Universitat de Girona

A HUMAN PANCREATIC RIBONUCLEASE VARIANT KILLS CANCER CELLS BY APOPTOSIS AND REDUCES THE EXPRESSION OF P-GLYCOPROTEIN IN MDR CELL LINES

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Tesi Doctoral

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Jessica Castro Gallegos

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Als meus pares

A la meva germana

A l'Edu

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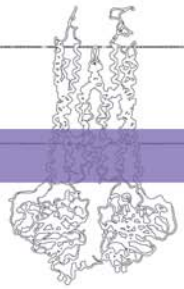

"Qualssevol que hagin estat els nostres èxits,

algú ens ha ajudat sempre a assolir-los"

Althea Gibson

Diuen que tot allò que té un inici també té un final... I això sembla. Però el final d'aquesta tesi hauria estat molt diferent sense l'ajuda i el recolzament d'un bon munt de persones que m'han acompanyat (en un moment o altre) al llarg d'aquest camí.

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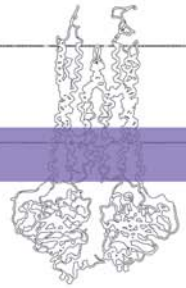
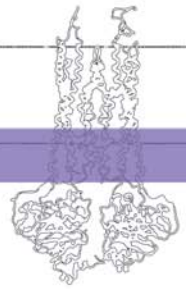
CONTENTS



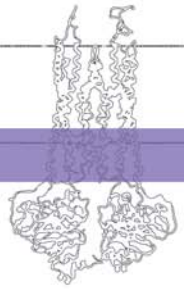
ABBREVIATIONS



ε	molar extinction coefficient
ABC transporter	ATP binding cassette transporter
Bax	bcl-2–associated X protein. Protein of the Bcl-2 family that promotes apoptosis by competing with other Bcl-2 related family members
Bcl-2	B-cell lymphoma 2 protein. Anti-apoptotic protein that promotes cell survival through protein-protein interaction with other Bcl-2 family members
BCRP	breast cancer resistance protein. ATP-dependent efflux transporter involved in multidrug resistance
BS-RNase	bovine seminal ribonuclease
CDK	cyclin-dependent kinase. Protein kinase involved in the regulation of the cell cycle which is activated by association with a cyclin, forming a cyclin-dependent kinase complex
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GST-π	glutathione S-transferase-π. Protein involved in the detoxification of both exogenous carcinogens and chemotherapeutic agents
HP-RNase	human pancreatic ribonuclease
I	interaction index
IC_{50}	half maximal inhibitory concentration. Concentration of a compound required to inhibit cell proliferation by 50%
JNK	c-Jun NH_2-terminal kinase. Protein that coordinates cell responses to stress and influences regulation of cell growth and transformation
MDR	multidrug resistance
MDR1	multidrug resistance 1 gene which encodes the multidrug transporter P-gp
MRP	multidrug resistance-associated protein. ATP-dependent efflux transporter involved in multidrug resistance
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLS	nuclear localization signal

p21$^{WAF1/CIP1}$	cyclin-dependent kinase inhibitor. Protein that binds to and inhibits the activity of cyclin-CDK2, -CDK4 or –CDK1 complexes and thus functions as a regulator of cell cycle
p27^{KIP1}	cyclin-dependent kinase inhibitor. Protein that binds to and prevents the activation of cyclin A or cyclin E-CDK2 and cyclin D-CDK4 or -CDK6 complexes and thus functions as a regulator of cell cycle
p53	tumor suppressor protein
P-gp	P-glycoprotein. ATP-dependent efflux transporter involved in multidrug resistance
PI	propidium iodide
RI	ribonuclease inhibitor
RNase	ribonuclease
XIAP	X-linked inhibitor of apoptosis. Protein that suppresses apoptotic cell death pathways by inhibiting caspases -3, -7 and -9

SUMMARY

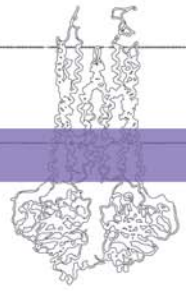


Ribonucleases are promising agents for use in anticancer therapy. In contrast to many chemotherapeutic agents, which act by interfering with DNA synthesis and cell division, cytotoxic ribonucleases are non-mutagenic agents that exert their effects by interfering with RNA functions such as protein synthesis or gene regulation and are able to kill non-dividing cells. The mechanism of cytotoxicity of PE5, a variant of human pancreatic ribonuclease carrying a nuclear localization signal, has been investigated and compared to that of onconase, a ribonuclease with antitumor activity that is currently in Phase IIIb clinical trials in patients with malignant mesothelioma. The results obtained in this study show that PE5 does not require the pro-apoptotic activity of p53 to trigger cell death. In addition, the cytotoxic effect is not prevented by a multiple drug resistance phenotype. Using different complementary techniques, it is demonstrated that the cytotoxicity of PE5 is produced through apoptosis. The data also show that *in vitro* PE5 is selective for tumor cells and that both ribonucleases induce cell death to the same extent although onconase also arrest the cell growth. The cytotoxic effects of both ribonucleases and the cytostatic effect of onconase have been compared by measuring their effects on the cell cycle, on the activation of different caspases and on the expression of different apoptosis- and cell cycle-related proteins. 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 underphosphorylation of p46 forms of JNK. At 5 μM, onconase is highly cytotoxic. While this effect is accompanied by the reduced expression of XIAP and Bcl-2, the overphosphorylation of JNK is not produced. On the other hand, cell growth arrest induced by onconase produces a prolongation of the overall cell cycle, which is accompanied by overphosphorylation of p46 forms of JNK and reduced expression of

cyclins D_1 and E. The present results show that PE5 and onconase kill the cells through mechanisms with significant differences.

In addition, PE5 cytotoxicity is synergic with doxorubicin on the doxorubicin-resistant NCI/ADR-RES cell line but not on the MCF-7 sensitive cell line. Since this result could be explained by a reduction in the level of P-glycoprotein induced by PE5, this thesis investigates whether PE5 could specifically inhibit the accumulation of P-glycoprotein in multidrug-resistant cells. The results show that PE5, but not onconase, is able to reduce the amount of P-glycoprotein in two different multidrug-resistant cell lines -NCI-H460/R and NCI/ADR-RES- while glutathione S-transferase-π is not affected. The reduction in P-glycoprotein accumulation, which has been functionally confirmed by flow cytometry analysis, may be caused by the previously reported underphosphorylation of JNK induced by PE5.

RESUM

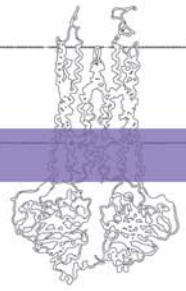


Les ribonucleases són proteïnes amb un gran potencial com a agents antitumorals. A diferència de la majoria d'agents quimioterapèutics, que actuen interferint amb la síntesi de DNA i la divisió cel·lular, les ribonucleases citotòxiques són agents no mutagènics que interfereixen amb les funcions del RNA com són la síntesi proteica o la regulació gènica i són capaces de matar cèl·lules que no estan en divisió. S'ha estudiat el mecanisme de citotoxicitat de PE5, una variant de la ribonucleasa pancreàtica humana que incorpora un senyal de localització nuclear, i s'ha comparat amb el de l'onconasa, una ribonucleasa amb activitat antitumoral que actualment es troba en fase IIIb d'assaigs clínics per al tractament del mesotelioma maligne. Els resultats obtinguts en aquest estudi mostren que la mort cel·lular induïda per PE5 és independent de l'activitat antiapoptòtica de p53. A més, el seu efecte citotòxic no es veu afectat per un fenotip de resistència a múltiples drogues. Utilitzant diferents tècniques complementàries s'ha demostrat que PE5 indueix l'apoptosi de les cèl·lules tractades. Les dades també mostren que l'activitat citotòxica de PE5 és selectiva per a cèl·lules tumorals *in vitro* i que l'efecte de PE5 i l'onconasa sobre la mort cel·lular és semblant, tot i que l'onconasa té la capacitat addicional d'aturar el creixement cel·lular. S'han comparat els efectes citotòxics de PE5 i l'onconasa i s'ha estudiat l'efecte citostàtic d'aquesta última mesurant l'acció de les dues ribonucleases sobre el cicle cel·lular, l'activació de diferents caspases i l'expressió de diferents proteïnes relacionades amb l'apoptosi i el cicle cel·lular. PE5 incrementa el nombre de cèl·lules en fase S i G_2/M del cicle cel·lular al mateix temps que augmenta l'expressió de la ciclina E i de $p21^{WAF1/CIP1}$ i disminueix la fosforilació de la isoforma p46 de JNK. A 5 µM, l'onconasa és altament citotòxica i disminueix l'expressió de XIAP i Bcl-2, mentre que no incrementa els nivells de fosforilació de JNK. D'altra banda, l'aturada de la proliferació cel·lular induïda per l'onconasa produeix una prolongació de la

durada global del cicle juntament amb un increment de la fosforilació de la p46 JNK i una disminució de l'expressió de les ciclines D_1 i E. Els resultats obtinguts mostren que PE5 i l'onconasa maten les cèl·lules a través de mecanismes diferents.

A més, la citotoxicitat de PE5 és sinèrgica amb la doxorubicina sobre la línia cel·lular resistent a doxorubicina NCI/ADR-RES però no sobre la línia MCF-7, que és sensible a doxorubicina. Ja que aquests resultats es podrien explicar per una reducció en l'expressió de la glicoproteïna-P induïda per PE5, en aquesta tesi s'ha estudiat si PE5 pot disminuir específicament l'acumulació de la glicoproteïna-P en cèl·lules resistents a múltiples drogues. Els resultats mostren que PE5, però no l'onconasa, és capaç de reduir la quantitat de glicoproteïna-P en dues línies cel·lulars diferents resistents a múltiples drogues, la NCI-H460/R i la NCI/ADR-RES, sense afectar els nivells de la proteïna glutatió S-transferasa-π. La reducció en l'acumulació de la glicoproteïna-P, que ha estat funcionalment confirmada per citometria de flux, podria ser causada per la disminució de la fosforilació de la p46 JNK descrita anteriorment.

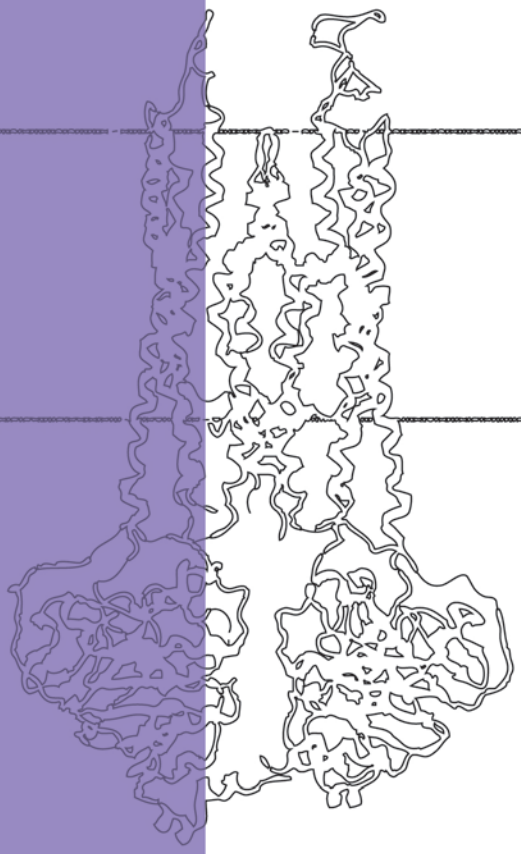
RESUMEN



Las ribonucleasas son proteínas con un gran potencial como agentes antitumorales. A diferencia de la mayoría de agentes quimioterapéuticos, que actúan interfiriendo con la síntesis de DNA y la división celular, las ribonucleasas citotóxicas son agentes no mutagénicos que interfieren con las funciones del RNA como la síntesis proteica o la regulación génica y son capaces de matar células que no están en división. Se ha estudiado el mecanismo de citotoxicidad de PE5, una variante de la ribonucleasa pancreática humana que incorpora una señal de localización nuclear, y se ha comparado con el de la onconasa, una ribonucleasa con actividad antitumoral que actualmente se encuentra en fase IIIb de ensayos clínicos para el tratamiento del mesotelioma maligno. Los resultados obtenidos en este estudio muestran que la muerte celular inducida por PE5 es independiente de la actividad antiapoptótica de p53. Además, su efecto citotóxico no se ve afectado por un fenotipo de resistencia a múltiples drogas. Mediante diferentes técnicas complementarias se ha demostrado que PE5 induce apoptosis en las células tratadas. Los datos también muestran que la actividad citotóxica de PE5 es selectiva para células tumorales *in vitro* y que el efecto de PE5 y onconasa sobre la muerte celular es similar, aunque la onconasa tiene la capacidad adicional de parar el crecimiento celular. Se han comparado los efectos citotóxicos de PE5 y de la onconasa y se ha estudiado el efecto citostático de ésta última determinando el efecto de las dos ribonucleasas sobre el ciclo celular, la activación de diferentes caspasas y la expresión de diferentes proteínas relacionadas con la apoptosis y el ciclo celular. PE5 incrementa el número de células en fase S y G_2/M del ciclo celular al mismo tiempo que aumenta la expresión de la ciclina E y de $p21^{WAF1/CIP1}$ y disminuye la fosforilación de la isoforma p46 de JNK. A 5 μM, la onconasa es altamente citotóxica y disminuye la expresión de XIAP y Bcl-2, mientras que no incrementa los niveles de fosforilación de JNK. Por otro lado, la

parada de la proliferación celular inducida por la onconasa produce una prolongación de la durada global del ciclo junto a un incremento de la fosforilación de p46 JNK y una disminución de la expresión de las ciclinas D_1 y E. Los resultados obtenidos muestran que PE5 y la onconasa matan las células a través de mecanismos diferentes.

Además, la citotoxicidad de PE5 es sinérgica con la doxorubicina sobre la línea celular resistente a doxorubicina NCI/ADR-RES pero no sobre la línea MCF-7, que es sensible a doxorubicina. Ya que estos resultados se podrían explicar por una reducción en la expresión de la glicoproteína-P inducida por PE5, en esta tesis se ha estudiado si PE5 puede disminuir específicamente la acumulación de la glicoproteína-P en células resistentes a múltiples drogas. Los resultados muestran que PE5, pero no la onconasa, es capaz de reducir la cantidad de glicoproteína-P en dos líneas celulares diferentes resistentes a múltiples drogas, la NCI-H460/R y la NCI/ADR-RES, sin afectar los niveles de la proteína glutatión S-transferasa-π. La reducción en la acumulación de la glicoproteína-P, que ha sido funcionalmente confirmada por citometría de flujo, podría ser causada por la disminución de la fosforilación de p46 JNK descrita anteriormente.



1. INTRODUCTION

Carcinogenesis, the process by which cancers are generated, is a multistep mechanism resulting from the accumulation of errors in vital regulatory circuits that govern normal cell proliferation and homeostasis. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs.

Cancer treatment, when it is localized, includes surgical removal accompanied by radiation treatment to kill residual cancer and adjuvant drug treatment. Drug treatment is the most widely used alternative therapy to surgery. Compounds used for treatment are described as being either cytotoxic or cytostatic depending, respectively, on whether they kill the cells or only stop their proliferation. Cytotoxic drugs have the potential to cure a patient whereas cytostatic ones can prevent further growth but they do not always eliminate cancer. The term "chemotherapy" is used to describe treatments based on drugs that affect a broad spectrum of cells because they affect processes such as DNA synthesis and cell proliferation common to all cells. More specific treatments like hormone therapy have been developed for major cancers, such as those of the prostate, breast and endometrium, that produces less side effects than chemotherapy. The DNA-damaging property of many chemotherapeutic agents also means they are mutagens that can actually cause cancers as well as cure them. This is an acceptable risk in comparison with the certainty of dying from the existing cancer if it is left untreated.

In recent years the use of ribonucleases (RNases) as non-mutagenic anticancer drugs has attracted the attention of many researchers.

1.1. RNases as potential antitumor drugs

Members of the pancreatic RNase superfamily display an array of biological activities ranging from cytotoxicity to angiogenesis. Among them, antitumor activity is one of the most attractive since such enzymes could be used, alone or conjugated to ligands or antibodies, as non-mutagenic therapeutic agents for cancer treatment. The molecular basis of the cytotoxicity of these RNases is not fully understood, but a

general mechanism of action is widely accepted. Cytotoxicity requires RNase interaction with the cell membrane and internalization to occur by endocytosis. Then, at a precise point, cytotoxic RNases are translocated to the cytosol where they cleave the cellular RNA, inducing apoptosis. How efficiently a particular RNase carries out each of these steps determines its potency as a cytotoxin (Benito et al., 2005; Benito et al., 2008).

1.1.1. Cytostatic and cytotoxic properties of onconase

As mentioned earlier, in contrast to most chemotherapeutic agents, which interfere with DNA synthesis and cell division, cytotoxic 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. Among the different natural or engineered RNases described to be cytotoxic, the case of onconase (P30 protein, Ranpirnase) is paradigmatic. Onconase is the smallest member (11.8 kDa) of the pancreatic RNase A superfamily, originally isolated from oocytes and early embryos of the Northern leopard frog (*Rana pipiens*). It exhibits aspermatogenic, embryotoxic and immunosuppressive activity (Matousek et al., 2003), but also selective cytotoxic and cytostatic activity against tumor cells. This activity has been demonstrated *in vitro* and *in vivo* in several tumor models (Darzynkiewicz et al., 1988; Lee et al., 2007a; Spalletti-Cernia et al., 2003). The antitumor selectivity of onconase may be due, at least in part, to the greater electronegativity displayed by the membrane surface of cancer cells compared to normal cells, thus facilitating electrostatic interactions with this protein, which is very basic. Furthermore, this negative charge is higher in membranes of cancer cells with high metastatic potential than in membranes of cells with low metastatic potential (James et al., 1956). However, the mechanism of the antitumor activity of onconase is not fully understood. There is controversy about the way onconase enters the cells. Onconase enters cells using AP-2/clathrin mediated endocytosis and is then routed, together with transferrin, to the receptor recycling compartment (Rodríguez et al., 2007). Although no cell-surface receptor for onconase

has been identified to date, these results suggest that onconase enters the cytosol by receptor-mediated endocytosis. These results are in agreement with previous work (Wu et al., 1993). However, it has also been reported that an onconase variant labeled with Oregon Green binds in a non-saturable manner to the cell surface in the HeLa cell line, indicating that onconase binds to the cell non-specifically (Haigis and Raines, 2003). Then, from the endosomes, onconase translocates to the cytosol, where it evades the cytosolic ribonuclease inhibitor (RI) and degrades RNA. RI is a potent protein inhibitor of some pancreatic-type RNases found in the cytosol of mammalian cells (Lee and Vallee, 1993). It is thought to act as a safeguard against extracellular RNases that could accidentally reach the cytosol. The inhibition occurs because some of the residues of the RNase that are important for RI binding are involved in the ribonucleolytic activity of the protein (Kobe and Deisenhofer, 1996).

Although the ribonucleolytic activity of onconase is very low compared to that of RNase A, it is essential for its cytotoxicity (Ardelt et al., 1991; Wu et al., 1993). Onconase can exhibit its ribonucleolytic activity in the cytoplasm, at least in part, because it is not inhibited by the RI and also because it has a remarkably high conformational stability (Notomista et al., 2000). Indeed, the midpoint of the thermal denaturation of this enzyme has been calculated to be around 87^{o}C. Studies have demonstrated that *in vivo* onconase degrades tRNA leaving mRNA and rRNA unaffected (Iordanov et al., 2000a; Lin et al., 1994; Saxena et al., 1996; Saxena et al., 2002). Most recently, it has also been shown that onconase is able to degrade double-stranded RNA (Saxena et al., 2009). This finding is consistent with the hypothesis that although onconase is able to arrest protein synthesis, it induces cell apoptosis through a mechanism that is independent of the inhibition of the translation (Iordanov et al., 2000a), likely targeting the RNA interference system involved in gene expression regulation via siRNA and miRNA (Ardelt et al., 2003; Ardelt et al., 2009). In fact, the silencing of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene by a specific siRNA in A549 cells is reported to be prevented by onconase (Zhao et al., 2008), indicating that siRNA is a target of onconase. Moreover, it has recently been

shown that onconase decreases miRNA-155 levels in malignant mesothelioma cells (Altomare et al., 2010), providing evidence that one of the targets of onconase is miRNA. Specifically targeting the family of miRNA may help to explain both the high selectivity of this RNase against tumor cells and the synergisms encountered with other antitumor agents that have different mechanisms of action (see below) (Ita et al., 2008). The development of many tumors has recently been associated with early alterations at the level of miRNA genes, which are located in the genome hot spots associated with cancer (Hernando, 2007; Tarasov et al., 2007; Volinia et al., 2006). Thus, onconase may kill tumor cells preferentially over normal cells by specifically cleaving these miRNAs. It has also been reported that onconase enhances the cytotoxicity of several chemotherapeutic agents with diverse mechanisms of action such as vincristine (Rybak et al., 1996), tamoxifen (Lee et al., 2003), cisplatin (Mikulski et al., 1992), lovastatin (Mikulski et al., 1992), tumor necrosis factor α (Deptala et al., 1998), interferons (Tang et al., 2005; Tsai et al., 2002) and ionizing radiation (Lee et al., 2007b). Targeting miRNA may also explain the synergisms of onconase with these chemotherapeutics since some families of miRNAs have been shown to regulate cell defenses against biological stress and protect against apoptosis (Ardelt et al., 2008). In addition, researchers have also postulated that the ability of onconase to sensitize cells to other antitumor agents could be mediated by targeting the transcription factor NF-kB. The reduced expression of NF-kB could suppress translation of the "survival genes" that inhibit apoptosis and cause the resistance of tumor cells to treatment (Deptala et al., 1998; Tsai et al., 2004; Wang et al., 1996).

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 because many cancer cells lack p53 or express a defective p53 protein that reduces or eliminates the apoptotic response induced by several chemotherapeutic drugs. Onconase activates the caspase cascade in the HeLa cell line (Iordanov et al., 2000a), although in some cases onconase leads to a caspase-independent apoptosis with features similar to autophagy (Michaelis et al., 2007) or cell senescence (Juan et al., 1998). The processing

of procaspases-9, -3, and -7 but not of procaspase-8 has been described in the HeLa cell line (Iordanov et al., 2000a). However, it is not clear to what extent the mitochondrial apoptotic pathway is involved: whereas no cytochrome c release or sustained decrease of Bax 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). Indeed, for the HL-60 human promyelocytic leukemia cell line, onconase induces the expression of Bax while decreasing that of Bcl-2 (Ardelt et al., 2007b).

The effect of onconase on the cell cycle has also been shown to be 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; Deptala et al., 1998; Halicka et al., 2000; Juan et al., 1998); but there are exceptions. For example, onconase arrests NIH/3T3 cells in the G_2/M cell cycle phase (Smith et al., 1999), but for Jurkat cells the arrest of proliferation induced by onconase could not be attributed to alterations in the cell cycle phase progression (Tsai et al., 2004). The effect of onconase on the expression of different key regulators of the cell cycle has been also investigated. The treatment of lymphoma U-937 cells by onconase decreases cyclin D_3 protein levels, increases $p27^{KIP1}$, $p16^{INK4A}$ and $p21^{WAF1/CIP1}$ levels and decreases the amount of the phosphorylated form of retinoblastoma protein (Juan et al., 1998). The increased expression of these cyclin inhibitors by onconase is another indication that its cytotoxic effect is not merely due to a general arrest of protein translation.

Although onconase shows renal toxicity at high concentrations (Vasandani et al., 1999), different clinical studies have demonstrated that it is effective as an anticancer drug in the treatment of several human cancers. At present, it is in Phase IIIb clinical trials for the treatment of malignant mesothelioma. The combined effect of onconase and doxorubicin has been compared to that of doxorubicin alone in patients with this disease. Although final results of these clinical trials are not yet available, preliminary results suggest that such a combination could be more effective than doxorubicin

alone (Porta et al., 2008). In these clinical trials, onconase seems to act as a cytostatic agent rather than a cytotoxic drug, and increase patient survival (Porta et al., 2008). In addition, onconase is currently in a Phase I/II clinical trial for the treatment of non-small cell lung carcinoma and other solid tumors. Moreover, onconase has shown good efficacy against neuroblastoma, rhabdomyosarcoma and chemotherapy-resistant variants of tumors in several preclinical studies.

1.1.2. Other natural cytotoxic RNases

Other natural cytotoxic RNases have been described as selective for cancer cells. Bovine seminal ribonuclease (BS-RNase) is a dimeric protein from bovine sperm with aspermatogenic, embryotoxic and immunosuppressive activity, as well as antitumor activity *in vitro* and *in vivo* (Laccetti et al., 1992; Laccetti et al., 1994; Vescia et al., 1980). This enzyme is endocytosed in malignant and non-malignant cells to the same extent, but it is much more toxic for cancer cells (Mastronicola et al., 1995; Vescia et al., 1980). 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, its ribonucleolytic activity is essential for this cytotoxicity and, therefore, for the anticancer activity of this enzyme (Kim et al., 1995). BS-RNase is a dimeric protein in which two identical subunits are held together by covalent and non-covalent interactions. Two dimeric forms coexist in nature, one with an N-terminal domain swapping between the monomers. The covalent dimeric form of BS-RNase is unique among the members of the RNase A superfamily and prevents RI binding by steric hindrance (Murthy et al., 1996). BS-RNase degrades intracellular rRNA, which leads to inhibition of protein synthesis and, finally, to cytotoxicity (Mastronicola et al., 1995). The dimeric structure of the BS-RNase is maintained during its internalization (Bracale et al., 2003) and seems to be required to cross the lipid bilayer (Mancheno et al., 1994). However, it is still not clear whether the dimer is maintained in the cytosol. Cells in which the RI has been silenced have been shown to be more sensitive to BS-RNase action (Monti and D'Alessio, 2004), indicating

that the RI inhibits BS-RNase to some extent. In fact, under the reducing conditions of the cytosol part of the BS-RNase, molecules could be dissociated and consequently BS-RNase could be partially inhibited. This effect may explain why BS-RNase is 10-30-fold less cytotoxic than onconase (Matousek et al., 2003; Mosimann et al., 1994). Nevertheless, this enzyme is more immunogenic than onconase (Matousek et al., 2003).

Other amphibian RNases show selective cytotoxic activity for tumor cells (Liao et al., 1996; Nitta et al., 1994a). Some of these RNases were isolated from *Rana catesbeiana* and *Rana japonica* eggs and show lectin activity towards cells with sialic acid- rich glycoproteins (Nitta et al., 1987; Sakakibara et al., 1979). Like onconase, the lectin from *Rana catesbeiana* evades the RI (Nitta et al., 1993) and its ribonucleolytic activity is essential for its cytotoxicity (Huang et al., 1998). However, prolonged exposure of cancer cells to this enzyme reportedly contributes to the development of receptor-based resistance (Nitta et al., 1994b).

Rana pipiens oocytes also contain four variants of another RNase, called amphinase (Singh et al., 2007). This enzyme is more basic than onconase and the largest among amphibian RNases (Singh et al., 2007). It is cytotoxic and cytostatic against tumor cells (Ardelt et al., 2007a; Singh et al., 2007) and ribonucleolytic activity is essential for its anticancer effects (Singh et al., 2007). Amphinase induces cell cycle arrest in the G_1 phase and apoptosis, which seems to involve activation of endonucleases, caspases, serine proteases and transglutaminases (Ardelt et al., 2007a). Like onconase, it does not interact with the RI (Singh et al., 2007), one of the key factors involved in its cytotoxicity. Although amphinase is considerably less active than onconase (Singh et al., 2007), its cytotoxic effect is similar to that of onconase (Ardelt et al., 2007a; Singh et al., 2007).

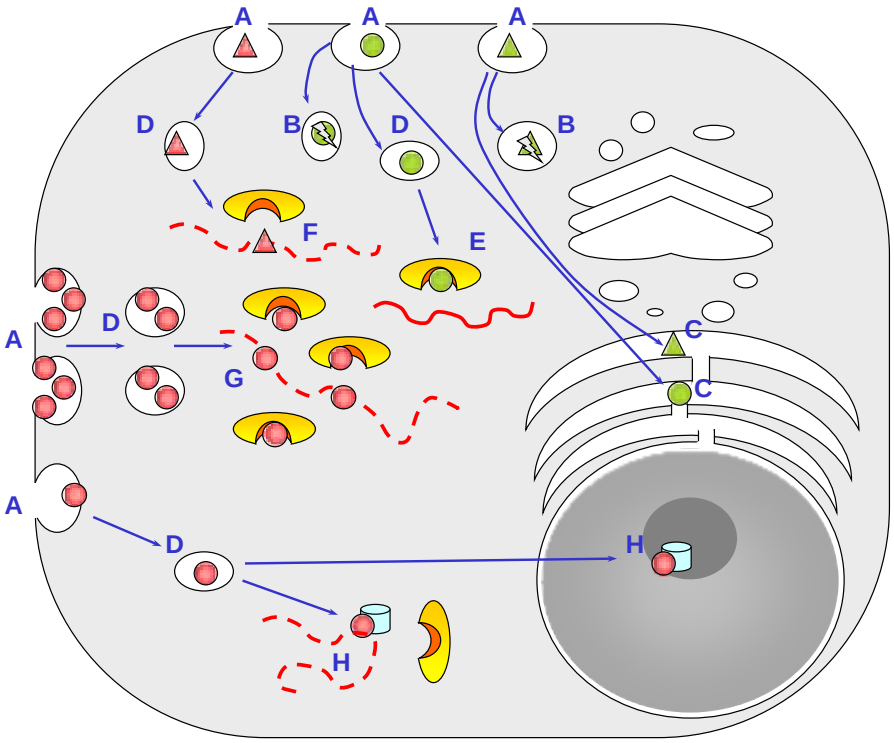


Figure 1.1. Different productive and non-productive intracellular pathways of an RNase. Cytotoxic RNases are depicted in red and non-cytotoxic RNases are depicted in green. RNases inhibited *in vitro* by RI are depicted as spheres and RNases that are non-inhibited *in vitro* by RI are depicted as triangles. RNA is drawn as a red curved line and degraded RNA as a discontinuous red curved line. The RNases are endocytosed (A), the amount of endocytosed molecules can determine the cytotoxicity of the RNase. Once internalised, the protein can: be degraded by the cellular proteases (B); follow a non-productive route that does not lead to the cytosol (C); or be conducted to an organelle from which the RNase can reach the cytosol (D). In the figure, the vesicles from which RNases reach the cytosol are a simplification and can mean the endocytic system, the trans-Golgi network, or even the Golgi apparatus. Once in the cytosol, some RNases are captured (E) by the RI (depicted in yellow), but cytotoxic RNases can follow three different mechanisms to avoid the inhibition: the inability to bind to the RI (F), the titration of the RI due to the high amount of RNase molecules that reach the cytosol (G), or interaction with other molecules (depicted in blue) that hampers the RNase–RI interaction (H). Extracted from (Benito et al., 2005).

1.1.3. Engineered cytotoxic RNases

Knowledge of the molecular basis of the cytotoxicity of natural or engineered RNases (Figure 1.1) led to the development of new RNase variants with cytotoxic activity as an alternative to conventional DNA-damaging cancer therapy. The human pancreatic RNase (HP-RNase) does not show renal accumulation *in vivo* (Vasandani et al., 1999; Vasandani et al., 1996) and shows a 10^3-10^4-fold increase in RNase activity compared to onconase (Leland et al., 2001). Thus, generating a cytotoxic variant of the HP-RNase would undoubtedly provide a potentially useful therapeutic agent that would be expected to have lower immunogenicity and renal toxicity than onconase. This explains why many of the cytotoxic variants described have been constructed onto the HP-RNase scaffold (for a review see (Rybak and Newton, 1999)). As discussed earlier, the ability of onconase and BS-RNase to evade the RI is one of the features that endow these drugs with cytotoxic properties. While other members of the RNase A superfamily are inhibited by the RI, onconase and BS-RNase are distinguished by their low affinity for the RI. Thus, the construction of RNase variants designed to evade the RI is one of the strategies that has been developed to endow them with cytotoxic activity. This effect has been achieved either by using site-directed mutagenesis to introduce selected changes and create steric or electrostatic incompatibilities in the interface of the RI-RNase complex or, alternatively, by inserting those residues necessary to generate a variant able to adopt a dimeric structure spontaneously and in this way allow the multimerization of the enzyme (for a review, see (Rutkoski and Raines, 2008)). On the other hand, more cationic RNases have been shown to be more efficiently delivered into cells, making cationization another strategy to enhance RNase cytotoxicity. This cationization may be achieved by either site-directed mutagenesis (Ilinskaya et al., 2002; Ilinskaya et al., 2004; Johnson et al., 2007) or chemical modification (Futami et al., 2001). As mentioned above, efficient interaction with the cell membrane ensures a more efficient internalization of the RNase. Provided that enough RNase reaches the cytosol, all the free RI could be depleted, enabling the new molecules reaching the cytosol to degrade the RNA even if

they could be inhibited *in vitro* by the RI. Many RNase variants have been described as chemically or genetically linked to tumor-associated ligands that ensure an efficient and specific internalization. Transferrin, growth factors and antibodies are among the ligands used (for a review, see (Rybak and Newton, 1999)).

We had previously constructed a cytotoxic variant of HP-RNase, called PE5, that arrests the protein translation of a panel of diverse cell lines despite being sensitive to the RI (Bosch et al., 2004). This variant carries a non-contiguous extended bipartite nuclear localization signal (NLS) constituted by at least three different regions of the protein comprising Lys1 and the Arg clusters 31–33 and 89–91 (Rodríguez et al., 2006). Although these residues are distributed over 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). This NLS binds to α-importin (Rodríguez et al., 2006), which drives the protein to the nucleus, specifically to the nucleolus, in an energy-dependent and Ran-dependent manner (Bosch et al., 2004). In the nucleus, the RI is absent and PE5 cleaves nuclear RNA but leaves cytoplasmic RNA unaffected (Tubert et al., 2010).

1.2. Multidrug Resistance (MDR)

Multidrug resistance (MDR) is defined as the resistance of tumor cells to the cytostatic or cytotoxic effects of multiple and dissimilar drugs used in cancer chemotherapy. It remains a major obstacle to the successful treatment of cancer. Sometimes, resistance to chemotherapy occurs as a result of a tumor's intrinsic drug resistance to chemotherapeutic agents, but most tumors may initially be sensitive to treatment and acquire drug resistance after chemotherapy begins (Figure 1.2). Tumors not only resist the cytotoxic drug used for the treatment, but also a whole range of drugs with different structures and cellular targets. Once MDR appears, the use of drugs in high doses to overcome resistance is ineffective: toxic effects appear and resistance is further stimulated. The overexpression of plasma membrane ATP binding cassette

(ABC) transporter proteins, in particular the P-glycoprotein (P-gp; MDR1), the multidrug resistance-associated protein (MRP) and the breast cancer resistance protein (BCRP), which efflux anticancer drugs from cells, has been shown to play a critical role in the development of the MDR phenotype (Stavrovskaya and Stromskaya, 2008). In addition, the overexpression of other genes such as glutathione S-transferase-π (GST-π) may contribute to the development of tumor cell resistance to chemotherapeutics (Awasthi et al., 1994; Lewis et al., 1988; Schisselbauer et al., 1992). Although MDR extrusion pumps have been known for decades, inhibitors of ABC transporters show limitations associated with cytotoxic effects and adverse pharmacokinetic drug interactions and clinical trials have been disappointing.

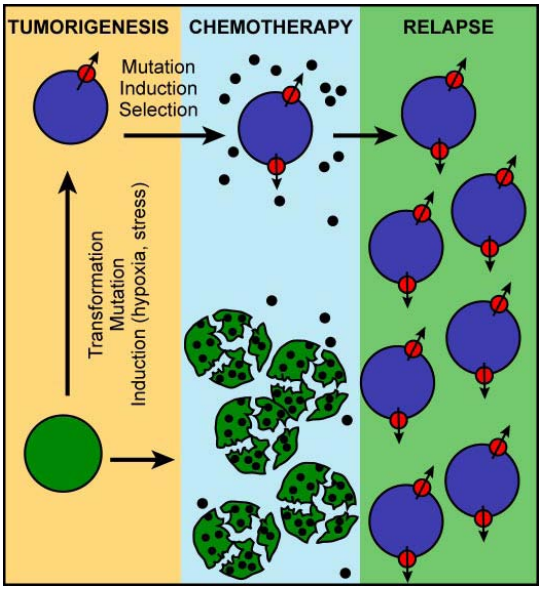


Figure 1.2. Evolution of MDR1/P-gp positive cancer. Tumor cells arise by a complex mutation and induction pathway. Cells that do not express multidrug transporters are sensitive to chemotherapy and are eliminated. In the course of chemotherapy, further mutations and selection may greatly increase the expression of multidrug transporters, which protect the tumor cells against chemotherapy. Extracted from (Sarkadi et al., 2006).

1.2.1. ABC transporters implicated in MDR

P-gp was the first identified ABC transporter (Juliano and Ling, 1976) and in humans it is one of the most clinically relevant proteins of the ABC transporter superfamily. It is a 170 kDa cell surface glycoprotein encoded by the ABCB1/MDR1 gene (Kartner et al., 1983). Because this transporter is expressed in a variety of normal tissues, tumors

derived from these tissues display an intrinsic MDR phenotype (Goldstein et al., 1989). One of the most interesting characteristics of P-gp is that it can expel a structurally broad spectrum of antitumor drugs. It is believed that these agents cross the cell membrane by passive diffusion and are eliminated by P-gp against a concentration gradient that requires energy supplied by ATP (Kuo, 2009). This transporter seems to be very important in preventing an accumulation of xenobiotics from entering the cell and facilitates the elimination of toxins from the body. Nevertheless, it has also been reported that P-gp plays a general anti-apoptotic role that extends beyond resistance to chemotherapeutics. This is because tumor cells overexpressing P-gp are resistant to a wide range of caspase-dependent apoptosis inducers, including tissue necrosis factor, Fas ligand, serum starvation and UV irradiation (Johnstone et al., 1999; Ruth and Roninson, 2000; Smyth et al., 1998).

Other members of the ABC superfamily have also been implicated in cancer MDR, including the multidrug resistance-associated protein-1 (MRP1) or ABCC1 and its homolog MRP2-6, which differ in substrate specificity and tissue distribution. The BCRP protein, also called the mitoxantrone-resistance protein or ABCG2, is another ABC transporter implicated in MDR. It is a 72 kDa plasma membrane glycoprotein expressed in many normal tissues that transports a broad spectrum of antitumor drugs through a process energized by ATP hydrolysis. Both the transport properties and the tissue distribution of BCRP seem to indicate a protective role against xenobiotics.

1.2.2. Other MDR mechanisms

Additional mechanisms may contribute to the MDR. They are sometimes linked to increases in drug detoxification caused by elevated intracellular glutathione concentrations or overexpression of enzymes such as GST-π. This enzyme is involved in the detoxification of both exogenous carcinogens and chemotherapeutic agents, and also seems to be involved in the regulation of cell proliferation by the suppression of

cellular reactive oxygen species (ROS) in cell division (McCaughan et al., 1994). Changes in membrane permeability or drug effectiveness linked to alterations in molecules involved in DNA repair, such as *O6*-methylguanine DNA methyl transferase or topoisomerase II, are also associated with MDR (el-Deiry, 1997). In addition, changes in the balance of proteins that control apoptosis, such as Bcl-2, can also reduce chemosensitivity since most chemotherapeutics exert their cytotoxic effects by inducing apoptosis (el-Deiry, 1997).

1.2.3. Approaches to MDR therapy

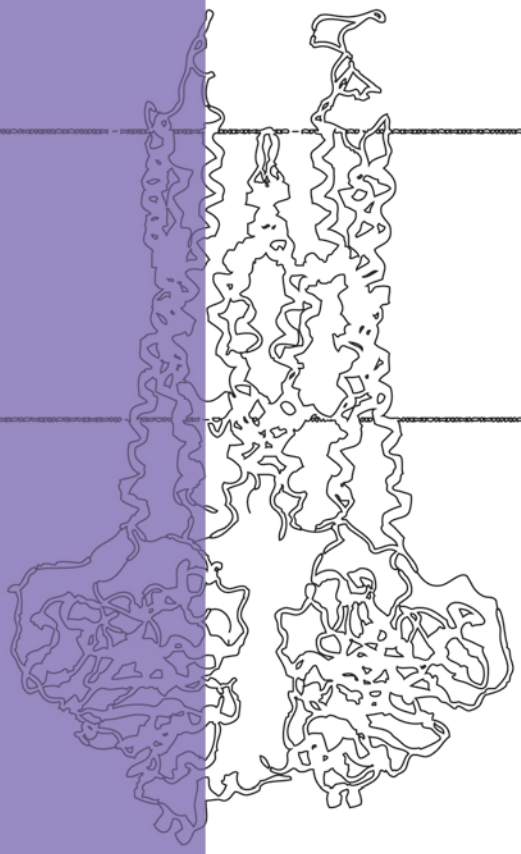
1.2.3.1. MDR inhibitors

In the search for novel approaches to overcome MDR and to develop more effective therapies, researchers have found that different pharmacological agents interfere with ABC proteins function. P-gp reversal agents act as competitive inhibitors, competing with antitumor drugs for the active site of the enzyme. These clinically assayed inhibitors include the calcium channel blocker verapamil, the immunosuppressant cyclosporine A and its analog valspodar (PSC 833) and, more recently, tariquidar (XR9576), laniquidar (R101933) or elacridar (GF-120918). In each case, clinical trials were unsuccessful due to problems with toxicity or pharmacokinetic interaction with the chemotherapeutic agents (Friedenberg et al., 2006; Greenberg et al., 2004; Szakacs et al., 2006; Thomas and Coley, 2003).

Many MRP1 and BCRP inhibitors have been identified, but clinical trials with them are still in the beginning stages. For example, a mycotoxin, fumitremorgin C, was shown to be a specific and high-affinity inhibitor of BCRP that reverses the MDR phenotype *in vitro* (Rabindran et al., 1998; Rabindran et al., 2000), but it shows an important neurotoxic effect when used *in vivo*. Nevertheless, the failure of P-gp inhibitors until now presents an unclear future for the MRP1 and BCRP inhibitors.

1.2.3.2. Alternative MDR therapies

In addition to these attempts with inhibitors of MDR transporters, other strategies have been developed to overcome MDR. These strategies are based on RNA technology (inhibiting the function of drug transporters by interfering with mRNAs using antisense oligodeoxyribonucleotides, ribozymes or RNA interference) (Crooke, 1992; Li et al., 2006; Masuda et al., 1998; Nagata et al., 2002; Stierle et al., 2005), the use of monoclonal antibodies directed against MDR transporters (Mickisch et al., 1992; Pearson et al., 1991; Tsuruo et al., 1989) and the development of new agents that are poor substrates for MDR transporters such as the anthracyclines idarubicin and annamycin (Lehne et al., 1996). However, despite promising preliminary results, most of these strategies are still being translated to the clinic.



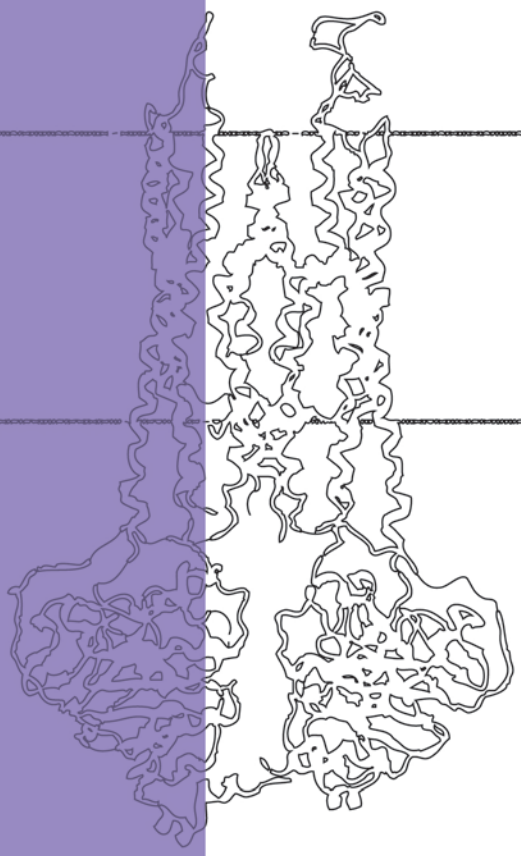
2. OBJECTIVES

Efforts to improve cancer therapy are aimed at designing new classes of antitumor agents with improved efficacy against tumor cells, but without compromising the function of normal cells and able to overcome tumor resistance. As mentioned in the introduction, RNases are especially attractive because of their selective toxicity for cancer cells. In contrast to many chemotherapeutic drugs currently used for cancer treatment that affect DNA metabolism, RNases are alternative non-mutagenic candidates for cancer therapy. Onconase is the only RNase undergoing clinical trials, but despite currently being in phase III of human clinical trials for the treatment of malignant mesothelioma, its use is limited due to its renal toxicity when administered at high concentrations. Thus, in recent years, many researchers have designed new cytotoxic human RNase variants to obtain lower renal toxicity and more immunological tolerance in order to overcome the limitations shown by onconase. Accordingly, we have constructed a cytotoxic variant of HP-RNase, called PE5, that carries a conformational bipartite NLS responsible for its import to the nucleus, where it degrades nuclear RNA.

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

1. To characterize the cytotoxic effects of PE5 and compare it with onconase.
2. To compare the specificity of PE5 and onconase towards cancer cells *in vitro.*
3. To analyze the effect of PE5 on the cell cycle phase distribution and to investigate its effect on the expression of different key regulators of the cell cycle.
4. To study the mechanism of cell death induced by PE5 and to identify the pathways involved in this type of cell death through the study of changes in the level of different proteins that control it.
5. To analyze the possible interaction between PE5 and doxorubicin on doxorubicin- sensitive and –resistant cell lines.

6. To investigate the effects of PE5 on the expression of proteins that may contribute to the development of resistance of tumor cells to chemotherapeutics such as P-gp and GST-π.



3. METHODS

3.1. RNase variants

Construction of plasmids pONC and pE5 expressing onconase and PE5, respectively, has been previously described (Bosch et al., 2004; Leland et al., 1998). Both plasmids are derivative of pET22b(+) and pET17b, respectively. Construction of PE5 and PM5 is shown in Figure 3.1. PE5 was constructed from PM5 (an HP-RNase variant carrying five substitutions at the N-terminus: Arg4Ala, Lys6Ala, Gln9Glu, Asp16Gly and Ser17Asn (Canals et al., 1999) replacing Gly89 and Ser90 by Arg (Bosch et al., 2004) and carries a conformational bipartite NLS (Rodríguez et al., 2006).

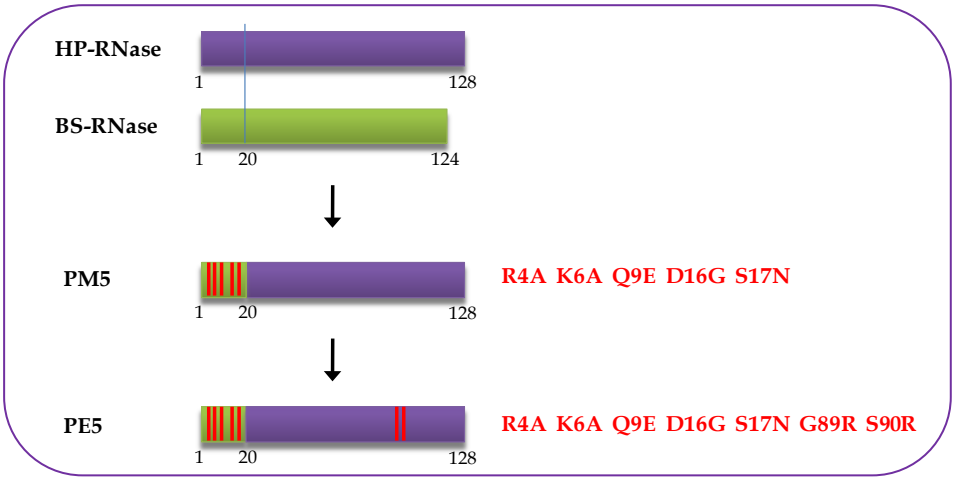


Figure 3.1. Localization of the substitutions within the sequence of PM5 and PE5 HP-RNase variants. Substituted amino acids which differ from the HP-RNase sequence are shown in red. PM5 incorporates the N-terminal sequence of BS-RNase (residues 1 to 20) which differs in 5 positions to the HP-RNase. PE5 replaces residues 89 and 90 of PM5 by Arg.

3.2. RNase expression and purification

Recombinant onconase and PE5 were produced and purified from *E. coli* BL21 (DE3) cells transformed with the corresponding vector essentially as described previously (Ribó et al., 2001; Ribó et al., 2004). Briefly, cells containing the pE5 plasmid were

grown in Luria-Bertani medium (LB) supplemented with 400 µg/ml ampicillin until an OD_{550} near 1.5 was reached. Cells containing the pONC plasmid were grown in Terrific Broth (TB) supplemented with 400 µg/ml ampicillin until an OD_{600} near 2 was reached. Protein expression was induced by addition of isopropyl thiogalactoside to 1 mM. After 3-4 h, cells were harvested by centrifugation. Pellets from 2 L of induced culture were resuspended in 30 ml of 50 mM Tris–HCl, (pH 8.0), 10 mM EDTA. Cells were lysed using a French press and inclusion bodies were harvested by centrifugation. Pellets were then resuspended in 10 ml of 6 M guanidinium-HCl, 10 mM EDTA, 50 mM Tris–acetate, (pH 8.0). 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 and solubilized protein was diluted dropwise into 0.5 M L-arginine, 1 mM oxidized glutathione, 2 mM EDTA, 0.1 M Tris–acetate, (pH 8.5), and incubated at 4°C for at least 24 h. To stop oxidation, the pH was then lowered to 5 with acetic acid and the proteins were concentrated by ultrafiltration using a Prep/Scale TFF cartridge (Millipore, Bedford, MA, 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 (Boix et al., 1996; Mosimann et al., 1994; Notomista et al., 2001). This is accomplished at this step by dialysis against 200 mM sodium phosphate (pH 7.2) at room temperature for a period of 48 h (Ribó et al., 2004). Both onconase and HP-RNase variants were afterwards dialyzed against 50 mM sodium acetate (pH 5), centrifuged to remove insoluble material and loaded onto a Mono-S HR 5/5 FPLC column (Amersham Biosciences, Piscataway, NJ, USA). Fractions containing pure RNases were dialyzed against 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 at 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 UV spectroscopy using an extinction coefficient of ε_{278}=7950 M^{-1} cm^{-1} for PE5 and of ε_{278}=10470 M^{-1} cm^{-1} for onconase, calculated using the method devised by Pace et al. (Pace et al., 1995).

3.3. Cell lines and culture conditions

Human ovarian cancer cell line NCI/ADR-RES (formerly known as MCF-7/Adr) (Liscovitch and Ravid, 2007) and MCF-7 cell line transfected with HER2 (MCF-7/HER2) (Menéndez et al., 2007) were a generous gift from *Institut Català d'Oncologia de Girona, Hospital Universitari de Girona Dr. Josep Trueta* (Girona, Spain). Normal human fibroblasts N1, cervical cancer HeLa cell line (Gey et al., 1952) and human breast cancer cell lines MCF-7 (Soule et al., 1973), SK-BR-3 (Fogh, 1975) and MDA-MB-231 (Cailleau et al., 1974) were obtained from *Eucellbank* (*Universitat de Barcelona*, Barcelona, Spain). Human lung cancer line NCI-H460/R (Pesic et al., 2006) was a generous gift from Dr. Sabera Ruzdijic, *Institute for Biological Research "S. Stankovic"* (Belgrade, Servia). NCI/ADR-RES, HeLa, MCF-7, MCF-7/HER2, MDA-MB-231 and N1 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, Berlin, Germany), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco, Berlin, Germany). For NCI/ADR-RES cells complete DMEM medium contained also 1 µg/mL (1.84 µM) doxorubicin (Tedec-Meijic Farma, Madrid, Spain) to ensure that high levels of P-gp expression were maintained. SK-BR-3 cells were grown on McCoy's medium (Gibco, Berlin, Germany) supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin. NCI-H460/R cells were grown on RPMI medium (Gibco, Berlin, Germany) supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin and also 100 nM doxorubicin. Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2, remained free of *Mycoplasma* and were propagated in adherent culture according to established protocols.

3.4. Cell proliferation assay

Cells were seeded into 96-well plates at the appropriate density: 7000 for NCI/ADR-RES and MCF-7, 6000 for SK-BR-3, 4500 for MCF-7/HER2, 4000 for MDA-MB-231, 3000 for NCI-H460/R and N1, and 2200 for HeLa. After 24 h incubation, cells were treated for 72 h with various concentrations of PE5 (0.1–30 μM), onconase (0.001–10 μM), doxorubicin (0.5-128 μM for NCI/ADR-RES or 0.002-0.6 μM for MCF-7) or combinations of these compounds as specified. Drug sensitivity was determined using an assay that monitors the reduction of Thiazolyl Blue Tetrazolium Blue (MTT) (Sigma, St. Louis, MO, USA) to formazan essentially as described by the manufacturer's instructions. Data were collected by reading at 570 nm in a multi-well plate reader (Anthos Labtec, Cambridge, UK). The IC_{30}, IC_{40} and IC_{50} values represent the concentrations of the assayed enzymes required to inhibit cell proliferation by 30, 40 and 50%, respectively, and were calculated by extrapolation from the obtained growth curves. The results for a single experiment were the average of three determinations and the experiments were repeated at least two times.

3.5. Analysis of cytostatic and cytotoxic effects of PE5 and onconase

NCI/ADR-RES cells (55000 per well) were seeded into 24-well plates and then treated with different concentrations of PE5 (2-35 μM) or onconase (0.3-5 μM) for 24, 48 and 72 h. After treatment, attached and floating cells were harvested at 600 xg and washed in cold phosphate buffered saline (PBS). The proliferation rate and viability in control and RNase-treated cultures was estimated by cell count, using a hemocytometer combined with the trypan blue exclusion assay based on the dye exclusion by the viable cells.

3.6. Cell cycle phase analysis

Cell cycle phase analysis was performed by propidium iodide (PI) staining. NCI/ADR-RES cells (1.2×10^6 cells/100-mm dish) were treated with 35 μM PE5 or 1

and 5 μM onconase for 72 h. Attached and floating cells were then harvested at 1500 xg and fixed with 70% ethanol for at least 1 h at -20°C. Fixed cells were harvested by centrifugation and washed in cold PBS. These collected cells were resuspended in PBS (1-2 x 10^6 cells/ml) and treated with RNase A (100 μg/ml) and PI (40 μg/ml) (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min prior to flow cytometric analysis. A minimum of 10,000 cells within the gated region were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle distribution was analyzed using the FlowJo program (FreeStar, Ashland, OR, USA).

3.7. Apoptotic morphology analysis

Experiments were carried out in serum-starved medium, at 37°C and in a 5% CO_2 atmosphere. NCI/ADR-RES cells (2.2 x 10^3 per well) were treated with 35 μM PE5 or 5 μM onconase for 72 h followed by the addition of 4 μM calcein AM (Molecular Probes, Eugene, OR, USA) and 20 μg/ml PI (Molecular Probes, Eugene, OR, USA). The effect of PE5 and onconase was analyzed using a laser scanning confocal microscope (Leica, Wetzlar, Germany). Images of cells stained with calcein AM and PI were acquired using a 488-nm argon laser and a 561-nm HeNe laser, respectively.

NCI/ADR-RES cells (2.2 x 10^4 per well) were also seeded in 35-cm^2 plates with a glass coverslip and treated with 35 μM PE5 or 5 μM onconase. After 72 h cells were incubated with 0.5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C in the dark. Confocal images of the cultures were captured using a laser scanning confocal microscope and a 405-nm blue diode laser.

3.8. Phosphatidylserine exposure assay

Quantitative analysis of apoptotic cell death caused by PE5 and onconase treatment was performed by flow cytometry using the Alexa Fluor 488 annexin V/PI Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OR, 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 35 μM PE5 or 5 μM onconase for 24, 48 and 72 h in serum-starved medium. After treatment, attached and floating cells were harvested, washed 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 using CellQuest Pro software. A minimum of 10,000 cells within the gated region were analyzed.

3.9. Caspase activation assay

Caspase-3, -8 and -9 catalytic activities were measured using the APOPCYTO Caspase-3, -8 and -9 colorimetric assay kits (MBL, Nagoya, Japan) following the manufacturer's protocol. 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 cells (1.2×10^6 cells/100-mm dish) were incubated with 35 μM PE5 or 5 μM onconase for 24, 48 and 72 h in serum-starved medium. Then, attached and floating cells were lysed and centrifuged. The supernatant was recovered and the protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Afterwards, 50 μl of the cell lysate corresponding to 110 μg of total protein, 50 μl of 2X reaction buffer containing 10 mM DTT and 5 μl of the 10 mM DEVD-*p*NA, IETD-*p*NA or LEHD-*p*NA substrates were added to each well of the 96-well plates. To confirm the specific hydrolysis of each substrate, samples were treated with the caspase inhibitors DEVD-FMK, IETD-FMK or LEHD-FMK, which are specific inhibitors of caspases-3, -8 and -9, respectively, following the manufacturer's protocol. Then the plate was incubated at 37°C for 4 h. The reaction was measured by changes in absorbance at 405 nm. Results are expressed as percentage of control values without RNase addition.

3.10. Western blot analysis

NCI/ADR-RES and NCI-H460/R cells were lysed in lysis buffer (Cell Signalling Technology, Beverly, MD, USA) and a sample was taken for protein concentration

determination using the Bradford protein assay according to the manufacturer instructions. Protein samples were separated on a 12.5% SDS-polyacrylamide gel (immunoblot analysis of Bcl-2, Bax, cyclin D_1, cyclin E, p21, p27, JNK, p-JNK, XIAP, GST-π and GAPDH) or 4–12% SDS-polyacrylamide gel (immunoblot analysis of P-gp and β-actin) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h at room temperature in blocking buffer [3% powdered-skim milk in 10 mM Tris–HCl (pH 7.5), 100 mM NaCl and 0.1% Tween-20] to prevent non-specific antibody binding. Antibody dilution was prepared in blocking buffer and blots were incubated with the corresponding monoclonal antibody overnight at 4°C. Primary antibodies against Bcl-2 (clone C-2; 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (clone B-9; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D_1 (clone DCS-6; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin E (clone HE12; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (clone F-5; 1: 50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p27 (clone F-8; 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), JNK (clone D-2; 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-JNK (clone G-7; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), XIAP (clone 28; 1:1000 dilution; BD Transduction Laboratories, San Jose, CA, USA), P-gp (clone C219; 1:500 dilution for NCI-H460/RES cells or 1:2000 for NCI/ADR-RES cells; Calbiochem, La Jolla, CA, USA), GST-π (clone 3; 1:80000 dilution for NCI-H460/RES cells or 1:400000 for NCI/ADR-RES cells; BD Transduction Laboratories, BD Biosciences, San Jose, CA), β-actin (clone C-2; 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (clone 6C5; 1:12000000 dilution; Chemicon/Millipore, Billerica, MA, USA) were used. Afterwards, membranes were incubated for 1 h at room temperature with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:30000 dilution; Calbiochem, La Jolla, CA, USA). Blots were developed with Immobilon Chemiluminiscent horseradish peroxidase substrate (Millipore, Bedford, MA, USA) (Bcl-2, Bax, cyclin D_1, cyclin E, p21, p27, JNK, p-JNK, XIAP, GST-π

and GAPDH) and images were captured by a FluorChem SP system (Alpha Innotech, San Leandro, CA, USA) or were revealed employing the commercial kit SuperSignal West Pico Chemiluminescent Substrate from Pierce/Thermo Scientific (Rockford, IL, USA) (P-gp and β-actin).

Quantitative analysis is based on the intensity of the band using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The linearity of the assay was preliminarily checked for each monoclonal antibody by submitting different amounts of untreated cell extracts to Western blotting.

3.11. Determination of synergism and antagonism: Isobologram analysis

The nature of the interaction between doxorubicin and the RNases was evaluated by the isobologram technique, a dose-oriented geometric method of assessing drug interactions that requires the determination of a given biological effect when the concentration ratio of two agents varies (Berenbaum, 1989; Chou and Talalay, 1984). In the isobologram method the concentration of one agent producing a desired (*e.g.*, 30% inhibitory) effect is plotted on the horizontal axis, and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a straight line joining these 2 points represents zero interaction (additivity) between two agents. The experimental isoeffect points are the concentrations (expressed relative to the IC_{30} value) of the two agents which when combined decreased cell viability by 30%. When the experimental isoeffect points fall below that line, the combination effect of the two drugs is considered to be supra-additive or synergistic whereas antagonism occurs when the points lie above it. A quantitative index of these interactions is provided by the isobologram equation

$$I = (a/A) + (b/B)$$

where I represents an index of drug interaction (Interaction Index), A and B represent the respective concentrations of doxorubicin and the assayed RNase required to produce a fixed level of inhibition (IC_{30}, IC_{40} or IC_{50}) when administered alone, and a

and *b* represent the concentrations required for the same effect when doxorubicin and the assayed RNase were administered in simultaneous combination. *I* values of < 1 indicate synergy, a value of 1 represents additivity, and values of > 1 indicate antagonism. Each experiment was carried out with triplicate cultures for each data and was repeated independently at least three times with similar results.

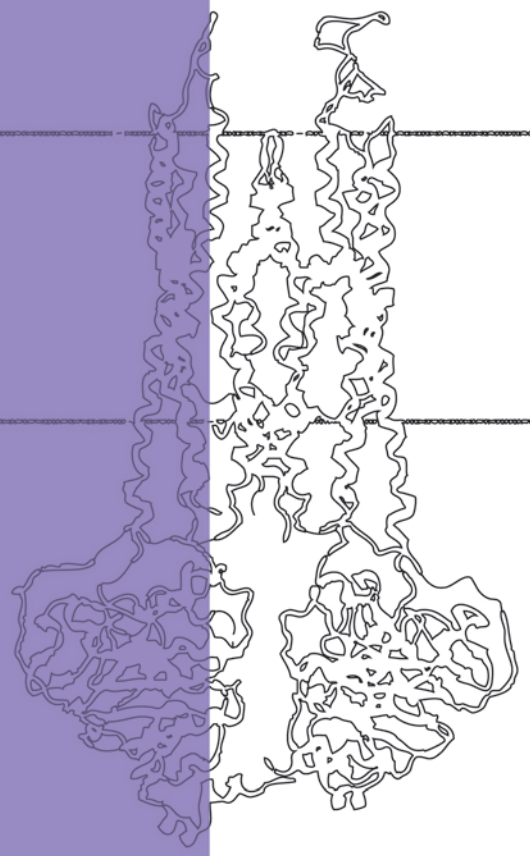
3.12. Doxorubicin accumulation assay

Intracellular doxorubicin levels were determined by flow cytometry. Briefly, NCI-H460/R cells (1.0×10^5 per well) were seeded into 6-well plates and then treated with PE5 (1, 1.6, 2.6 and 4.3 μM) or onconase (0.145, 0.245, 0.39 and 0.625 μM). NCI/ADR-RES cells (2.2×10^5 per well) were also seeded into 6-well plates and then treated with 3.6 μM PE5 or 0.4 μM onconase. After 72 h of treatment, cells were incubated with 10 μM doxorubicin for 1 h at 37°C in 5% CO_2. In some experiments 10 μM verapamil (Sigma-Aldrich, St. Louis, MO, USA), 25 μM fumitremorgin C (Sigma-Aldrich, St. Louis, MO, USA) or 60 μM MK571 (Alexis, Farmingdale, NY, USA), which are competitive inhibitors of P-gp, BCRP and MRP1, respectively, were added in combination with the doxorubicin. Cells were treated with trypsin–EDTA and the cell suspensions were washed twice in ice-cold PBS and resuspended in this same buffer. Cellular uptake was measured on a Becton Dickinson FACSCalibur (San Jose, CA, USA) using CellQuest Pro software. Excitation was set at 484 nm and emission at 560 nm. A minimum of 10,000 events were assayed for each sample.

3.13. Statistical analysis

All of the *in vitro* experiments were done in triplicate and shown as mean ±SE unless otherwise indicated. Results were analyzed by one-way ANOVA or by repeated-measures ANOVA using a Sidak test as a post-test. For interaction index analysis (*I*), Student's *t*-test were applied to each set of data points to formally evaluate whether synergism or antagonism was evident for a particular schedule and effect level (as

compared to a null hypothesis *I* of 1). P-values below 0.05 were considered statistically significant.



4. RESULTS

Quan surts per fer el viatge cap a Ítaca,
has de pregar que el camí sigui llarg,
ple d'aventures, ple de coneixences.
Has de pregar que el camí sigui llarg,
que siguin moltes les matinades
que entraràs en un port que els teus ulls ignoraven,
i vagis a ciutats per aprendre dels que saben.
Tingues sempre al cor la idea d'Ítaca.
Has d'arribar-hi, és el teu destí,
però no forcis gens la travessia.
És preferible que duri molts anys,
que siguis vell quan fondegis l'illa,
ric de tot el que hauràs guanyat fent el camí,
sense esperar que et doni més riqueses.
Ítaca t'ha donat el bell viatge,
sense ella no hauries sortit.
I si la trobes pobra, no és que Ítaca
t'hagi enganyat. Savi, com bé t'has fet,
sabràs el que volen dir les Ítaques.

"Ítaca"

Konstantinos Kavafis - Lluís Llach

4.1. PE5 and onconase toxicity to immortalized cell lines and to primary cells

We had previously found that PE5 is cytotoxic against different cell lines by measuring the incorporation of [^{35}S]methionine into the cells (Bosch et al., 2004). We were interested in broadening this study to other cell lines by measuring how this RNase affects the metabolic status of the cells (MTT assay). Onconase was used as a control.

Table 4.1 shows that PE5 is cytotoxic against the indicated tumor cell lines, the IC_{50} of PE5 being 6-10-fold higher than that of onconase.

Table 4.1. IC_{50} values for PE5 and onconase for the indicated cell lines. Data are presented as mean ±SE of at least three independent experiments made in triplicates.

Cell line	PE5 (μM)	Onconase (μM)
SK-BR-3	33.1±3.7	4.2±0.6
MCF-7	20.0±1.5	2.2±0.1
MDA-MB-231	12.7±2.0	1.9±0.5
NCI/ADR-RES	6.9 ±0.8	1.1 ±0.1
NCI-H460/R	4.3±0.4	0.6±0.1
HeLa	8.2 ±0.6	0.8 ±0.1

We also investigated the effect of the overexpression of HER2/neu on the cytotoxicity of PE5. To this end, we tested the cytotoxicity of PE5 and, for comparison, of onconase on MCF-7 cells stably transfected with HER2/neu containing vector (Menéndez et al., 2007). MCF-7/HER2 expresses 70-fold more HER2/neu than the parental cell line (Menéndez et al., 2007). Figure 4.1 shows that neither PE5 nor onconase were less toxic against this transfected cell line than against the non-transfected cells, which indicates that overexpression of HER2/neu does not result in an increase of resistance to the action of PE5.

The most sensitive cell lines were NCI/ADR-RES and NCI-H460/R, both corresponding to MDR cell lines (Table 4.1).

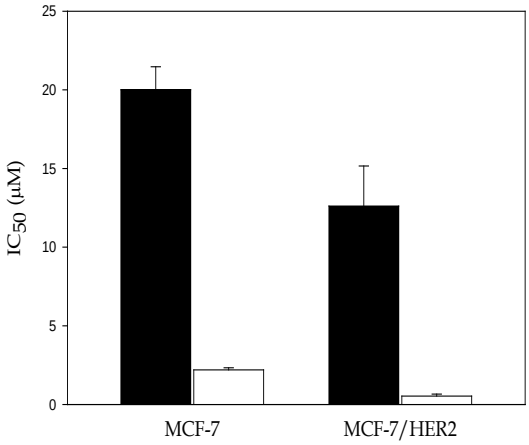


Figure 4.1. Effect of the overexpression of HER2/neu on the sensitivity of the MCF-7 cell line to the PE5 and onconase cytotoxic effects. IC_{50} values for PE5 (black bars) and onconase (white bars) are indicated for MCF-7 and MCF-7/HER2 cells. Data are presented as mean ±SE of at least two independent experiments made in triplicates.

We also examined the cytotoxic effect of PE5 and onconase on N1 normal human fibroblasts (Figure 4.2). Again, both RNases were cytotoxic for N1 fibroblasts, being the IC_{50} for PE5 and onconase of 19.5±1.4 and 1.1±0.2, respectively. In his case the difference in IC_{50}s between both RNases was increased to nearly 20-fold.

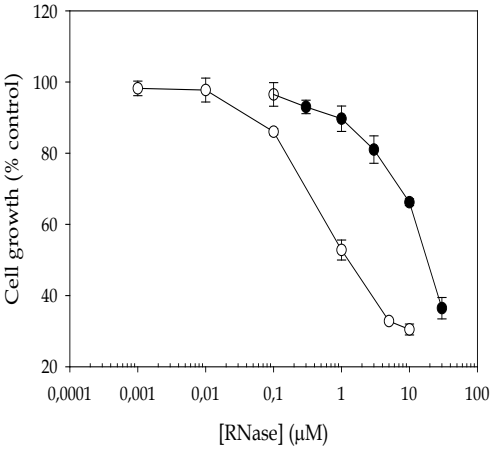


Figure 4.2. Effect of PE5 (●) and onconase (○) on N1 cells. Control and RNase-treated cell cultures were maintained for 72 h and metabolic activity was determined by MTT assay as described in the methods section. Cell growth was expressed as the percentage of control activity using the absorbance values. The curves in the figure are from one representative experiment. Equivalent results were found in at least three independent experiments.

4.2. PE5 and onconase induce cell death at similar concentrations

We performed a more detailed evaluation of the cytotoxic properties of PE5 using one of the most affected cell lines investigated. Again, for comparison, onconase was included in these experiments.

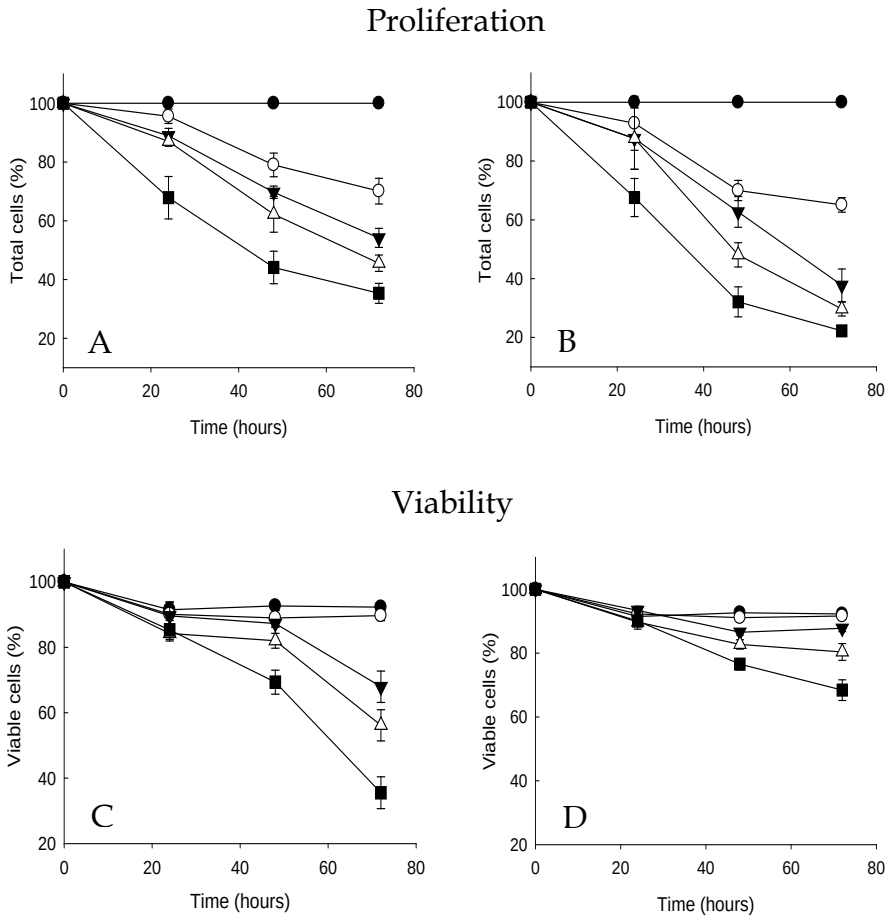


Figure 4.3. Proliferation and viability curves of NCI/ADR-RES cells treated with PE5 or onconase. Cells were treated with 0 (●), 2 (○), 7 (▼), 14 (Δ) and 35 μM (■) of PE5 (A and C) or 0 (●), 0.3 (○), 1 (▼), 2 (Δ) and 5 μM (■) of onconase (B and D). Trypan blue dye exclusion cell viability was estimated at 0, 24, 48 and 72 h after administration of the RNases. The plotted points represent means of at least three independent experiments.

Figure 4.3 shows the effect on NCI/ADR-RES proliferation and viability when incubated with four different concentrations of PE5 or onconase for up to 72 h. Even at 24 h of intoxication an effect of both RNases on proliferation could be observed at the highest concentrations assayed. At this same time the viability of NCI/ADR-RES cells was reduced only by treatment with 14 or 35 μM PE5. Consistently with the cytotoxicity results (Table 4.1), cells treated with 7 μM PE5 or 1 μM onconase for 72 h (which correspond to the IC_{50} values described in Table 4.1) decreased the proliferation to around 50% respective to untreated cells (Figures 4.3A and 4.3B).

It was apparent that both RNases behaved in different ways: the effect of PE5 was mainly cytotoxic, although at low concentrations a minimal cytostatic effect could be observed. On the contrary, onconase was highly cytostatic. Although a 7-fold lower concentration of onconase was necessary to reduce cell growth to 50%, the percentage of viable cells at each incubation time was similar when using either enzyme (Figures 4.3C and 4.3D). From this observation we concluded that both RNases induced a similar cell death at similar concentrations but that onconase presented an additional capacity of arresting proliferation.

4.3. Analysis of the RNase treatments on the cell cycle phase distribution

We investigated the effect of 35 μM PE5 or 5 μM onconase on the NCI/ADR-RES cell cycle progression. At these concentrations PE5 is mainly cytotoxic and onconase presents both cytotoxic and cytostatic activities (see above). Cell cycle phase distribution in each case is presented in Figure 4.4. After 72 h of PE5 exposure, a clear increase of S and G_2/M cell cycle phases, concomitant with a decrement of G_0/G_1 cell cycle phase cells was observed. In contrast, the cell cycle phase distribution of cells treated with 5 μM onconase was very similar to that of untreated growing cells. We also assayed the effect of 1 μM onconase. At this concentration, onconase is exclusively cytostatic. In this case we also could not observe any change in the cell population distribution (Figure 4.4D).

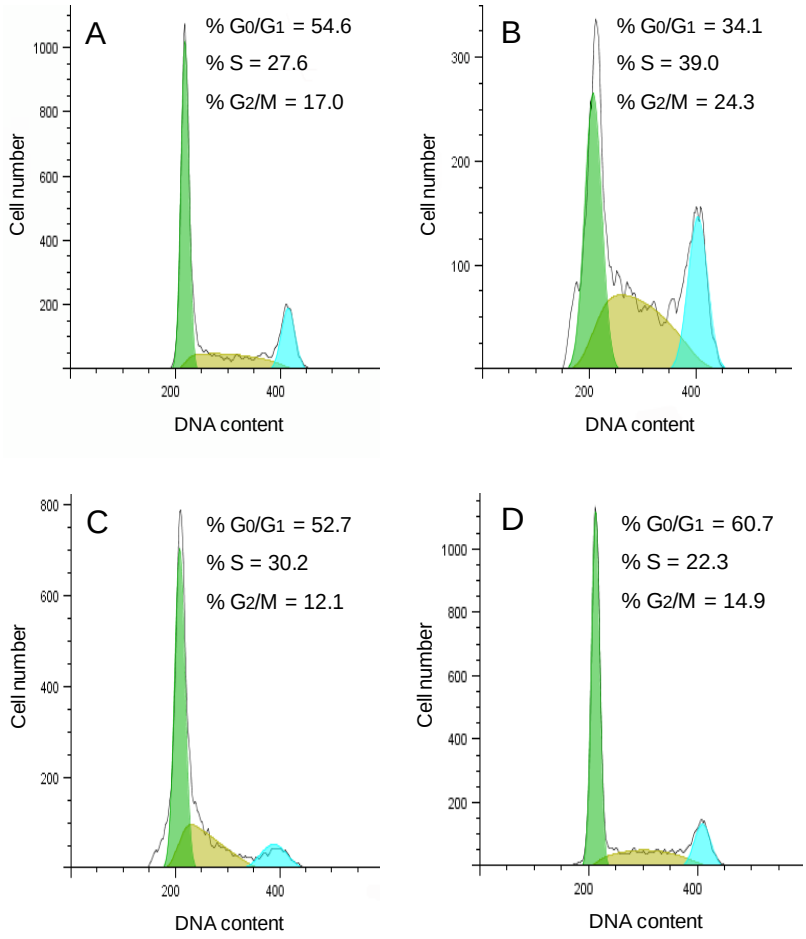


Figure 4.4. Effects of PE5 and onconase on the NCI/ADR-RES cell cycle phase distribution after treatment for 72 h. Untreated cells (A), 35 µM PE5-treated cells (B), 5 µM onconase-treated cells (C) and 1 µM onconase-treated cells (D) were permeabilized and stained with PI. Cell DNA content was analyzed by flow cytometry as described in the experimental section. Data are representative of three independent assays. Values were analyzed from 10,000 total events.

Since differences in cell cycle phase distribution were observed between both RNases, we used Western blot to also analyze the expression of cyclin D_1, cyclin E, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Figure 4.5). PE5 at 7 µM increased the expression of cyclin E to a 209%±25 of that of untreated cells and also of $p21^{WAF1/CIP1}$ to a minor extent (175%±37 of untreated cells), whereas the amount of cyclin D_1 and $p27^{KIP1}$ remained unchanged

respective to untreated cells. Onconase at 1 μM reduced the expression of cyclin D_1 to a 34%±7 of that of untreated cells and also that of cyclin E (62%±7 of untreated cells). The amounts of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ remained nearly unchanged respective to untreated cells.

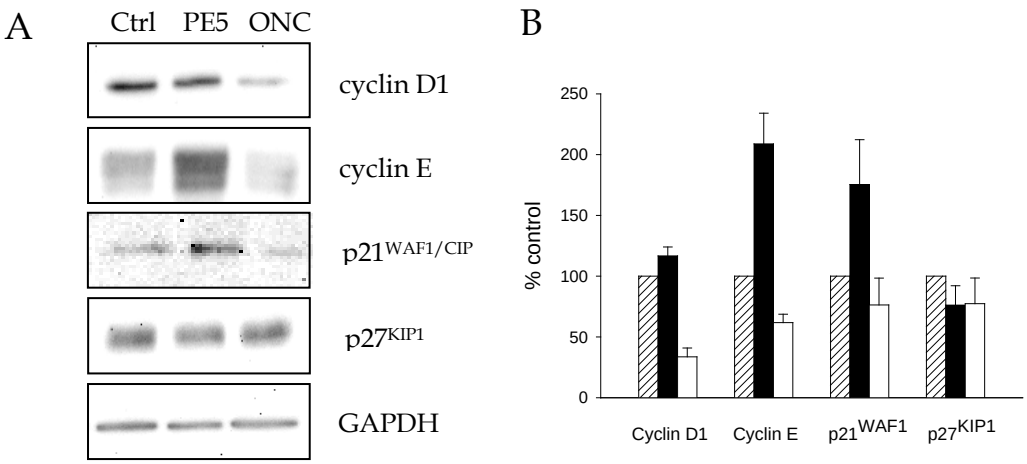


Figure 4.5. Expression of cyclin D_1, cyclin E, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in 7 μM PE5- or 1 μM onconase (ONC)-treated cells. 72 h after the treatments, cells were harvested and analyzed by Western blot using the antibodies described in the experimental section. A) Western blot of a representative experiment. B) Densitometric analysis of the GAPDH-normalized immunoblots of each treatment respective to that of untreated cells. Striped bars indicate untreated cells (control) and black bars and white bars indicate PE5- and onconase-treated cells, respectively.

4.4. PE5 induce apoptosis in NCI/ADR-RES cells

We studied the cell death induced by the treatment with PE5. To determine whether the RNase-treated cells displayed characteristic features of apoptosis, NCI/ADR-RES cells were treated with 35 μM PE5 and 5 μM onconase as control for 72 h and then analyzed, using a confocal microscope, in fresh cultures after staining with Hoechst 33342. In both treatments but not in the untreated control cells, nuclear morphological changes typical of apoptosis, such as the characteristic chromatin condensation and

the nuclear fragmentation, could be observed (Figure 4.6). The PE5 and onconase effect on NCI/ADR-RES cell morphology was also studied in fresh cultures using calcein AM and PI staining. Calcein AM is a lipid-soluble fluorogenic diester that passively crosses the cell membrane. Once inside the cells, the virtually non-fluorescent calcein AM is hydrolyzed by intracellular esterases into the intensely fluorescent calcein. The fluorescent calcein is retained in the cytoplasm in live cells. When the plasma membrane becomes compromised, PI can react with nucleic acids, producing red fluorescence. Thus, calcein AM allows for the determination of cellular viability and the visualization of changes in cell morphology while PI allows verification of the changes in the cell membrane permeability and nuclei morphology. Cells treated with 35 μM PE5 or 5 μM onconase show typical apoptosis features like membrane blebbing, apoptotic body formation, chromatin condensation and apoptotic nuclear fragmentation (Figure 4.6).

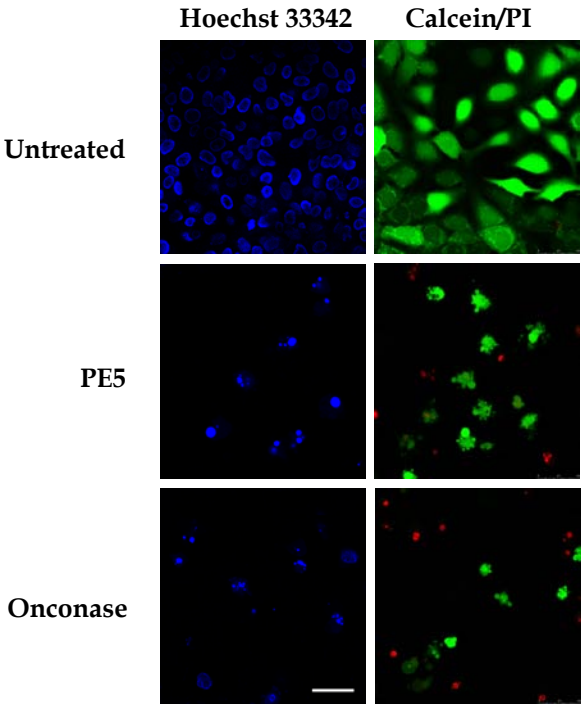


Figure 4.6. Nuclear and cellular morphological effects of PE5 and onconase on the NCI/ADR-RES cell line. Overnight serum-starved NCI/ADR-RES cells were treated with 35 μM PE5 or 5 μM onconase for 72 h. Subsequently, Hoechst 33342 and calcein/PI staining were performed and observed under a confocal laser microscope. Bar 50 μm.

We sought to quantify the percentage of NCI/ADR-RES cells in early and late apoptosis after 24, 48 and 72 h of incubation with 35 μM PE5 or 5 μM onconase. The results of the FACS analysis of these cells stained with Alexa Fluor 488 annexin V and PI, presented in Table 4.2, show that the percentage of necrosis induced by the treatment was negligible in both cases. Again, induction of apoptosis could be demonstrated by the translocation of phosphatidylserine to the external hemi-membrane. Apoptosis was evident at 48 h of treatment in both cases and further increased at 72 h. Interestingly, the percentage of cells treated with PE5 that were at early apoptosis was nearly 50% higher than that of those treated with onconase.

We were interested in investigating whether the cytotoxic mechanism of both RNases could be different. In order to characterize apoptosis of NCI/ADR-RES cells induced by PE5 and onconase, activation of procaspases-3, -8 and -9 was investigated (Figure 4.7). PE5 induces the activation of the procaspases-3 and -8 even at 24 h of drug incubation ($p<0.05$). Activation reaches its maximum at 48 h. Activation of procaspase-9 is also induced by PE5 but it can be only detected after 48 h of incubation with the RNase and continues to increase at 72 h. For onconase, procaspase-8 and -9 activation was delayed to 48 h of RNase incubation ($p<0.05$). The pattern of activation is very similar to that found for PE5. At 72 h of incubation the three caspases were active although to a minor extent when compared to PE5 treatment. Caspase activity was also investigated after 72 h of RNase incubation in the presence of the caspase-specific inhibitors DEVD-FMK, IETD-FMK or LEHD-FMK. In the presence of these inhibitors the activity dropped to the level of the control extracts. In this assay we could not detect caspase activity in extracts of NCI/ADR-RES cells treated with a cytostatic concentration of onconase (1 μM), excepting for caspase-3, for which an approximately 1.5-fold increase was detected after 12 h of substrate incubation.

Table 4.2. Apoptosis measured by Alexa Fluor 488 annexin V/PI staining. NCI/ADR-RES cells were treated with 35 μM PE5 or 5 μM onconase for 24, 48 and 72 h, 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+. All data were expressed as mean ±SE of three different experiments. Values were analyzed from 10,000 total events.

	Control			PE5			Onconase		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Early apoptotic cells (%)	4.1 ± 0.2	4.2 ± 0.5	4.5 ± 0.7	3.9 ± 0.1	17.7 ± 1.9	28.3 ± 1.3	6.8 ± 2.1	11.9 ± 1.1	20.4 ± 2.0
Late apoptotic cells (%)	5.9 ± 0.2	7.2 ± 0.9	5.7 ± 0.1	0.7 ± 0.3	6.5 ± 1.4	26.0 ± 4.4	2.3 ± 0.1	11.6 ± 2.6	25.9 ± 1.4
Necrotic cells (%)	2.4 ± 0.3	2.4 ± 0.7	1.6 ± 0.2	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
Viable cells (%)	87.6 ± 0.6	86.2 ± 1.1	88.2 ± 1.0	95.4 ± 0.4	75.8 ± 0.5	45.7 ± 5.3	90.9 ± 2.0	76.5 ± 3.7	53.7 ± 0.6

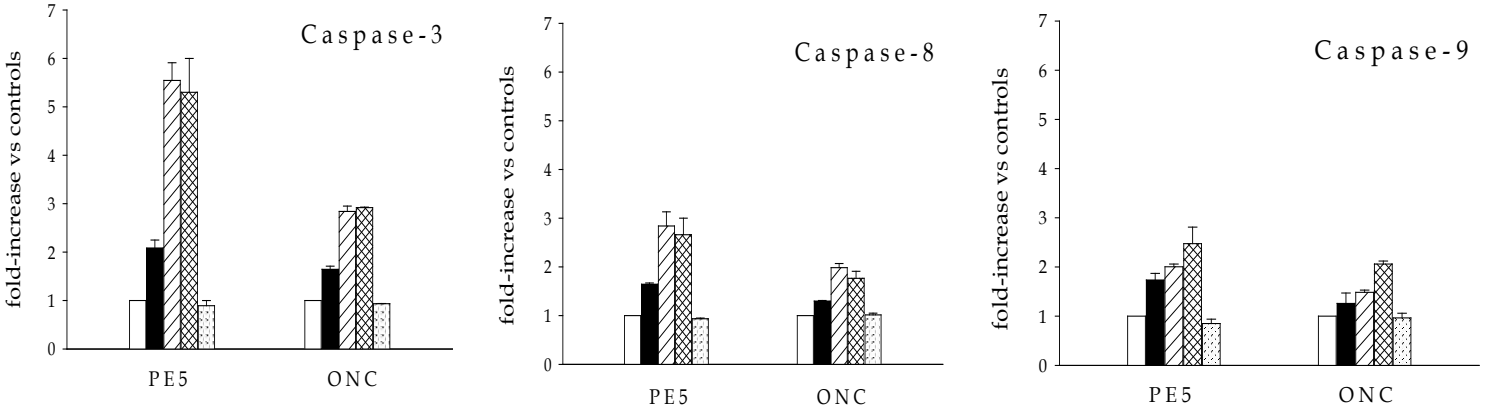


Figure 4.7. Caspase-3, -8 and -9 activities in NCI/ADR-RES cells treated with PE5 or onconase. Cells were treated with 35 μM PE5 or 5 μM onconase for 24 (black bars), 48 (striped bars) and 72 h (checkered bars). White bars indicate untreated cells (control). Parallel samples were treated with the specific inhibitors of caspase-3 (z-DEVD-FMK), caspase-8 (z-IETD-FMK), caspase-9 (z-LEHD-FMK) (stippled bars) as a negative control. Caspase-3, -8 and -9 activation was quantified in whole cell lysates using a quantitative colorimetric assay as described in the text. Results are expressed as mean ±SE of three independent experiments.

4.5. Analysis of the expression of apoptosis related proteins

We investigated by Western blot the effect of PE5 and onconase on the level of different apoptosis-related proteins. For onconase, we tested its effect when it induced a cytostatic effect on the NCI/ADR-RES cells (1 μM) and also when it induced a significant cytotoxic activity in these cells (5 μM). Cellular extracts from NCI/ADR-RES treated for 72 h with 7 μM PE5 or with 1 or 5 μM onconase were subjected to immunoblotting using antibodies against Bcl-2, Bax, XIAP, JNK and the phosphorylated form of p46 JNK.

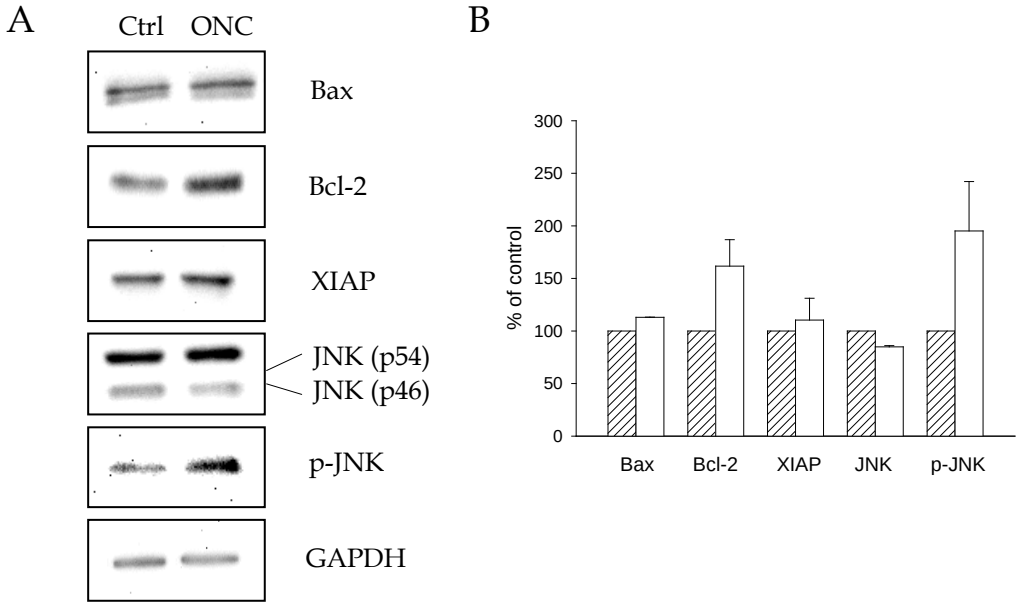


Figure 4.8. Effect of 1 μM onconase on the expression of Bcl-2, Bax, JNK and XIAP and on the phosphorylation levels of p46 JNK in NCI/ADR-RES cells after treatment for 72 h. Protein levels were determined by Western blot analysis (see experimental section). A) Western blot of a representative experiment. B) Densitometric analysis of the GAPDH-normalized immunoblots of each treatment respective to that of untreated cells. JNK and p-JNK refers to the unphosphorylated and phosphorylated p46 JNK isoforms, respectively. Striped bars indicate untreated cells (control) and white bars indicate onconase-treated cells.

The effect of 1 μM onconase on the expression of these proteins in NCI/ADR-RES is shown in Figure 4.8. In this case, the amount of Bax and XIAP respective to that of untreated cells remained unaltered after the treatment, while for Bcl-2 a slight increase (around 62%) was observed. Finally, when the effect of onconase on the expression of JNK was investigated we could not detect any change in the expression of either p54 or p46 JNK isoforms when compared to untreated cells. However, the treatment produced a 2-fold increase in the amount of the phosphorylated form of p46 JNK.

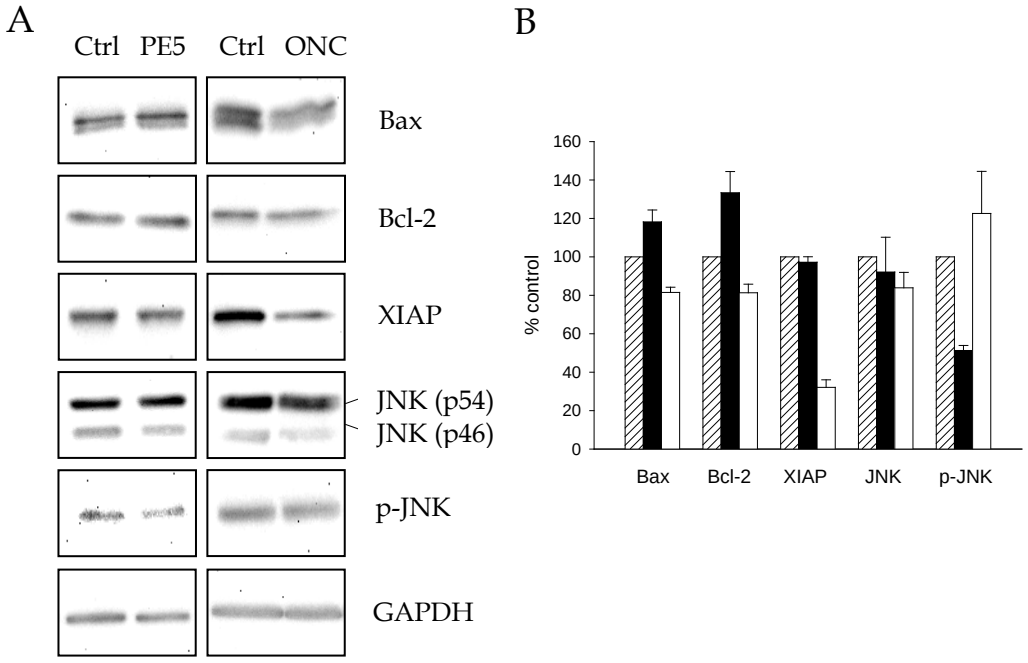


Figure 4.9. Effects of 7 μM PE5 or 5 μM onconase on the expression of Bcl-2, Bax, JNK and XIAP and on the phosphorylation levels of p46 JNK in NCI/ADR-RES after treatment for 72 h. Protein levels were determined by Western blot analysis (see experimental section). A) Western blot of a representative experiment. B) Densitometric analysis of the GAPDH-normalized immunoblots of each treatment respective to that of untreated cells. JNK and p-JNK refers to the unphosphorylated and phosphorylated p46 JNK isoforms, respectively. Striped bars indicate untreated cells (control) and black bars and white bars indicate PE5- and onconase-treated cells, respectively.

In Figure 4.9 we show the effects of the treatment of NCI/ADR-RES with cytotoxic concentrations of either PE5 (7 μM) or onconase (5 μM) on the expression of these apoptotic-related proteins. PE5 did not change the expression levels of any of the assayed proteins. However, PE5 induced a clear 2-fold decrease of the level of the phosphorylated form of p46 JNK respective to untreated cells. For onconase, important differences could be detected when compared with the effects produced using a cytostatic concentration. Although again Bax remained unaltered, Bcl-2 clearly decreased to half the level found under the cytostatic conditions. Also, an important decrease of XIAP compared to untreated cells was observed in this case, and finally, we could not detect changes in the levels of phosphorylated and dephosphorylated forms of JNK respective to untreated cells (Figure 4.9).

Interestingly, the effect of PE5 was different to that of onconase under cytotoxic conditions (Figure 4.9).

4.6. PE5 presents synergistic cytotoxicity with doxorubicin in NCI/ADR-RES

We have previously shown that the cell lines most sensitive to PE5 presented an MDR phenotype (see Table 4.1). NCI/ADR-RES is especially resistant to doxorubicin (IC_{50} of 76.4 μM), compared to the non-MDR MCF-7 cell line or to the NCI-H460/R MDR cell line (IC_{50} of 0.41 and 1.16 μM, respectively) (Figure 4.10). We wondered whether a response in NCI/ADR-RES cells to PE5 could be exploited to increase the growth inhibitory effects of doxorubicin. For comparison, interaction between PE5 and doxorubicin in the non-MDR MCF-7 cell line was also investigated.

Both cancer cell lines were treated with PE5 or doxorubicin or with a combination of both drugs. The nature of the interaction between doxorubicin and PE5 was evaluated by the isobologram technique. Isobolographic analysis was performed for each combination and the interaction index was used to determine the kind of interaction between the drugs (Table 4.3).

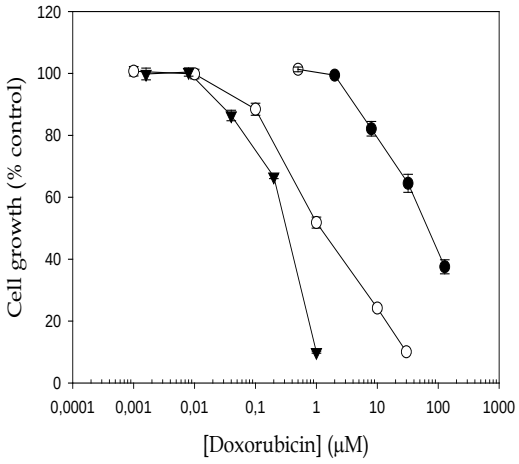


Figure 4.10. Effect of doxorubicin on NCI/ADR-RES (●), NCI-H460/R (○) and MCF-7 (▼) cells. Control and doxorubicin-treated cell cultures were maintained for 72 h and metabolic activity was determined by MTT assay as described in the methods section. Cell growth was expressed as the percentage of control activity using the absorbance values. The curves in the figure are from one representative experiment. Equivalent results were found in at least three independent experiments.

A synergistic interaction was found between doxorubicin and PE5 in the NCI/ADR-RES cell line. The interaction index at each dose was significantly lower than the value of 1 (around 0.45). In MCF-7, at IC_{50} and IC_{40} PE5 was antagonistic but at IC_{30} it was additive to the effect of doxorubicin (Table 4.3). For comparison, interaction between onconase and doxorubicin was also evaluated in these cell lines. Onconase was synergistic with doxorubicin in both cell lines at the different doses assayed.

Table 4.3. Interaction index (I_x) values for the combination of PE5 or onconase and doxorubicin at 30%, 40% and 50% of inhibition of cell proliferation. Data are presented as mean ±SE.

	PE5			Onconase		
Cell line	I_{30}	I_{40}	I_{50}	I_{30}	I_{40}	I_{50}
NCI/ADR-RES	0.48*±0.03	0.42*±0.06	0.45*±0.08*	0.45*±0.09	0.39*±0.05	0.40*±0.03
MCF-7	0.91±0.07	1.13*±0.03	1.14*±0.03	0.54*±0.06	0.73*±0.08	0.82*±0.06

* Significantly different (P<0.05) from 1 by Student's t-test.

4.7. PE5 reduces the accumulation of P-gp in MDR cell lines

One explanation of the synergism with doxorubicin in NCI/ADR-RES, but not in the MCF-7 cell line, could be that PE5 modulates the expression of certain MDR genes involved in doxorubicin resistance. We tested this possibility by incubating this cell line with each RNase and monitoring the expression of P-gp and GST-π proteins by Western blot. It has been shown that NCI/ADR-RES overexpresses both P-gp and GST-π proteins (Batist et al., 1986; Fairchild et al., 1987; Gehrmann et al., 2004). Expression of β-actin or GAPDH was used as an internal control of the samples. Cells were treated with 3.6 μM PE5 for different incubation times (24, 48 and 72 h) and equal amounts of total protein extracts were applied onto the electrophoresis gel. A control experiment was included in which NCI/ADR-RES cells were treated with 0.4 μM onconase. Treatment of NCI/ADR-RES for 72 h with these RNase concentrations induced a decrease of cellular viability of around 40% in both cases.

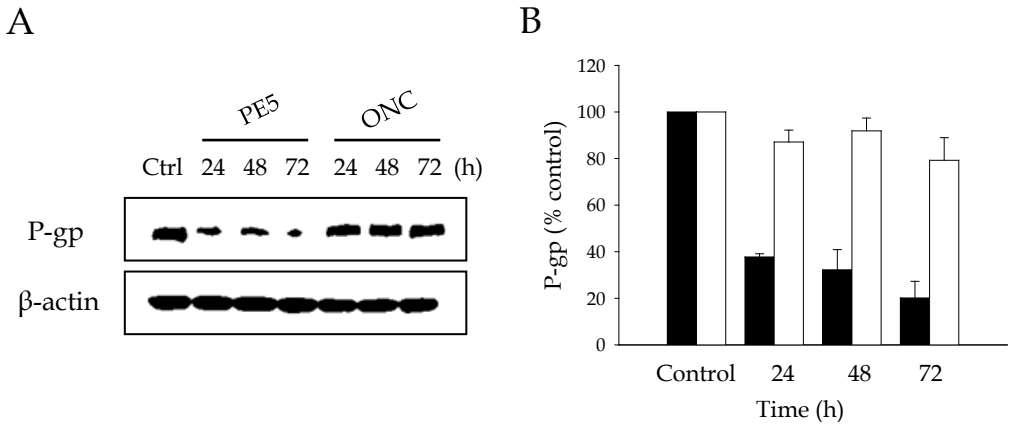


Figure 4.11. Amount of accumulated P-gp in NCI/ADR-RES cells treated with 3.6 μM PE5 or 0.4 μM onconase for the indicated times. A) The P-gp protein level was determined by Western blot using anti-P-gp C219 antibody. B) The amount of P-gp was quantified by densitometric analysis and related to that of β-actin. Data are depicted as histograms with ±SE. Black bars and white bars indicate PE5- and onconase-treated cells, respectively. Experiments were performed at least in duplicate with similar results. Representative blots are presented.

Figure 4.11 shows that PE5, but not onconase, reduces in a time-dependent manner the amount of P-gp. After 24 h of incubation with PE5, the amount of P-gp (normalized with the intensity of the β-actin band) decreased to around 40% and after 72 h of incubation this amount decreased to around 20% with respect to untreated cells.

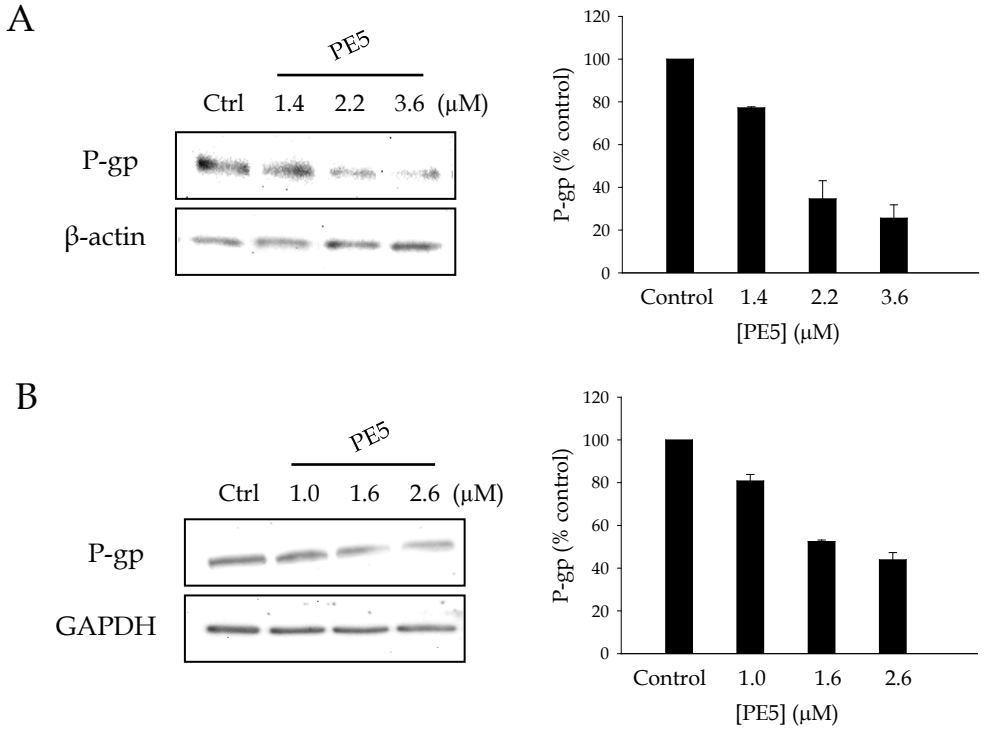


Figure 4.12. Amount of accumulated P-gp in NCI/ADR-RES and NCI-H460/R cells treated for 72 h with the indicated concentrations of PE5. The P-gp protein level was determined by Western blot (left) and quantified by densitometric analysis. The amount of P-gp was related to that of β-actin or GAPDH and data are depicted as histograms with ±SE (right). A) PE5 causes concentration-dependent decrease of P-gp expression in NCI/ADR-RES cells. 72 h after the treatment with the indicated concentrations of PE5, NCI/ADR-RES cells were lysated and subjected to Western blot analysis. B) PE5 induces concentration-dependent decrease of P-gp expression in NCI-H460/R cell line. Cells were incubated with the indicated concentrations of PE5 for 72 h and then P-gp protein levels were determinate by Western blot analysis. All experiments were performed at least in duplicate with similar results. Representative blots are presented.

Figure 4.12A shows that the treatment of NCI/ADR-RES cells with PE5 for 72 h also reduces in a dose-dependent manner the amount of accumulated P-gp. Treatment of NCI/ADR-RES with 1.4, 2.2 and 3.6 μM PE5 reduced the P-gp levels to 75, 35 and 26% of those of untreated cells, respectively.

Figure 4.13A shows a representative Western blot of the effect of PE5 or onconase treatment on the accumulation of GST-π in NCI/ADR-RES cells. As can be seen, we could not detect any change in the amount of GST-π when compared to untreated cells at any of the RNase concentrations assayed.

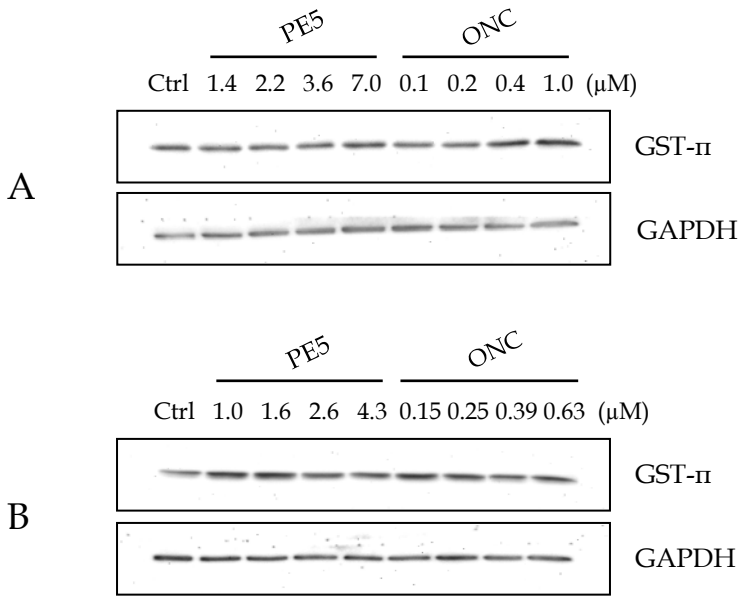


Figure 4.13. Effects of PE5 or onconase on the expression of GST-π in NCI/ADR-RES and NCI-H460/R cells. NCI/ADR-RES (A) and NCI-H460/R cells (B) were treated for 72 h with the indicated concentrations of PE5 and onconase and GST-π levels after the treatments were determined by Western blot. GAPDH protein was used as an internal control. Each blot is representative of at least two independent experiments.

The effect of PE5 on the amount of accumulated P-gp was confirmed in the NCI-H460/R cell line. This cell line is more sensitive to doxorubicin (see Figure 4.10) and

expresses lower levels of P-gp as assessed by Western blot (result not shown). Again, intoxication of NCI-H460/R cells with different concentrations of PE5 for 72 h produced a dose-dependent decrease of the P-gp level to around 40% of that of untreated cells (Figure 4.12B). Neither PE5 nor onconase changed the accumulation of GST-π in NCI-H460/R cells (Figure 4.13B).

4.8. Effect of PE5 on doxorubicin uptake in MDR cell lines

The ability of PE5 to decrease the P-gp expression was further evaluated by investigating whether a decrease in the P-gp function could be observed. Doxorubicin is considered a classical substrate for P-gp and has been previously used to evaluate its drug-pumping activity via flow cytometric analysis (Azzariti et al., 2006; Li et al., 2001). Although NCI-H460/R cells showed poor uptake of doxorubicin, when these cells were previously incubated for 72 h with PE5, doxorubicin uptake was clearly increased. Incubation with 1.0, 1.64, 2.6 and 4.33 μM PE5 produced a 51, 72, 94 and 104% increase of doxorubicin accumulation (Figure 4.14). As expected, treatment of NCI-H460/R cells with 0.15, 0.25, 0.39 and 0.63 μM onconase for 72 h did not produce any change in the doxorubicin accumulation. Treatment with 10 μM verapamil for 1 h produced a 266% increase of doxorubicin accumulation.

The effect on doxorubicin accumulation after treatment of NCI/ADR-RES cells with PE5 was also investigated. In this case we could not observe a significant difference in the accumulation of doxorubicin between PE5-treated and control cells. We postulated that the discrepancy between the FACS and the Western blot results was because NCI/ADR-RES overexpressed other extrusion pumps which could mask the effect of PE5 on the P-gp function. We assayed the effect of inhibiting P-gp, BCRP and MRP1 on the percentage of doxorubicin accumulation in this cell line, using 10 μM verapamil, 25 μM fumitremorgin C and 60 μM MK571, respectively. These extrusion pumps are those most commonly expressed in MDR cell lines (Stavrovskaya and Stromskaya, 2008). Figure 4.15A shows that the highest increase of doxorubicin

accumulation is produced after the treatment with verapamil (increase of 340%) but that fumitremorgin C also produces a significant increase. The specific MRP inhibitor MK571 does not change the intracellular accumulation of doxorubicin.

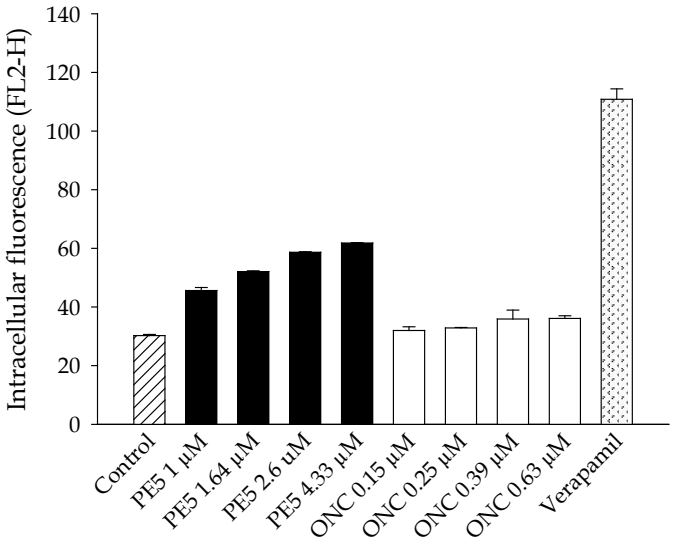


Figure 4.14. Effect of PE5 and onconase on doxorubicin accumulation in NCI-H460/R cells. Cells were treated with the indicated concentrations of PE5 or onconase for 72 h and then exposed to 10 µM doxorubicin for 1 h. 10 µM verapamil was used as a positive control. Intracellular fluorescence of doxorubicin was measured by flow cytometry. Data were expressed as mean ±SE of at least two independent experiments. Values were analyzed from 10,000 total events for each experimental sample.

The effect of PE5 on the doxorubicin accumulation in cells treated with 25 µM fumitremorgin C is shown in Figure 4.15B. In this case, the amount of accumulated doxorubicin in the cells treated with a combination of PE5 and fumitremorgin C increased by 44%. Again, onconase did not promote a specific change in the intracellular accumulation of doxorubicin in the fumitremorgin C-treated cells.

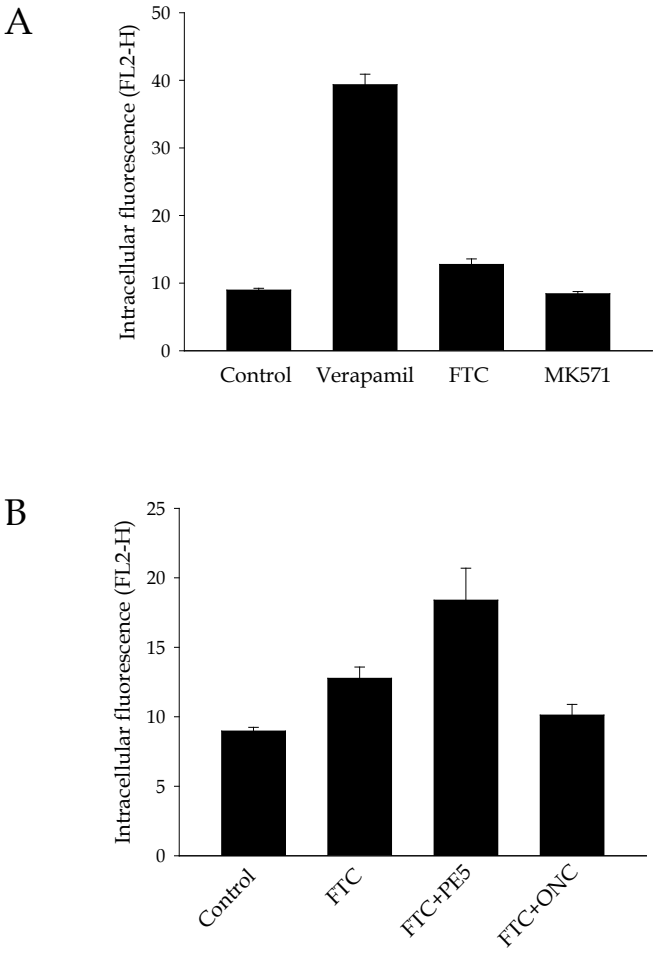
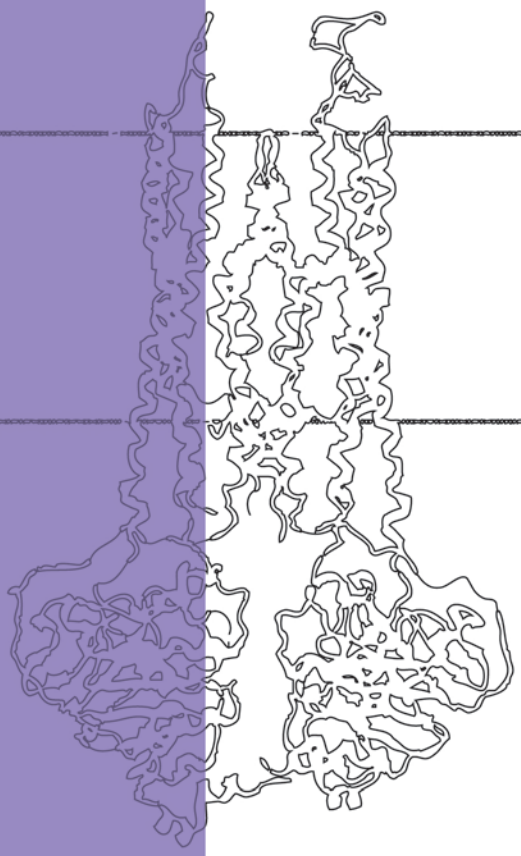


Figure 4.15. Intracellular doxorubicin accumulation in NCI/ADR-RES cells treated with PE5, onconase and modulators of MDR function. A) Cells were incubated in culture media containing 10 μM doxorubicin for 1 h in the presence or absence of MDR modulators: verapamil (10 μM), fumitremorgin C (25 μM) or MK571 (60 μM). B) Cells were treated with 3.6 μM PE5 or 0.4 μM onconase for 72 h and then exposed to 10 μM doxorubicin and 25 μM fumitremorgin C for 1 h. Intracellular accumulation of doxorubicin was evaluated by flow cytometry. Results are expressed as mean ±SE of at least two independent experiments. Values were analyzed from 10,000 total events for each experimental sample.



5. DISCUSSION

In this work we have studied the cytotoxic properties of an HP-RNase variant directed to the nucleus and we have compared them with those of onconase as a paradigmatic example of cytotoxic RNases. Unlike many chemotherapeutic drugs currently used for cancer treatment, RNases are alternative non-mutagenic candidates for cancer therapy. In contrast to onconase, PE5 is a human-derived RNase and displays 10^3-10^4 times higher ribonucleolytic activity (Leland et al., 2001). In addition, the fact that PE5 and onconase cleave RNA on different cellular compartments (Tubert et al., 2010) opens the possibility of using both RNases in combination as chemotherapeutical agents. We had previously shown (Bosch et al., 2004) that PE5 inhibits protein synthesis of a large panel of eukaryotic cell lines representative of different types of cancer. In a preliminar experiment we have investigated the effect of this RNase and onconase on the metabolic state of human tumor cell lines representative of breast, lung, ovarian and cervical cancers (MTT-based assay). The breast cancer cell lines used in this work were selected because they were representative of different stages in the epithelial-mesenchymal transition hypothesis that describe the progression of breast cancer from atypical hyperproliferation to metastatic disease (for a review, see (Lacroix and Leclercq, 2004)). MCF-7 is representative of a first stage in which cells are poorly invasive and display luminal epithelial-like traits. SK-BR-3 can be included in a second stage in which the weakly luminal epithelial-like cells present a reduced expression of some epithelioid markers, and MDA-MB-231 is representative of a third stage corresponding to a stromal-like group which is highly invasive. The data obtained showed that the most affected breast cell line was the MDA-MB-231. The other cell lines assayed were the ovarian cancer cell line NCI/ADR-RES, the lung cancer line NCI-H460/R and the cervical cancer HeLa cell line.

The sensitivity of the breast cancer cell lines to the RNases inversely correlated with the expression of HER2/neu (SK-BR-3>>MCF-7>MDA-MB-231) (Menéndez et al., 2004). This overexpression has been associated with poor disease-free survival in breast cancer patients (Esteva et al., 2005; Slamon et al., 1987; Slamon et al., 1989) and

with resistance to certain chemotherapeutic agents (Muss et al., 1994; Paik et al., 1998). Thus, we were interested in investigating whether overexpression of HER2/neu could be predictive of a poor cytotoxicity of both PE5 and onconase on a given cell line. Our results show that overexpression of HER2/neu does not result in an increased resistance to the action of these RNases.

Moreover, p53 status differs between the affected breast cancer cell lines: MCF-7 express wild type p53 whereas SK-BR-3 and MDA-MB-231 express mutant p53. NCI/ADR-RES and NCI-H460/R cell lines also contain mutated p53. Although the HeLa cell line has wild type p53 alleles, it has been reported (Matlashewski et al., 1986) that it contains no detectable p53 tumor suppressor protein. This is likely because in the HeLa cell line (a human papillomavirus 18-positive human cervical carcinoma cell line) any p53 protein that is synthesized is rapidly degraded by E6 oncoprotein encoded by the papillomavirus genome (Scheffner et al., 1990). Among the assayed cell lines, NCI/ADR-RES and NCI-H460/R were the most affected by PE5. These results show that the cytotoxicity of PE5 is not prevented by a mutated p53. NCI/ADR-RES and NCI-H460/R cell lines overexpress P-gp and GST-π multidrug resistance proteins (Batist et al., 1986; Fairchild et al., 1987; Gehrmann et al., 2004; Ogretmen and Safa, 1997). MDR phenotype and mutated p53 are features that are among the most important mechanisms contributing to the MDR phenotype (Fojo and Bates, 2003). P-gp overexpression is shown to correlate with poor prognosis for a number of human cancers (Gottesman et al., 2002). In addition, NCI/ADR-RES also overexpresses XIAP and survivin anti-apoptotic proteins (Shi et al., 2007). The data presented here suggest that these RNases are more cytotoxic for cell lines representative of poor prognosis cancers. The MDR cell lines were also very affected by onconase. These results were expected since it was previously reported that onconase induces cell death in other MDR cell lines and that the pro-apoptotic activity of p53 is not required for onconase-induced apoptosis (Iordanov et al., 2000a; Michaelis et al., 2007; Rybak et al., 1996).

We have also investigated the cytotoxicity of PE5 onto non-tumor human N1 fibroblasts. We observed that N1 cells treated with PE5 were clearly less affected than those treated with onconase. We observed a 20-fold increase in IC_{50} respective to that of onconase, whereas this difference was reduced to 6-10 times in the immortalized cells (see Table 4.1 and Figure 4.2). This is indicative that the specificity of PE5 for tumor cells could be almost equal to that of onconase. Although *in vivo* onconase presents clear antitumor activity, the cytotoxicity of this RNase for non-tumor cell lines has been described to be variable. For example, Haigis and coworkers (Haigis et al., 2003) reported the cytotoxicity of onconase for different tumor and non-tumor cell lines. They found that the IC_{50} of IMR-90 lung fibroblast cell line was similar to those of tumor cell lines whereas the IC_{50} of WI-38 lung fibroblasts increased 10-fold.

Analysis of cell viability and proliferation of NCI/ADR-RES cells incubated with either PE5 or onconase show that both RNases behave dissimilarly. PE5 is mainly cytotoxic although at low concentrations a minimal cytostatic effect can be observed. On the contrary, onconase arrests proliferation at low concentrations (below to 1 μM) but begins to be cytotoxic at higher concentrations, as it has been previously described for other cell lines (Darzynkiewicz et al., 1988). Remarkably, at similar concentrations the RNases induce the same percentage of cell death.

We demonstrate here that PE5-treated cells display classical hallmarks of apoptosis such as plasmatic membrane blebbing, apoptotic body formation, chromatin condensation, nuclear fragmentation, phosphatidylserine translocation and caspase activation. This is very interesting since a non-apoptotic mode of cell death could hamper the potential application of PE5 as an anticancer drug because it could produce inflammatory or immune complications in patients. As expected, onconase also induces the apoptosis in NCI/ADR-RES, as it has been previously reported for different cell lines (Ardelt et al., 2007a; Darzynkiewicz et al., 1988; Deptala et al., 1998; Grabarek et al., 2002; Iordanov et al., 2000a; Juan et al., 1998; Tsai et al., 2004). We show here that in NCI/ADR-RES cells PE5 induce the activation of initiation of procaspases-8 and -9 which lead to the activation of executioner procaspase-3. We also

show that no changes in the proportion of Bcl-2 amounts respective to those of Bax are detected in PE5-treated NCI-ADR-RES cells. These results suggest that the treatment of NCI/ADR-RES with PE5 induces both extrinsic and intrinsic apoptotic pathways, the latter being independent of Bcl-2 and Bax.

During apoptosis cell cycle progression is stopped (Jacotot et al., 2000). The FACSCalibur flow cytometric analysis showed that cell death induced by PE5 is accompanied by a 42% accumulation of cells in the S- and G_2/M-phases in the NCI/ADR-RES cell line. We used Western blot analysis to study the effect of PE5 on different cell cycle protein regulators. We show that PE5 induces the elevation of cyclin E and $p21^{WAF1/CIP1}$ protein levels, whereas the accumulation of cyclin D_1 and $p27^{KIP1}$ remains nearly unaltered. Overexpression of cyclin E and $p21^{WAF1/CIP1}$ explains the observed reduction of the percentage of cells in G_0/G_1 cell cycle phase together with the increase of the percentage of cells in S and G_2/M cell cycle phase. Cyclin E is a G_1 cyclin expressed near the G_1 to S transition and drives entry into the S-phase by binding to CDK2. $p21^{WAF1/CIP1}$ acts as a dual inhibitor of cyclin-dependent kinases (CDKs) (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993) and proliferating-cell nuclear antigen (Waga et al., 1994). As a cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ interacts with several cyclins and CDKs leading to inhibition of these kinases with subsequent cell cycle arrest (Harper et al., 1993). Overexpression of $p21^{WAF1/CIP1}$ results in G_1-, G_2- (Niculescu et al., 1998) or S-phase arrest (Ogryzko et al., 1997). As a proliferation inhibitor, $p21^{WAF1/CIP1}$ is poised to play an important role in preventing tumor development. For example, it has been show that celecoxib leads to upregulation of $p21^{WAF1/CIP1}$ along with downregulation of cyclin B1 promoting G_2/M arrest and induction of apoptosis in a p53-independent pathway (Dvory-Sobol et al., 2006).

It has been shown that $p21^{WAF1/CIP1}$ acts as a non-enzymatic inhibitor of stress activated protein kinases (SAPKs)/JNKs (Patel et al., 1998; Shim et al., 1996). Accordingly, JNK is underphosphorylated in PE5-treated cells. Although JNK has been implicated largely in stress-induced apoptosis, under some circumstances, JNK

plays a protective role and supports cell survival, and evidence is accumulating that JNK plays a role in cell proliferation, cell transformation and tumor progression (Davis, 2000; Liu and Lin, 2005). The anti-apoptotic function of JNK has been related to the status of p53, exerting its anti-apoptotic activity in p53-deficient tumor cells (Liu and Lin, 2005). The $p21^{WAF1/CIP1}$ mediated inhibition of JNK in PE5-treated cells would promote the apoptosis of NCI/ADR-RES cells. Similar examples of induction of apoptosis by JNK inhibition have been previously reported. For example, treatment with antisense oligonucleotides to JNK2 suppresses growth and induces apoptosis of tumor cell lines with mutant but not wild-type p53 (Potapova et al., 2000). It has been also reported that the pharmacological inhibition of JNK using SP600125 is associated with an increase in the G_2/M cell cycle phase and apoptosis in KB-3 cell line (Du et al., 2004). Finally, it has been shown (Iordanov et al., 2000b) that cells immortalized via retrovirus-mediated transfer of E6 HPV gene product, which targets p53 for degradation, are more sensitive to onconase when both JNK1 and JNK2 alleles are disrupted.

Regarding to onconase, we found that it arrests proliferation of NCI/ADR-RES at low concentrations (below to 1 μM) but begins to be cytotoxic at higher concentrations as previously described for other cell lines (Darzynkiewicz et al., 1988). At IC_{50}, the cytotoxic effect of onconase is negligible and, consequently, very low amounts of caspase activity could be detected in cells treated with 1 μM onconase. We show that for this cell line, onconase does not arrest the proliferation at a defined cell cycle phase. These results suggest that the proliferation inhibitory effect of onconase on NCI/ADR-RES cells is due to a prolonged overall cell cycle rather than a specific arrest at a particular phase of the cell cycle. Although a general consensus in the literature indicates that onconase arrests proliferation at the G_0/G_1 cell cycle phase (Deptala et al., 1998; Halicka et al., 2000; Juan et al., 1998; Michaelis et al., 2007), this is far from always being the rule. Onconase arrests NIH/3T3 cells at the G_2/M cell cycle phase (Smith et al., 1999), but for Jurkat cells the arrest of proliferation induced by onconase could not be attributed to alterations in the cell cycle phase progression (Tsai

et al., 2004). Proliferation arrest is accompanied by a decrease in the expression of both cyclin D_1 and cyclin E in this cell line while it does not affect the expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. It has been previously reported that onconase upregulates the expression of $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p16^{INK4A}$ in histiocytic lymphoma U937 cell line (Juan et al., 1998). This upregulation is accompanied by the arrest of proliferation at the G_1 cell cycle phase. Therefore, the effects of onconase on the cell cycle and on the expression of these protein regulators are dependent of the cell line used as a model. Interestingly, we have observed that proliferation arrest induced by onconase promotes the increase of the level of Bcl-2 anti-apoptotic protein and the overphosphorylation of the p46 JNK form in NCI/ADR-RES which, as discussed above, seems to support cell survival in a p53 mutant context. These observed effects may reflect a situation in which the NCI/ADR-RES cells are able to efficiently respond to the metabolic effects of onconase by inducing different survival mechanisms.

The mode by which onconase induces cell death has been shown to be dependent on the cell line used in the study. Onconase has been shown to induce apoptosis by activating caspases and serine proteases in some cases (Grabarek et al., 2002; Iordanov et al., 2000a) but, recently, onconase-induced caspase-independent death of neuroblastoma cell lines have been reported (Michaelis et al., 2007). Although processing of procaspases-9, -3, and -7 but not of procaspase-8 has been described (Iordanov et al., 2000a), it is not clear to what extent the mitochondrial apoptotic pathway is involved since no cytochrome c release or sustained decrease of Bax has been observed (Iordanov et al., 2000a). In contrast, for the HL-60 human promyelocytic leukemia cell line, onconase induced the expression of Bax while decreasing that of Bcl-2 (Ardelt et al., 2007b). We show here that cytotoxic concentrations of onconase (5 μM) induce the activation of initiation of procaspases-8 and -9 which lead to the activation of executioner procaspase-3. Analysis of the cell cycle distribution of cells treated with these concentrations of onconase also indicates that this drug does not preferentially kill NCI/ADR-RES at any specific cell cycle phase. Nevertheless, the effect of 5 μM onconase on NCI/ADR-RES cells is clearly

different to that of 1 μM onconase treatment. First of all, treatment with 5 μM onconase decreases 2-fold the concentration of Bcl-2 and 3-fold the concentration of XIAP respective to the treatment with 1 μM. On the other hand, under these conditions we have not detected significant differences in the level of the phosphorylated form of JNK respective to untreated control cells. It is therefore clear that at cytotoxic concentrations onconase is bypassing three different mechanisms of survival (Bcl-2, XIAP and overphosphorylation of JNK), two of which are induced at cytostatic concentrations.

The effects of PE5 on NCI/ADR-RES cells are clearly different to those induced by the treatment with onconase. The cell cycle phase distribution and the proportion of cells at early and late apoptosis are very different. On the other hand, PE5 and onconase seem to be bypassing different mechanisms of survival, mediated by XIAP and JNK, respectively. These results are very indicative that both RNases induce apoptosis by different mechanisms.

In this work we show that PE5 reduces the amount of accumulated P-gp in MDR cell lines. This reduction is time- and dose-dependent and is not restricted to a single cell line. The reduction seems to be specific, since we do not observe changes in the accumulation of the overexpressed GST-π MDR protein. Onconase produces no change in the P-gp levels either in NCI/ADR-RES or in NCI-H460/R cell lines. Accordingly, Ryback and colleagues (Rybak et al., 1996) reported that the treatment of the HT-29^{mdr1} MDR cell line with onconase did not affect the cellular accumulation of vincristine. It is possible that the different effects of PE5 and onconase on P-gp levels could be, at least in part, a consequence of their differential ribonucleolytic activity on cytoplasmatic and nuclear RNA, respectively (Tubert et al., 2010).

Treatment with 10 μM verapamil for 1 h produced an approximately 266% and 340% increase of doxorubicin accumulation in NCI/ADR-RES and NCI-H460/R, respectively. Doxorubicin accumulation induced by PE5 in these cell lines was less pronounced (100% for NCI-H460/R and 50% for NCI/ADR-RES cells treated with fumitremorgin C) (see Figures 4.14 and 4.15), because a significant amount of P-gp

active molecules were still remaining in the PE5-treated cells. Similar differences in the doxorubicin accumulation have been reported when NCI/ADR-RES cells were treated with cyclohexylpiperazine derivative PB28, a σ_2 receptor agonist and σ_1 receptor antagonist that reduces the expression of P-gp (Azzariti et al., 2006).

As mentioned above, PE5 but not onconase induces the underphosphorylation of JNK in NCI/ADR-RES cells. It has been reported that JNK is activated in human carcinoma cells by treatment with a number of different anticancer drugs and that this activation of JNK correlates with increased MDR expression (Osborn and Chambers, 1996). It has been also shown that glucose depletion or hypoxia increases MDR1 expression which seems to be mediated by activation of the JNK signaling pathway (Comerford et al., 2004; Ledoux et al., 2003). On the other hand, opposite results were found in MDR cell lines when adenovirus-mediated enhancement of JNK was used (Zhou et al., 2006). It is tempting to speculate that PE5 could decrease the accumulation of P-gp through decreasing the expression of MDR1 via deactivation of JNK. JNK is known to phosphorylate and activate c-Jun, which, together with c-Fos, constitutes the heterodimeric AP-1 transcription factor (Hibi et al., 1993). It is also known that there are AP-1 binding sites on the promoter of MDR1 (Ikeguchi et al., 1991) and a positive correlation between AP-1 activation and MDR1 transcription has been reported (Volm, 1993).

One of the objectives of our study was to determine the nature of the interactions between selected RNases and the broadly used chemotherapeutic agent doxorubicin. Since the most affected cell lines contained an MDR phenotype, we explored what kind of interaction was produced between doxorubicin and PE5 or onconase in NCI/ADR-RES. Previously, onconase was shown to be synergistic with vincristine in another MDR cell line (HT-29^{mdr1}) (Rybak et al., 1996).

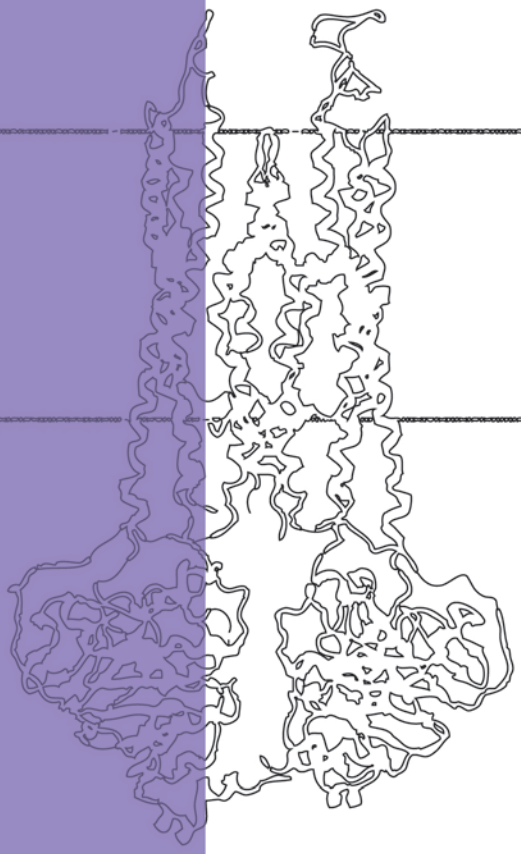
The effect of the combination of onconase and doxorubicin has been previously reported *ex vivo* in chronic lymphocytic leukemia (CLL) and acute myeloblastic leukemia (AML) cells (Smolewski et al., 2008a; Smolewski et al., 2008b). A synergistic effect of the combination of doxorubicin and onconase could be demonstrated for the

CLL cell (combination index of 0.67) (Smolewski et al., 2008b) but not for the AML cells (Smolewski et al., 2008a). Here, we show that the combination of onconase at the assayed concentrations and doxorubicin is synergistic for both MCF-7 and NCI/ADR-RES cell lines.

The synergistic effect of combinations of onconase and various different antitumor drugs with diverse structures and modes of action has been attributed to onconase's ability to specifically cleave the family of miRNAs shown to enhance tumor resistance to cytotoxic anticancer therapy by mobilizing the cell defense mechanisms (Ardelt et al., 2003). In some cases this synergistic effect has been explained by the cleavage of the mRNA of genes implicated in survival pathways by onconase (Deptala et al., 1998; Ita et al., 2008; Ramos-Nino and Littenberg, 2008).

The combination of PE5 at the assayed concentrations and doxorubicin is synergistic in NCI/ADR-RES but not in MCF-7 cell line. Although PE5 reduces significantly the amount of P-gp, no differences are observed in doxorubicin accumulation in the NCI/ADR-RES cell line after PE5 treatment. This might be due to functional redundancy with other transport systems that mask the effect on P-gp produced by PE5. Thus, the observed synergy between PE5 and doxorubicin in this cell line cannot be attributed to PE5 increasing the intracellular concentration of doxorubicin by decreasing the P-gp levels. Several reports provide evidence that P-gp might promote cell survival independently of cytotoxic drug efflux. For example, tumor cells overexpressing P-gp are resistant to caspase-dependent apoptotic inducers like tissue necrosis factor, serum starvation or UV irradiation (Johnstone et al., 1999; Robinson et al., 1997; Ruth and Roninson, 2000). It has been also shown that the P-gp inhibitor PSC 833 promotes cell cycle arrest and apoptosis in human leukemia cells (Lehne et al., 1999; Lehne and Rugstad, 1998) and delays engraftment in a mouse leukemia xenograft model (Lehne et al., 2002). Furthermore, the inhibition of P-gp function enhanced Fas-mediated activation of caspase-3 in MDR leukemia cells (Smyth et al., 1998) and the silencing of the MDR1 gene by using an RNA interference method suppressed tumor cell proliferation *in vitro* (Katoh et al., 2008). Therefore, it is possible

that the synergistic effects between doxorubicin and PE5 in NCI/ADR-RES could be mediated by the suppression of the survival mechanisms induced by P-gp.



6. CONCLUSIONS

1. PE5 is cytotoxic for different human tumor cell lines representative of breast, lung, ovarian and cervical cancer, the IC_{50} of PE5 being 6-10-fold higher than that of onconase for the cell lines assayed. PE5 and onconase are more cytotoxic against those cell lines representatives of poor prognosis cancers. The multidrug-resistant cell lines NCI/ADR-RES and NCI-H460/R are the most affected by both RNases.

2. The cytotoxicity induced by PE5 or onconase does not require the pro-apoptotic activity of p53.

3. The cytotoxic effects of PE5 and onconase are not affected by the overexpression of HER2/neu.

4. Even though PE5 and onconase are cytotoxic for N1 human fibroblast cells, the IC_{50} of PE5 is 20-fold higher than that of onconase. This difference suggests that PE5 presents a selective toxicity against cancer cells almost equal to that of onconase *in vitro*.

5. PE5 is cytotoxic for NCI/ADR-RES cells even though at low concentrations it shows a slight cytostatic effect. Onconase arrests the cell growth of NCI/ADR-RES cells at low concentrations (around 1 μM). When cells are treated with higher concentrations of onconase it displays significant cytotoxic activity.

6. The percentage of cell death induced by PE5 or onconase at a given concentration is very similar.

7. PE5 increases the percentage of cells in S and G_2/M cell cycle phases in NCI/ADR-RES, which is associated with an increase in the cyclin E and $p21^{WAF1/CIP1}$ expression. The amount of cyclin D_1 and $p27^{KIP1}$ remains unchanged in PE5-treated cells.

8. The growth inhibitory effect of onconase results in a prolongation of the overall cell cycle in NCI/ADR-RES, which is accompanied by a reduction in the cyclin D_1, cyclin E. The levels of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ remain nearly unaltered.

9. NCI/ADR-RES cells treated with PE5 show typical morphological hallmarks of apoptosis such as chromatin condensation, nuclear fragmentation, membrane blebbing and apoptotic body formation, as observed for cells treated with onconase.

10. PE5 and onconase cause translocation of phosphatidylserine to the outer face of the cells and induce the activation of caspases-8, -9 and -3, which are characteristic markers of apoptosis induction. The proportion of cells in early and late apoptosis after the treatment with both RNases is different.

11. Treatment with PE5 changes neither the proportion of Bcl-2 amounts respective to those of Bax nor the expression levels of XIAP but reduces the phosphorylation levels of the p46 forms of JNK.

12. Cytostatic concentrations of onconase produce a slight increase in Bcl-2 accumulation and an overphosphorylation of p46 forms of JNK, whereas the expression levels of Bax and XIAP remain unaltered.

13. Cytotoxic concentrations of onconase decrease the level of the XIAP protein. Nevertheless, changes in the amount of Bax and Bcl-2 and the phosphorylation levels of p46 JNK are not detected respective to untreated cells.

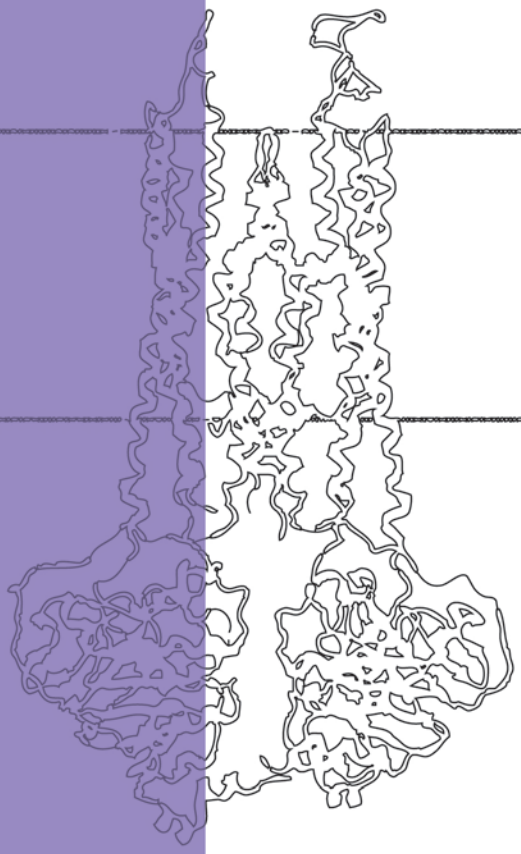
14. The combination of PE5 with doxorubicin is synergistic in the doxorubicin-resistant NCI/ADR-RES cell line but not in the doxorubicin-sensitive MCF-7 cell

line, whereas onconase presents synergistic cytotoxicity with doxorubicin in both the NCI/ADR-RES and the MCF-7 cell lines.

15. PE5, but not onconase, reduces in a time- and dose-dependent manner the levels of P-gp. This reduction is not restricted to a single cell line. PE5 treatment does not change the amount of the GST-π MDR protein in NCI/ADR-RES.

16. Treatment with PE5 produces a 100% and 50% increase in doxorubicin accumulation in NCI-H460/R and NCI/ADR-RES cells treated with fumitremorgin C, respectively.

17. PE5 decreases the P-gp levels in the NCI/ADR-RES cell line. However, this is not accompanied by an increase in doxorubicin accumulation. Therefore, the observed synergistic effect between PE5 and doxorubicin might be mediated by the suppression of the survival mechanisms induced by P-gp.



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