mRNA and protein, in addition to increase cisplatin-sensitivity in resistant cells (Yang et al., 2012).

PE5 changes the expression of different miRNAs related to metabolism

Several studies have revealed that miRNAs participate in the control of cancer cell metabolism by directly modulating the expression of metabolic transporters or enzymes, or alternatively, by regulating transcription factors or signaling proteins that in turn regulate the metabolic machinery (Reviewed in Chen et al., 2012).

Our results indicate that PE5 alters the expression level of some miRNAs involved in regulation of metabolism, but this effect do no exactly match with those observed on gene expression microarrays. Indeed, gene expression microarrays showed that PE5 affects enzymes implicated in glucose, lipid, and amino acid metabolism, whereas PE5 differentially expressed miRNAs are involved in glucose metabolism, in addition to participate in OXPHOS. To our knowledge, practically no relationship with lipid or amino acid metabolism has been described for the miRNAs whose expression changes upon PE5 treatment. This result could indicate that the alteration of multiple metabolic pathways induced by PE5 is not mainly due to a regulation of miRNAs. Otherwise, it is also possible that the relationship between PE5 differentially expressed miRNAs and metabolism had not yet been fully studied and not enough literature information is available.

Among PE5 regulated miRNAs, miR-19a (hsa-miR-19a-3p), miR-210 (hsa-miR-210), and mir-378(*) (hsa-miR-378a-5p) are associated with abnormal glucose metabolism and OXPHOS in cancer cells. All of them were underexpressed in cells treated with PE5. These miRNAs are briefly described below.

As indicated above, **miR-19a** directly targets and inhibits the tumor suppressor PTEN, and accordingly, downregulation of this miRNA increases PTEN mRNA and protein expression levels (Jia et al., 2013). In turn, PTEN elevation has been described to trigger a metabolic reprogramming that results in reduced glucose uptake, decreased lactate secretion, inhibition of PPP, increased mitochondrial OXPHOS, generation of ROS, and reduction of glutamine uptake (Garcia-Cao et al., 2012; Hong et al., 2013). On the other hand, **miR-210** decreases mitochondrial respiration, activates the generation of ROS, and upregulates the glycolysis through

targeting and repressing iron-sulfur cluster assembly enzyme (ISCU) and cytochrome c oxidase assembly homolog 10 (COX10), which are two important factors of the mitochondria electron transport chain and the TCA cycle (Chen et al., 2010), and glycerol-3-phosphate dehydrogenase 1-like (GPD1L), an enzyme that negatively regulates HIF-1α stability (Kelly et al., 2011). Therefore, downregulation of this miRNA could reverse all these metabolic pathways. Similarly, **mir-378(*)** reduces cellular respiration and increases lactate production in cancer cells. This miRNA performs these functions by inhibiting estrogen-related receptor gamma (ESRRG) and GA binding protein transcription factor, alpha subunit (GABPA), which are transcriptional regulators of oxidative energy metabolism (Eichner et al., 2010). Also, downregulation of miR-378(*) might generate the opposite effects.

Onconase regulates the expression of a more limited number of miRNAs than PE5

At the treatment conditions established in our experiments, microarray analysis showed that onconase regulates the expression of fewer miRNAs in NCI/ADR-RES cell line than PE5.

We only identified three upregulated miRNAs, namely miR-20a* (hsa-miR-20a-3p), miR-181a* (hsa-miR-181a-3p), and miR-424* (hsa-miR-424-3p). To our knowledge, the expression pattern of these miRNAs, physiological function, and relationship with carcinogenesis are currently unknown. On the other hand, we found ten miRNA downregulated in cells treated with onconase. Among them, miR-503 (hsa-miR-503), miR-744 (hsa-miR-744-5p), miR-24 (hsa-miR-24-3p), miR-99b (hsa-miR-99b-5p), miR-18a (hsa-miR-18a-5p), and miR-138 (hsa-miR-138-5p) can exert an oncogenic activity promoting proliferation, survival, invasion, and metastasis. A brief description is presented below.

Supporting their oncogenic role, these miRNAs are overexpressed in a diversity of cancers (Özata et al., 2011; Nurul-Syakima et al., 2011; Chan et al., 2012; Chen, L. et al., 2013; Liu, S.G. et al., 2013), where they promote tumor development and progression via targeting and regulating different mRNAs. For instance, **miR-744** enhance cell proliferation through induction of cyclin B1 expression, a cell cycle regulator that controls the G_2/M

transition (Huang et al., 2012). **miR-24** promotes cancer cell proliferation, invasion, and migration, as well as tumor growth and metastasis in mice by targeting and reducing the expression of the tumor suppressors suppression of tumorigenicity 7 like (ST7L) and MAX interactor 1(MXI1) (Chen, L. et al., 2013; Xu et al., 2013), the phosphatases that act over EGFR known as protein tyrosine phosphatase, non-receptor type 9 (PTPN9) and protein tyrosine phosphatase, receptor type, F (PTPRF) (Du et al., 2013), and also the cyclin-dependent kinase inhibitors $p27^{KIP1}$ and $p16^{INK4A}$ (Giglio et al., 2013). Moreover, **miR-138** promotes growth and survival of cancer cells through targeting and inhibiting the pro-apoptotic caspase-3 and the tumor suppressors bladder cancer associated protein (BLCAP) and MAX dimerization protein 1(MXD1) (Chan et al., 2012). This miRNA also have a role promoting resistance to chemotherapeutic drugs, such as cisplatin (Nordentoft et al., 2012) and gemcitabine (Kozinn et al., 2013).

The target genes described above do not coincide with the onconase differentially expressed genes obtained in gene expression microarray experiments. However, it is interesting to note that the general effects caused by miRNAs and genes regulated by onconase are greatly similar.

Taken together, the results of microarrays experiments presented here indicate that PE5 mediates its antitumor activity by regulating the expression of numerous genes and miRNA, and modulating important cellular processes like cell proliferation, metastasis, apoptosis, chemotherapy resistance, and metabolism. Interestingly, onconase affects the expression of different and more limited number of genes and miRNAs, which are implicated in cell proliferation, cell cycle progression, apoptosis, and response to stress. Thus, we can conclude that these two RNases exert their cytotoxic mechanism by different ways. However, we are aware that the microarray results described in this work have to be validated by an alternative technique, such as quantification of the expression changes by RT-PCR.

PE10 and NLSPE5 are nuclear-directed RNases with improved properties compared to PE5

In the present work we were also interested in improving the properties of PE5 as an antitumor drug candidate. One major concern in the development of recombinant

antitumor drugs is the generation of an immunogenic response against the drug in treated patients. In order to decrease the potential immunogenicity of PE5, we constructed PE9 and PE10. In PE9, the five N-terminal residues mutated in PE5 (positions 4, 6, 9, 16 and 17), that are not involved in the NLS function, were reverted to those of the wild type enzyme, while in PE10, only three of these residues (positions 9, 16 and 17) were back-mutated (Figure 1). As a result, PE9 carries two additional basic residues in comparison to PE10: Arg4 and Lys6. Since both variants maintain the residues involved in the NLS of PE5 (Lys1, Arg31-33 and Arg89-91), it was expected that they exhibited the same cytotoxicity than PE5. Surprisingly, PE9 was much less cytotoxic than PE5 or PE10 (Table 15). In order to account for this difference in cytotoxicity, we measured the thermal stability and the catalytic efficiency of these RNases. PE9 displayed the same catalytic efficiency but it was 4ºC less thermally stable than PE5 or PE10. Protein stability is an important factor in the cytotoxic potency of RNases (Klink and Raines, 2000). However, the slight decrease in thermal stability observed for PE9 cannot explain the low cytotoxicity of this RNase, since this effect could be counteracted by the fact that PE9 carries two additional basic residues in comparison to PE5 and PE10, and it has been described that the increase of basicity raises the cytotoxicity of RNases (Futmai et al., 2001; Ilinskaya et al., 2002). This latter strategy is based on the rational fact that a more efficient internalization could be achieved through electrostatic interactions to bind highly cationic proteins to the negatively charged membrane surface. We believe that unexplored reasons may be the cause of the difference in cytotoxicity between PE9 and PE5 or PE10. For instance, it could be postulated that in PE9 Arg4 and Lys6 lower the strength by which α-importin binds to the RNase and/or increase the binding by RI. Indeed, in the nuclear magnetic resonance structure of wild type HP-RNase, Lys1, which belongs to the NLS of PE5, has a high mobility that is most likely due to the presence of a fixed cluster of positive charges (Arg4, Lys6, and Lys7) in its neighborhood (Kövér et al., 2008).

PE5 is an attractive antitumor RNase not only because of its human origin, but also because it exerts its cytotoxic activity on a different cell compartment compared to other RNases like onconase or RI evading RNases. The nuclear routing offers to PE5 new RNA substrates, and therefore new targets to kill tumor cells. Indeed, a previous study (Castro et al., 2011a) and the microarrays analysis performed in the present

work have shown that the mechanism of cytotoxicity of PE5 is different from that of onconase.

We were conscious that the effect of PE5 on cell cultures was lower than that of onconase (Castro et al., 2011a), and we were therefore interested in improving the cytotoxicity of PE5. We reasoned that increasing the capacity of the RNase to reach the nucleus would lead to an increase of its cytotoxicity. In order to increase the ability of PE5 to reach the nucleus, we tried to increase its affinity for the α-importin. One strategy to improve the binding of a molecule to a partner is the insertion of additional new binding sites on the molecule. Natural and synthetic multivalent ligands can bind to receptors with high avidity and specificity and therefore can function as potent effectors or inhibitors of biological processes (Reviewed in Kiessling et al., 2000). To this end, we fused a known and efficient NLS (the NLS of SV40 large T-antigen) either at the N- or C-terminus of PE5 to increase its binding to α-importin (Figure 1). Among the different constructions assayed, only NLSPE5, carrying the NLS at its N-terminus, was clearly more cytotoxic than PE5 (Table 16), which indicates that the position where the NLS is inserted into the RNase is critical for its cytotoxic activity. This fact could be due to intramolecular NLS masking, a phenomenon previously described for other proteins that prevents the recognition of the NLS by the nuclear import machinery (Henkel et al., 1992). It is worth mentioning that the insertion of the same NLS at the N-terminus of PE3, a low cytotoxic HP-RNase variant that has not an NLS, also causes a clear increase of cytotoxicity, showing that the increase of cytotoxicity promoted by the insertion of the N-terminal NLS is not restricted to PE5 (Ruiz, 2011).

The difference in cytotoxicity between NLSPE5 and scNLSPE5 (Table 16) indicates that the cytotoxicity of NLSPE5 is mainly due to its routing into the nucleus. We are aware that the increase of cytotoxicity observed for NLSPE5 respective to PE5 might be also due in part to the cationization of the protein since the scNLSPE5 is also slightly more cytotoxic than PE5. As mentioned above, cationization of an RNase by chemical or genetic modification has been previously described as a strategy to increase its internalization efficiency (Futmai et al., 2001; Ilinskaya et al., 2002). Nevertheless, we have shown that the additional basic stretch does not confer a

cytotoxic activity *per se* since the NLSPE5H119A variant, which carries the same stretch and does not have catalytic activity, is not cytotoxic.

We were interested in characterizing NLSPE5 and PE10, the two improved PE5 variants. To this end, we first analyzed the *in vitro* RI inhibition of the different variants. Although all the variants were inhibited by the RI, the binding between NLSPE5 and RI was slightly lower than for the rest of the variants (Figure 13). However, NLSPE5 does not fully evade the RI *in vivo*, because we observed that this RNase does not cleave cytosolic RNA, an indication that RI is protecting it (Figure 14). In addition, we observed that both NLSPE5 and PE10 cleave nuclear RNA, suggesting that the cytotoxicity of these RNases is dependent on the cleavage of nuclear RNA, as described for PE5 (Figure 14). We also investigated whether the cytotoxic properties of NLSPE5 and PE10 differ from those of PE5. We have obtained multiple data that indicate that NLSPE5, PE10, and PE5 share the same cytotoxic mechanism. Treatment of cancer cells with these RNases increases two-fold the levels of $p21^{WAF1/CIP1}$ and cyclin E, but not those of cyclin D1 (Figure 17), similarly increases the accumulation of doxorubicin inside MDR cells (Figure 18), and induces the same proportion of early and late apoptotic cells along the different incubation times (Figure 15). Also, the activation pattern of procaspase-3, -8, and -9 is very similar in cells treated with NLSPE5, PE10, or PE5 (Figure 16). All together, the results presented here indicate that NLSPE5 and PE10 are improved variants of the nuclear-directed PE5. On the one hand, NLSPE5 is much more cytotoxic than PE5, not because it exerts its cytotoxicity by a different pathway, but because it cleaves nuclear RNA more efficiently. On the other hand, PE10 is as cytotoxic as PE5 and displays the same cytotoxic mechanism, but it is expected that this variant would be less immunogenic than PE5 in treated patients due to its sequence is more similar to that of the wild type HP-RNase.

As a result of the present work, we envisage the construction of new HP-RNase variants in which a combination of the two strategies presented here will produce more effective and non-immunogenic cytotoxic drugs. It is worth mentioning that NLSPE5 has been humanized recently by replacing the NLS of SV40 T-large antigen by other NLSs of human origin. These new variants carry the NLSs of human interleukin 1 (HIL1PE5) and human serum response factor (SRFPE5). Interestingly,

the cytotoxicity of HIL1PE5 does not differ too much from that of NLSPE5 (Feu, 2013). Otherwise, since nuclear-directed RNases increase the accumulation of other antitumor drugs such as doxorubicin inside MDR cells, we believe that the chemotherapeutic efficacy of nuclear-directed RNases would likely increase as a component of a combination therapy regime. The fact that these RNases display multiple pleiotropic effects on the treated cells favors also the possibility of establishing synergistic effects between them and other drugs. Therefore, nuclear-directed RNases are non-RI-evading RNases that exert their cytotoxic action by cleaving nuclear RNA and are promising agents for the treatment of cancer.

Conclusions

1. The primary action of PE5 on NCI/ADR-RES cells is both to increase and to decrease the expression of genes involved in numerous cellular pathways, causing pleiotropic effects on the cells.

2. In NCI/ADR-RES cells, PE5 reduces the expression of multiple genes implicated in different glucose metabolic pathways, such as glycolysis, cataplerosis, lactate production and utilization of intermediate metabolites of glycolysis for the glycogenesis, pentose phosphate pathway or serine biosynthesis. It also diminishes the expression of genes implicated in lipid metabolic pathways like fatty acid, sphingolipid and cholesterol biosynthesis, as well as β-oxidation, and genes related to biosynthesis and catabolism of diverse amino acids and protein biosynthesis.

3. In NCI/ADR-RES cells, PE5 decreases the expression of different genes involved in the quenching of ROS, including genes associated with thioredoxin and glutathione antioxidant systems and NADPH production.

4. In NCI/ADR-RES cells, PE5 decreases the expression of oncogenes and increases that of tumor suppressors implicated in cell proliferation, metastasis and apoptosis. It also reduces the expression of some MDR-associated genes.

5. Onconase causes pleiotropic effects different from those of PE5 in NCI/ADR-RES cells, and affects the expression of distinct and more limited number of genes. In contrast to PE5, the primary action of onconase on these cells is mainly to activate gene expression.

6. In NCI/ADR-RES cells, onconase increases the expression of various genes that encode transcription regulators with tumor suppressor functions implicated in cell proliferation, cell cycle progression, apoptosis, and response to stress. The effect induced by onconase in OVCAR-8 cell line is similar but less intense.

7. In NCI/ADR-RES cells, PE5 and onconase cause significant changes in miRNA expression, mostly downregulating them. For many of the affected miRNAs, we have not identified validated targets whose expression is altered in the gene expression

microarray experiments. However, the general effects of the differentially expressed miRNA and genes are predicted to be very similar.

8. Half of the residues mutated in PE5 (Glu9, Gly16 and Asn17) can be back-mutated to those of HP-RNase without decreasing the cytotoxicity of the resulting variant, named PE10. Back-mutation of all the residues that are not important for the NLS function (Ala4, Ala6, Glu9, Gly16 and Asn17) clearly reduces the cytotoxicity of the resulting variant, named PE9.

9. The introduction of an additional NLS may increase the cytotoxicity of PE5 depending on the insertion site. The most effective variant is NLSPE5, which carries the NLS of SV40 large T-antigen at its N-terminus.

10. The cytotoxicity of NLSPE5 is similar to that of onconase and 6-14 times higher than that of PE5 in a large panel of human cancer cell lines.

11. The cytotoxicity of NLSPE5 and PE10 is dependent on the cleavage of nuclear RNA and it is not provoked by the acquisition of the ability to cleave cytosolic RNA.

12. The effects caused by NLSPE5 and PE10 on cancer cells are analogous to those of PE5. Both HP-RNase variants induce the same proportion of early and late apoptotic cells than PE5 at different incubation times, generate a comparable pattern of procaspase-3, -8, and -9 activation, lead to a similar increase of doxorubicin accumulation in MDR cells, and increase two-fold the levels of $p21^{WAF1/CIP1}$ and cyclin E, but not those of cyclin D1.

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