THE ROLE OF POLY (ADP-RIBOSE) POLYMERASE-1 IN THE CELLULAR RESPONSE TO SEVERAL MARINE-DERIVED COMPOUNDS

By

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ABSTRACT OF THE THESIS

The Role of Poly (ADP-Ribose) Polymerase-1 in the Cellular Response to Several Marine-Derived Compounds

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PARP-1 is a multi-functional protein that is involved in many DNA-dependent genomic processes under normal and pathophysiological conditions. It is well characterized as a DNA damage detector and responds by catalytic production and attachment of polymers of ADP-ribose (PAR) to nuclear protein targets, facilitating the chromatin changes that are a prerequisite to DNA repair. In this study, we tested whether PARP-1 is involved in the cellular response to Yondelis®, Zalypsis®, PSL1, and PSL2, novel chemotherapeutic agents with putative DNA damage- and transcription-targeted activities. We observed a dose-dependent activation of PARP-1 catalytic activity following treatment with all four compounds, while PARP-1 protein levels remained unchanged. Interestingly, cells derived from PARP-1 null mice were significantly sensitized to the agents, yet, with respect to Yondelis®, only moderate DNA damage was observed which was repaired with equal efficiency by both PARP-1 wildtype and PARP-1 null cells. While the mechanism of sensitization is unclear, it is of interest to determine whether inhibition of PARP in human cells could sensitize

cells to the four agents. Initial *in vivo* experiments testing this prediction using MX-1 breast carcinoma xenografts treated with Yondelis® alone or in combination with the PARP-1 inhibitor DIQ, demonstrate an additive effect between these two compounds with regard to tumor volume inhibition and tumor growth delay. However, corresponding *in vitro* experiments failed to corroborate this observation. The effects of PARP-1 on the transcription of genes impacting drug sensitivity, as well as the cyto-protective role of PARP-1 independent of its catalytic function are of interest to direct future efforts to clarify the mechanism of PARP-1-mediated sensitivity to the four agents. Taken together, these data suggest that PARP-1 plays an important role in the protection of cells to Yondelis®, Zalypsis®, PSL1, and PSL2, and suggest that PARP-1 status may determine the sensitivity or resistance of cells treated with these agents.

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It is with utmost humility that I offer my lasting gratitude to my mentor and thesis advisor, Dr. Kathleen Scotto. Her stellar accomplishments in the field of multi-drug resistance of cancer attracted me to her lab, but her disarming demeanor and generous patience easily convinced me to continue my academic training under her tutelage. In deed, the comfortable yet scientifically stimulating atmosphere that Dr. Scotto has created in her lab, encapsulates the perfect balance of intellectual prowess and genial disposition of its members. It is under the superb direction of Dr. Scotto that I have realized the true value of the expression, "I don't know." Initially a symbol of shameful ignorance, it has come to represent the excitement of discovery inherent to all science. Thus, although I thank Dr. Scotto with utmost sincerity for all of her guidance in my academic and scientific maturation, I am eternally grateful for her gift of altered perspective.

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The practice of basic science research is often insular. However, no man is an island and therefore no scientist can live on science alone. Words cannot express the degree to which my fellow lab-mates, both current and those whom have gone on to greener pastures but have never left my heart, have sustained

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Finally, but most importantly, I yield my heart as ever-present gratitude to my Hili. Her tolerance of my selfishness and her capacity for joy are immeasurable. I could not have come this far and cannot aspire for further success without her forming the essence of who I am. Teşekkür ederim, Hilalim.

Dedication

In loving memory of my father, Bipinchandra Patel.

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Chapter 1

Introduction

Over the last 50 years the marine environment has gradually changed from a transient source of marine-derived drugs, to one that is much more reliable and amenable to systemic searches for such compounds. The work by Bergman *et al.* on the sponge *Cryptotheca crypta*, which lead to the identification of the novel nucleosides, spongothymidine and spongouridine, (1-3), and the plethora of their subsequent derivatives (4), is considered the earliest and quintessential example of a marine-derived drug successfully reaching market. Since then, there has been growing scientific effort, especially by pharmaceutical companies, to develop newly discovered compounds of marine origin into marketable drugs. Although this dedication has resulted in many marine-derived agents targeting a wide range of diseases (5), the vast majority of the marine natural products that have entered clinical trials are anti-cancer agents (6).

Recently, a pharmaceutical company interested in targeting compounds of marine origin for therapeutic applications, PharmaMar SAU, has purified several novel, yet related, marine-derived compounds, Yondelis®, Zalypsis® (PM00104), PSL1, and PSL2, and has begun their pharmacological development as anti-cancer agents. Of the four compounds, Yondelis®, derived from the Caribbean tunicate *Ecteinascidia turbinate* (7), is the best studied and has been granted orphan drug status for the treatment of ovarian cancer in the EU and U.S.A., as well as marketing authorization for the treatment of advanced soft tissue sarcoma

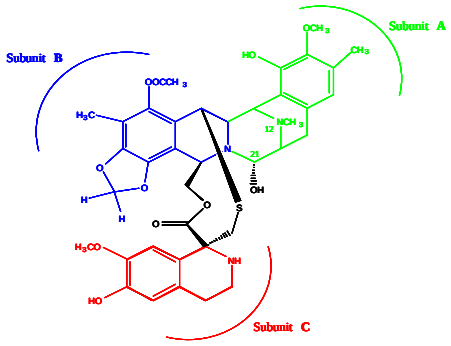

Figure 1. Stucture of Yondelis®

after failure of anthracyclines and ifosamide in the EU (personal communication). Currently, Yondelis® is in Phase III combination clinical trials for ovarian cancer (8,9), in Phase III clinical trials for monotherapy of soft tissue sarcoma (10), and in Phase II clinical trials for monotherapy of breast carcinoma (11). Structurally similar to the saframycin family of antibiotics, Yondelis® is comprised of 3 tetrahydroisoquinoline subunits (Figure 1); subunits A and B are responsible for binding to the minor groove of DNA, after which Yondelis® forms covalent adducts by bonding the N-2 of guanine of DNA to its highly reactive carbinolamine moiety, while subunit C is putatively responsible for promoter-specific inhibition of transcription (12,13). Yondelis® displays potent and broad-spectrum anti-tumor activity *in vitro* in a variety of tumor cells (14), has been granted orphan drug status for the treatment of refractory soft-tissue sarcomas in Europe (15), and is currently in Phase III and Phase I combination clinical trials, where it has demonstrated efficacy in a number of tumor types including sarcoma, breast and ovarian cancers (16-23). Although pre-clinical studies have confirmed that Yondelis® can in deed bind the minor groove of DNA at guanine residues *in vitro* (24), other observations have set Yondelis® apart from most DNA binding agents. First, Yondelis® possesses the unique ability to bend DNA towards the major groove (12), in contrast to most other DNA-interacting agents which bend DNA towards their binding groove. A

second unique feature of this compound, initially described by our laboratory (25,26) and others (27), is its ability to inhibit the transcriptional activation of a variety of promoters, without significant impact on their constitutive expression. Indeed, activation of the human multidrug-resistance (*MDR1)* and cyclin-dependent kinase inhibitor 1a (*p21)* promoters by a variety of inducers, is blocked by treatment with Yondelis®, while basal levels of transcription from these promoters are unaffected.

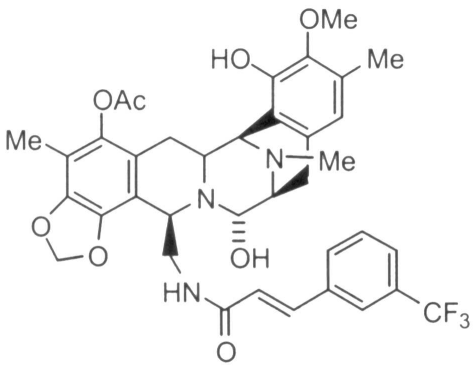

Figure 2. Structure of Zalypsis®

Zalypsis® (PM00104) is a more recent entry into the realm of anti-cancer agents of marine origin that enjoy clinical trial status (28,29). It is a tetrahydroisoquinoline (Figure 2) related to the antibiotic jorumycin, a compound originally derived from the nudibranch *Jorunna funebris* (30), and to the family of renieramycins, antibiotics derived from sponges and tunicates (31,32). Preliminary biological evaluation of the agent suggests that Zalypsis® displays potent *in vitro* cytotoxicity in human cancer cell lines, and *in vivo* cytotoxicity in murine models of human cancers (29). Previous studies in our lab investigating the mechanism of action of Zalypsis® indicate that the anti-cancer agent can induce cell cycle arrest and apoptosis, as well as bind (33) and damage DNA (data not shown). Other investigations have indirectly shown that Zalypsis® induces double strand DNA damage and a subsequent

DNA damage repair response (34). Moreover, using microarray analysis, *Ocio et al.* have demonstrated that Zalypsis® can both activate and inhibit expression of several genes, although it is not yet known whether this occurs at the level of transcription or post-transcriptionally (34). However, our work investigating the mechanism of Zalypsis®-mediated transcriptional regulation using the *MDR1* transcription system indicates that Zalypsis® can inhibit trichostatin A (TSA)-mediated activation of the *MDR1* promoter, without affecting its basal expression, similar to Yondelis®'s transcriptional impact on said gene (33). Further, we now show that Zalypsis® can also inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated activation of *MDR1*, without affecting constitutive transcription, thereby expanding the repertoire of transcriptional inducers whose activity is inhibited by Zalypsis®.

PSL1 and PSL2 are two of the newest PharmaMar compounds to be isolated from the marine environment that are targeted for anti-cancer treatment, specifically for solid and hematological tumors (35). However, due to their relatively recent discovery and purification, these putative anti-cancer agents are currently limited to preclinical status. In order to further their characterization, we have included these new marine-derived compounds in the current study of Yondelis® and Zalypsis®. We show for the first time that, similar to Zalypsis® and Yondelis®, PSL1 and PSL2 can inhibit the activation of the *MDR1* promoter without affecting its basal transcription.

Although the molecular basis underlying the transcriptional inhibition of *MDR1* mediated by the four compounds remains to be elucidated, our studies suggest that Yondelis® is not selective for a specific gene, transcription factor or inducer, since we and others have shown inhibition of multiple promoters activated by different inducers through different transcriptional regulators (25-27). Moreover, in the cases studied, Yondelis®-mediated inhibition of transcription is not facilitated via the direct displacement of transcription factors from their binding sites *in vivo* (27). Thus, it appears that Yondelis® targets a general factor/mechanism required for transcriptional activation. While it is possible that the DNA damage and unique but broad-spectrum transcriptional effects of Yondelis® or Zalypsis® are not linked, we have begun our search for mediators of Yondelis® or Zalypsis® action by investigating proteins that play a role in both activities of either agent.

Poly (adenosine diphosphate-ribose) polymerase (PARP-1) is a highly conserved, abundant nuclear enzyme that has been well characterized as an early sensor of DNA damage, and as an important signaling protein in the repair of DNA lesions (36). More than forty years of research have led to the characterization of PARP-1 as a dynamic protein involved in a multitude of genetic processes following DNA damage including: transcription, DNA repair, replication, and chromatin remodeling. Upon binding to damaged DNA, PARP-1's catalytic function becomes rapidly and potently activated, leading to the production of poly (adenosine diphosphate-ribose) (PAR) polymers using cellular

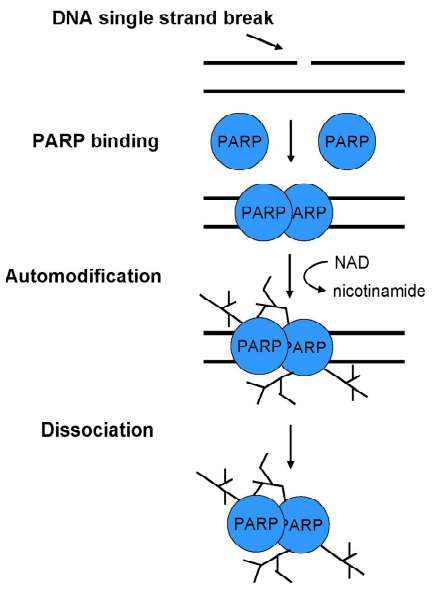

Figure 3. DNA damage-mediated mechanism of action of PARP-1. (Modified from *DNA Repair (Amst). 2008 Jul 1;7(7):1077-86. Epub 2008 May 12.*)

nicotanimide adenine dinucleotide (NAD^+) as a substrate. In a process known as poly (adenosine diphosphate-ribosyl)ation (PARylation), these highly negatively-charged molecules are attached to a variety of acceptor proteins at glutamic and aspartic acid residues. The ribosylation targets of PARP-1 following DNA damage are transcription factors, histones, both RNA and DNA polymerases, as well as the major target, PARP-1 itself (37). The addition of PAR polymers to these nuclear protein acceptors in response to DNA damage causes them to dissociate from the DNA through electrostatic repulsion and alleviates the strong steric hindrance caused by transcription and/or replication complexes, thereby allowing repair enzymes access to the damaged DNA (Figure 3). Overall, PARP-1 activation helps to insure that defective DNA is neither transcribed nor replicated in the event of DNA damage.

More recently, a role for PARP-1 in gene regulation under normal, non-pathophysiological conditions has begun to emerge (38). Considerable evidence is accumulating that suggests that PARP-1 is involved in the transcriptional activation (39-47), and in some cases inhibition (48), of a variety of promoters in the absence of DNA damage (Figure 4); indeed PARP-1 binding

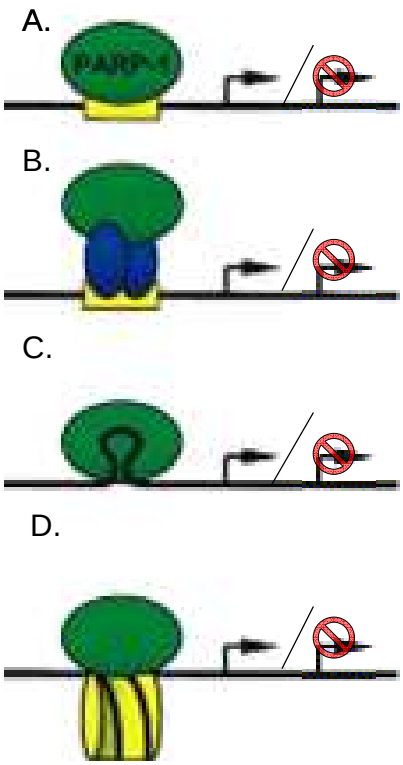

Figure 4. Modes of PARP-1-mediated transcriptional regulation. PARP-1 positively or negatively impacts transcription by A. directly binding to sequence-specific regions of promoters, B. acting as a co-activator or co-repressor, C. binding to secondary DNA structures (e.g. bent or cruciform DNA), and/or D. interacting with higher order DNA-protein structures such as nucleosomes and chromatin. (Modified *from Cell 2003 June 13;113(6):677-83. Epub 2003 April 15.*)

was shown to be an essential step in the formation of RNA polymerase II pre-initiation complexes (49). Moreover, basal ribosylation has been implicated as a post-translational modification important in transcriptional activation, as the addition of single units or oligomers of PAR to nuclear proteins confers a negative charge, not unlike phosphorylation or acetylation modifications. Studies on PARP-1 in *Drosophila* have recently demonstrated that PARP-1 is distributed widely throughout the genome, and can likely manipulate the structure of chromatin differentially in response to a variety of different stimuli (50). Indeed, PARP-1 was recently compared to histone acetyltransferases (HATs) and other NAD/ATP-dependent chromatin-modifying enzymes, in that it can affect many DNA dependent processes including transcription, replication and repair (34).

The current consensus among investigators is that PARP-1 acts as a molecular switch. Under normal conditions PARP-1 is intimately involved with active transcriptional complexes in order to enhance, stimulate or repress transcription;

in the event of DNA damage PARP-1 becomes more catalytically active (producing up to 500 times more PAR compared with normal cells) and quickly halts transcriptional complexes, alters chromosomal structure, and undergoes automodification in order to prevent the transcription of damaged DNA and to allow DNA repair machinery access to the DNA.

Given the dual role of PARP-1 in DNA repair and transcriptional regulation, we sought to determine whether this multifunctional protein is involved in the cellular response to Yondelis®, Zalypsis®, PSL1, or PSL2. We observed an increase in PARP-1 catalytic activity following exposure to the four compounds, as well as an attenuation of said PARylation upon pretreatment with the PARP-1 catalytic inhibitor 1,5-isoquinolinediol (DIQ). Further, we have investigated whether the loss or inhibition of PARP-1 could alter the sensitivity of cells to Yondelis®, Zalypsis®, PSL1, or PSL2. Using PARP-1 null mouse embryonic fibroblast cells, we demonstrate that loss or inhibition of PARP-1 hyper-sensitizes cells to all four agents. Taken together, these data strongly suggest that PARP-1 plays a cellular protective role against the compounds, and implicates PARylation as a surrogate marker for the activity of the putative antitumoral agents. Interestingly, the PARP-1-dependent sensitivity to Yondelis® that we observed does not directly correlate with DNA damage and repair, suggesting that another function of PARP-1 may be the critical determinant of Yondelis®- mediated cytotoxicity. As such, our data prompt us to further explore the role that PARP-1 may play in

the cellular response to not just Yondelis® but also to Zalypsis®, to PSL1, and to PSL2.

Chapter 2

Materials & Methods

Cell culture.

The pancreatic adenocarcinoma cell lines (HTB-147, MIA PaCa-2, Panc-1, AsPc-1), the colon carcinoma cell line SW620, the breast carcinoma cell line MX-1, and the ovarian adenocarcinoma cell line OVCAR-3 were maintained in RPMI 1640 media (Mediatech, Inc.). The ES-2 and SK-OV-3 ovarian adenocarcinoma cell lines, the osteosarcoma cell lines U2-OS and SaOS2, and the colon carcinoma cell line HCT-116 were maintained in McCoy's 5A media (Invitrogen). Mouse embryonic fibroblasts (MEFs) were maintained in DMEM (Mediatech Inc.). The growth medium used for OVCAR-3 was supplemented with 20% non-heat-inactivated FBS, and the growth medium used for SaOS-2 was supplemented with 15% heat-inactivated FBS. All cell lines were grown under 37ºC and 5% CO2 conditions. Unless otherwise indicated, all growth media were supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 2mM L-glutamine (Mediatech, Inc.), 100 U/mL penicillin (Invitrogen), and 100ug/mL streptomycin (Invitrogen). MEFs were kindly provided by Dr. Z.Q. Wang (International Agency For Research on Cancer, Lyon, France). All other cell lines, except the colon carcinoma cell lines and the SK-OV-3 cell line, were obtained from the American Type Culture Collection.

MTS cytotoxicity assay.

All drugs including the PharmaMar compounds were dissolved in DMSO and stored at -80ºC. Drugs or PharmaMar compounds were diluted to appropriate concentrations in the media corresponding to the cell lines under investigation. U2-OS or SaOS2 cells were seeded into 96-well plates at 15,000 cells per well or 8,000 cells per well, respectively, and, following a 24h growth incubation, were treated with indicated agent for 72h at 37ºC. All other cell lines were seeded into 96-well plates at 5,000 cell per well and treated as per the osteosarcoma cell lines. *In vitro* cytotoxicity to the agents was determined using the MTS CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Cat# G5430) as per manufacturer's protocol. Doxorubicin was obtained from Sigma. All other agents were provided under a contract with PharmaMar SAU (Madrid, Spain).

Western blotting.

Cells were harvested in whole cell lysis buffer containing 50mM Tris pH 7.4, 150mM NaCl, 0.5% nonyl phenoxylpolyethoxylethanol (NP-40), 0.5% sodium deoxycholate, 1mM EDTA pH 7.5, 8.5 X 105 g/mL PMSF, and one half tablet of complete® Mini, EDTA-free (Roche) per 5mL buffer. Thirty micrograms of protein were separated on a 10% SDS/PAGE gel and transferred to nitrocellulose membrane. Blots were subsequently blocked using ECL Advance™ blocking agent (GE Healthcare) in 2% TBS-Tween (0.1% (vol/vol) Tween 20) and incubated with anti-PAR, anti-PARP, or anti-tubulin antibody (Trevigen) at 1:1,000 dilution, 1:500 dilution, or 1:5,000 dilution respectively. The

Amersham™ ECL Advance™ system, as well as the Bio-Rad ChemiDoc™ XRS and its accompanying Quantity One® software were used for detection of relevant bands.

DNA damage/repair assays.

Single cell electrophoresis of DNA (comet assay) were performed using the Comet Assay Kit (Trevigen Inc.) following the manufacturer's protocol. Briefly, cells were treated in 6-well dishes as above, scraped into PBS, mixed with molten LMA agarose, then spotted onto comet slides that were allowed to polymerize for 30 minutes at 4ºC. Slides were immersed in cold lysis solution (provided with kit) for 1 hour, followed by immersion in alkaline solution (pH>13) for an additional hour. Cells were washed twice in 1 x TBE for 5 minutes and electrophoresed on a horizontal electrophoresis apparatus for 20 minutes at 20 V (1 V/cm). Following electrophoresis, DNA was immersed in 70% ethanol, dried and stained with SYBR green for visualization using fluorescence microscopy. Comet olive tail moment was measured using Auto Comet Software (TriTek). A minimum of 50 comets were scored for each treatment and the mean and standard deviation were determined and plotted. Each experiment was performed at least three times in duplicate. For assessment of DNA damage, cells were incubated with the indicated amount of Yondelis® or doxorubicin for 24 hours. For assessment of DNA repair, cells were incubated with 3 nM Yondelis® or 10 nM doxorubicin (the IC_{50} values for the PARP-1 +/+ cells) for 3

hours, at which time the compound -containing media was washed out and replaced with compound-free media.

Animal studies.

Athymic nude mice bearing the nu/nu gene (Taconic Farms) were used for the MX-1 xenografts. All mice were female, between 4-6 weeks old and weighed between 18-20 g at study initiation. Each mouse was injected s.c. with $5x10^6$ MX-1 cells in 100 uL of PBS. Tumors were given 14 days to reach palpable size, and mice were then randomized into five treatment groups. For Yondelis® treated groups, mice were injected i.v. via the tail vein q3dx3 with either 30 or 50 ug/kg Yondelis® in 100 ul volume. For DIQ injections, mice were injected i.p. with 3 mg/kg of drug in 100 ul volume q3dx3. For drug combinations, DIQ was injected i.p. 1 hour prior to Yondelis® injections. For control treatments, 100 ul of a 1% DMSO-PBS solution was injected i.p. one hour prior to a 100 ul i.v. tail vein injection of a 0.1% DMSO-PBS solution. Following treatments, tumor volumes were recorded using a digitial caliper with the calculation: (l x w x h) x (π/6). Animal weights were recorded in order to measure toxicity. Tumor volume inhibition was calculated as % tumor growth compared to control. Mice in each group were sacrificed when tumor volume reached 1600 m^3 or due to tumor necrosis. Tumor growth delay was measured as the time (days) to 1000 mm^3 for each treatment group, as compared to control.

Luciferase assay for MDR1 promoter activity.

SW620 cells were stably transfected as described (51) with the pMDR1(-1202) reporter construct, in which the MDR1 promoter sequence (-1202 to +118) was inserted upstream of the luciferase gene in the pGL2B vector (Promega). Cells were plated at a density of 3.75 x 10^5 cells/well in 6-well plates, and 24 hours later, media was replaced with drug-containing media. Drug treatments were conducted using 16mM TPA (Sigma), 50nM Yondelis®, 50nM Zalypsis®, 50nM PSL1, and/or 50nM PSL2. Following 24 hours of exposure to the drugs, cell extracts were prepared, and luciferase activity was determined using Thermo Scientific Luminoskan® Ascent. Activity was expressed as luminescence units normalized to protein concentration as determined by the BCA protein assay.

Chapter 3

Results

Cytotoxicity analysis of Yondelis® in multiple cancer cell lines.

To establish the general cytotoxicity profile of Yondelis®, cells of several cancer types in which Yondelis® has been demonstrated to be efficacious (osteosarcoma and ovarian adenocarcinoma) (22,23), as well as cancer types in which there is limited information on the potency of Yondelis® (pancreatic adenocarcinoma and colon carcinoma) were treated with titrated dosage of the agent (Table 1). As gemcitabine is a common front-line drug used against pancreatic and ovarian adenocarcinoma (52,53), its cytotoxicity was included as a reference. Of all cancer types tested, the colon carcinoma and the osteosarcoma cell lines were the most sensitive to Yondelis®, displaying IC50 values reaching less than 2,000 fold above the pancreatic and ovarian adenocarcinoma cell lines. Nevertheless, the latter two cell lines were exquisitely sensitive to Yondelis® compared to gemcitabine, and all the IC50 values of Yondelis® in all cell lines tested are consistent with the evaluation of the compound in the National Cancer Institute Developmental Therapeutics Program Human Tumor Cell Line Screen (NSC# 648766) (54).

Cancer	Cell Line	Treatment	IC50 (nM)
Pancreatic Adenocarcinoma	HTB147	Yondelis®	3
		Gemcitabine	600,000
	MIA PaCa-2	Yondelis®	0.07
		Gemcitabine	790
	Panc-1	Yondelis®	3.9
		Gemcitabine	400,000
	AsPc-1	Yondelis®	4.7
		Gemcitabine	3,500
Ovarian Adenocarcinoma	ES-2	Yondelis®	0.12
		Gemcitabine	5,000
	OVCAR-3	Yondelis®	2.8
		Gemcitabine	15,000
	SK-OV-3	Yondelis®	4
		Gemcitabine	60,000
Colon Carcinoma	SW620	Yondelis®	0.07
	HCT-116 p53 +/+	Yondelis®	0.24
	HCT-116 p53-/-	Yondelis®	0.006
Osteosarcoma	U2-OS	Yondelis®	0.02
	SaOS-2	Yondelis®	0.23

Table 1. Cytotoxicity profile of Yondelis® in various cancer cell lines.

Analysis of Yondelis®-mediated PARP-1 catalytic activity.

As mentioned in the introduction, Yondelis® has been demonstrated to target both DNA and activated transcription. Since PARP-1 has been implicated in both of these processes, we reasoned that PARylation and/or PARP-1 protein expression may be modulated by Yondelis®. Using mouse embryonic fibroblast (MEF) cells that expressed PARP-1 (PARP-1 +/+) or their isogenic counterparts (MEF PARP-1 -/-), we tested this prediction by treating cells with Yondelis® to establish their PARylation profile. As shown in Figure 5, Yondelis® induced PARylation in a concentration-dependent manner in PARP-1 +/+ MEFs without affecting PARP-1 protein expression. As expected, neither PARP-1 protein expression nor PARylation was observed in the PARP-1 -/- MEFs upon

Yondelis® treatment. These experiments were extended into human cancer cells and, as indicated in Figure 6, resulted in similar observations.

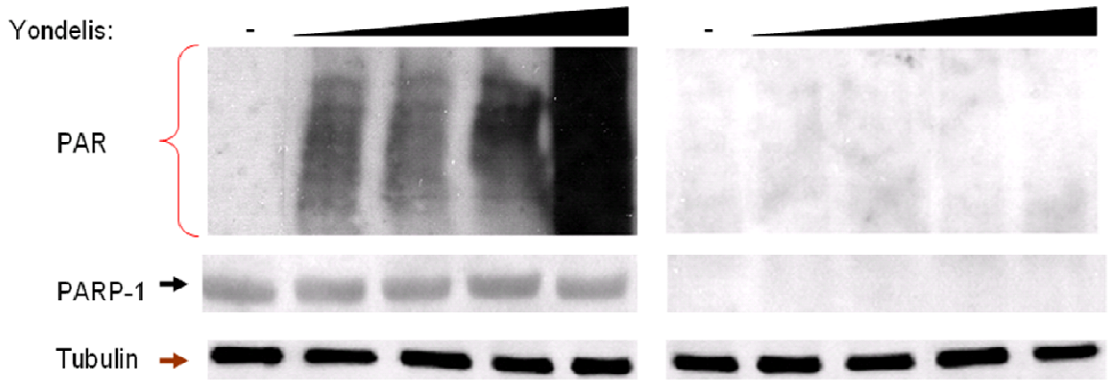

Figure 5. Yondelis® induces PARylation in MEF PARP-1 +/+ cells (left panel) but not PARP-1 -/- cells (right panel). Cells were treated with 1-1000nM of Yondelis® for 15 minutes and tested for PARylation via Western blot analysis (red brace). Tubulin was used as a loading control (brown arrow). Yondelis® did not affect PARP-1 protein expression (black arrow).

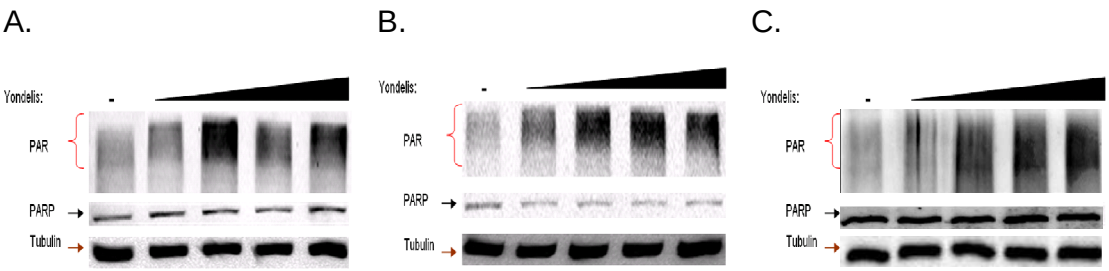

Figure 6. Yondelis® induces PARylation in osteosarcoma and breast carcinoma cells. Cells were treated with 1-1000nM of Yondelis® for 15 minutes and tested for PARylation via Western blot analysis (red brace). Tubulin was used as a loading control (brown arrow). A. Yondelis® induced PARylation in SaOS-2 osteosarcoma cells and did not affect PARP-1 protein expression (black arrow). B. Yondelis® induced PARylation in U2-OS osteosarcoma cells and did not affect PARP-1 protein expression (black arrow). C. Yondelis® induced PARylation in MX-1 breast carcinoma cells and did not affect PARP-1 protein expression (black arrow)

Cytotoxicity profile of Yondelis® in the absence of PARP-1.

Since Yondelis® activated PARP-1 catalytic activity without affecting PARP-1 protein expression, we investigated whether the loss of PARP-1 impacted on Yondelis®-mediated cytotoxicity. Doxorubicin was included as a positive control as it has been reported that PARP-1 null cells are sensitive to the agent,

presumably through a PARP-1-deficiency-dependent DNA repair defect (55). MTS viability assays revealed that Yondelis® is significantly more potent in PARP-1 -/- MEFs than in PARP-1 +/+ MEFs (Figure 7A), exhibiting an approximate 30,000 fold greater cytotoxicity in the former, which dwarfs the approximate 3 fold sensitivity observed using doxorubicin (Figure 7B) as well as other DNA-damaging drugs (56,57). Further, these observations are consistent with previous unpublished studies in our lab in which PARP-1 -/- MEFs exhibited significantly more apoptosis after 72 hours of exposure to Yondelis®, as measured by Guava-Nexin assays, than their wild-type, isogenic counterparts, while maintaining similar doubling time and cell cycle profiles to PARP-1 +/+ MEFs (data not shown).

A. B.

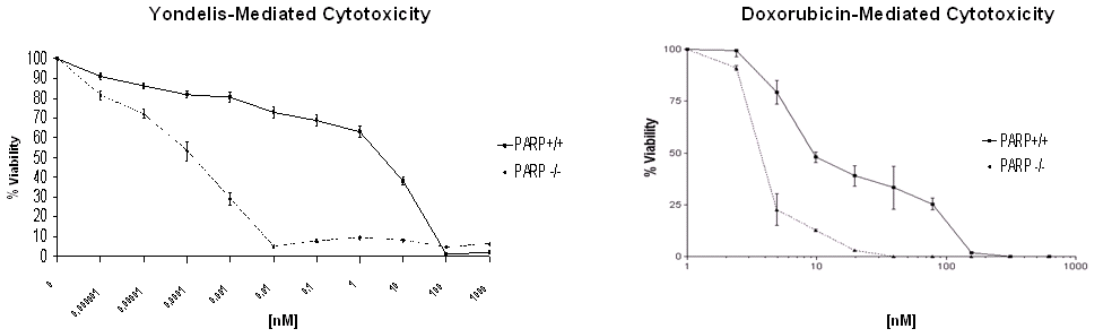

Figure 7. Loss of PARP-1 results in hypersensitivity to Yondelis® in MEF PARP-1 -/- cells. Cells were assayed via MTS following 72 hours of treatment with Yondelis®. A. Yondelis®-mediated cytotoxicity analysis of MEF PARP-1 isogenic cells. The IC_{50} concentration of PARP-1 null cells is 0.0001 nM while that of PARP-1 wild-type cells is 3 nM. B. Doxorubicin-mediated cytotoxicity analysis of MEF PARP-1 isogenic cells. The IC_{50} concentration of PARP-1 null cells is 3.5 nM while that of PARP-1 wild-type cells is 10 nM.

Yondelis®-mediated DNA damage in the absence of PARP-1.

Previous studies have found that the loss of PARP-1 can lead to a lack of efficient DNA repair signaling, and the subsequent accumulation of DNA damage in these cells after treatment with DNA damaging agents, including DNA

alkylators (58,59). Considering that PARP-1 is involved in DNA damage repair, and that Yondelis® has been demonstrated to both bind DNA and to induce breakage of DNA strands (60-62) as measured by single cell electrophoresis assays (comet assays), a technique used to quantify single- and double-strand DNA breaks (63), a previous member of the lab, Dr. Michael Mandola, investigated whether differences in rate of formation of DNA breakage and/or repair of these breaks could account for the observed sensitivity of PARP-1 -/- MEFs to Yondelis®. Comet assays conducted after treatment of PARP-1 +/+ and PARP-1 -/- MEFs with Yondelis® yielded a dose-dependent increase in DNA damage following 24 hours of exposure to the agent in both the PARP-1 wild-type and null cells; the slight increase in DNA damage observed in the PARP-1 -/- cells was not statistically significant (Figure 8A). To determine whether the PARP-1 -/- cells were impaired in their ability to repair the DNA strand breaks caused by Yondelis®, both cell lines were treated with Yondelis®, and subjected to DNA damage and DNA damage repair rate analysis via comet assay (Figure 8B). Expectedly, Yondelis® induced a similar but modest amount of DNA strand breaks in both cell lines; note the much higher degree of DNA damage incurred following exposure to equitoxic concentrations of doxorubicin. Surprisingly, while the rate of repair of doxorubicin-induced DNA damage was significantly impaired in the PARP-1 -/- cells, as has previously been reported (55,58,59), the rate of repair of Yondelis® -induced DNA damage was essentially indistinguishable between the two cell types. This suggests that the dramatic sensitivity of PARP-1 -/- cells to Yondelis® can not be explained by the DNA strand breaks induced

by Yondelis®, or by a compromised repair of Yondelis®-induced breaks in PARP-1 -/- cells. However, in light of the aforementioned evidence of the potential for Yondelis® to induce single- and double-strand DNA breaks in human cancer cells (60-62), DNA damage and cytotoxicity profiles of Yondelis®-treated human cancer cell lines, in the context of PARP-1 ablation, must be established to confirm that this seemingly DNA damage-independent cytotoxicity is not specific to the murine system under investigation.

A. B.

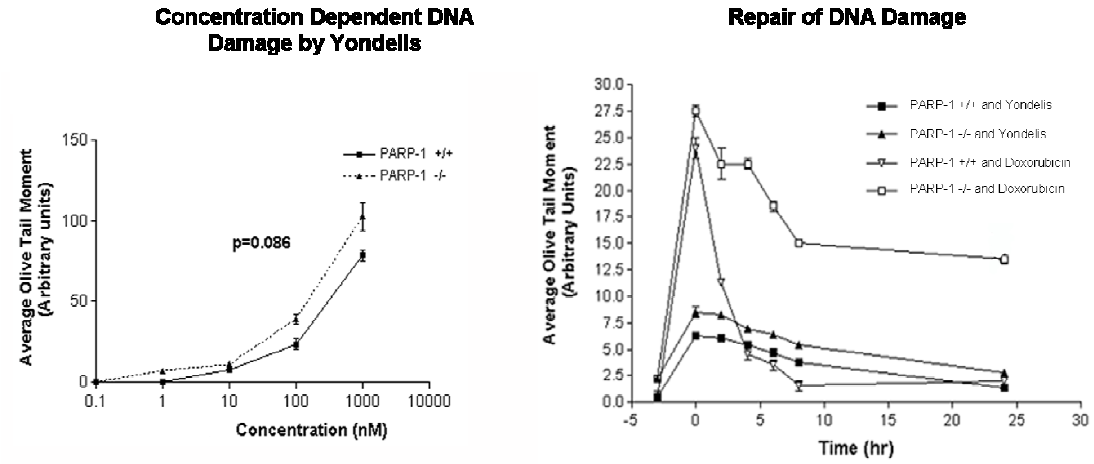

Figure 8. Yondelis® induces DNA strand breakage but not DNA break repair in PARP-1 null cells. A. PARP-1 +/+ and PARP-1 -/- cells were treated with increasing concentrations of Yondelis® for 24 hours. Following treatment, cells were mixed with LMA agarose and subject to single-cell electrophoresis. DNA was stained using SYBR green and comet olive tail moments were quantified using Auto Comet software. Statistical comparisons were calculated using a two-tailed student's t-test. p-values less than 0.05 were considered significant. Data shown is representative of at least three independent experiments performed in triplicate. B. PARP+/+ and PARP-/- cells were treated with 30 nM Yondelis® or 10 nM doxorubicin for 3 hours, after which media was replaced with fresh drug-free media. Cells were isolated and comet assays performed at the indicated times following the 3-hour wash-out.

Potentiation of Yondelis® via PARP-1 inhibition.

Although the increased sensitization of PARP-1 null cells to Yondelis® could not be explained by differences in accumulation of DNA stand breakage or repair, we sought to determine whether inhibition of PARP in human cells could lead to increased cellular sensitivity to Yondelis®. As PARP inhibitors are currently in clinical trials as combinatorial agents with DNA damaging/methylating compounds (64-68), we used the commercially available and potent PARP catalytic inhibitor DIQ. Since DIQ has been used successfully to achieve significant PARP inhibition in cell culture as well as in mice (69,70), we first confirmed DIQ's PARylation-inhibition activity in MEF PARP-1 +/+ cells as well as in various human cancer cells (Figure 9). Pretreatment with DIQ for 1 hour was sufficient to significantly attenuate both basal and Yondelis®-mediated PARP-1 catalytic activity, without affecting PARP-1 protein expression.

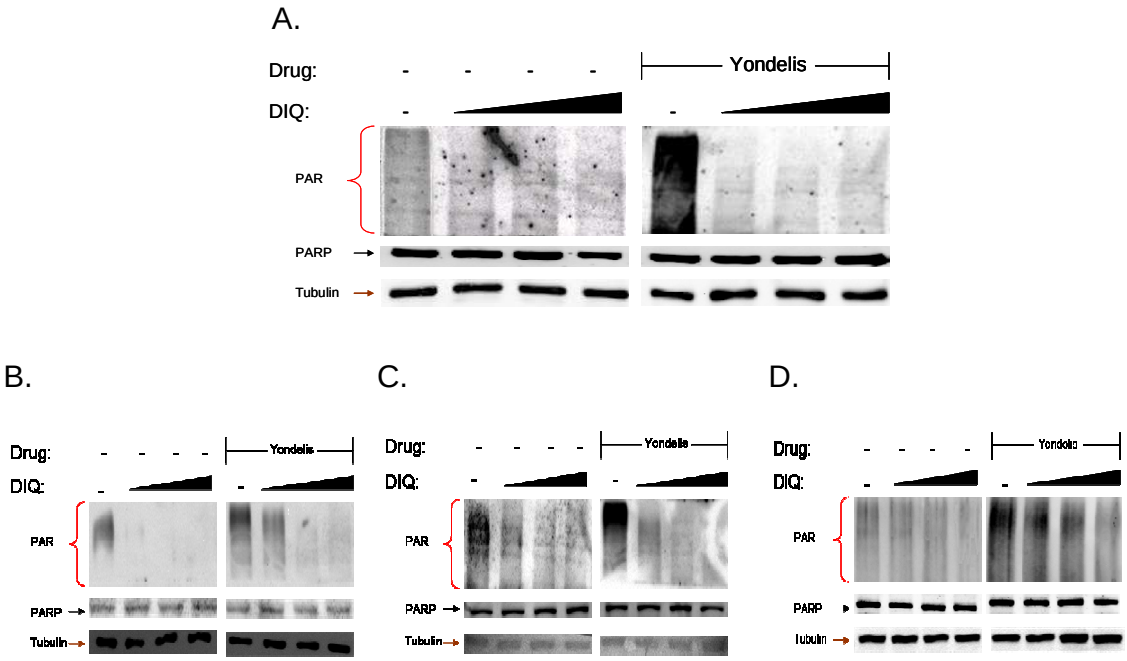

Figure 9. DIQ attenuates Yondelis®-mediated PARylation. Cells were treated with 1-100 µM DIQ for 1 hour prior to 15 minutes of treatment with Yondelis®-containing media. Western blot analysis for PARylation (red brace) was conducted following cell harvesting. Tubulin was used as a loading control (brown arrow). Neither DIQ nor Yondelis® affected PARP-1 protein expression (black arrow) in any of the three cell lines tested. A. MEF PARP-1 +/+ cells were pretreated with DIQ prior to treatment with 10 nM of Yondelis®. B. SaOS-2 osteosarcoma cells were pretreated with DIQ prior to treatment with 10 nM of Yondelis®. C. U2-OS osteosarcoma cells were pretreated with DIQ prior to treatment with 100 nM of Yondelis®. D. MX-1 breast carcinoma cells were pretreated with DIQ prior to treatment with 1000 nM of Yondelis®.

Since DIQ was found to be an effective inhibitor of Yondelis®-induced PARylation, we next sought to determine whether this inhibition of PARP-1 catalytic function could potentiate the cytotoxicity of Yondelis®. Combination studies of Yondelis® and DIQ were conducted in MX-1 breast carcinoma tumor xenografts in nude mice. Ncr nude mice (Taconic Farms) were inoculated subcutaneously with 5 x 10^6 MX-1 breast carcinoma cells and tumors were allowed to grow to palpable size for 14 days. Mice were randomized into 7 groups of 6 mice per group as follows: Control group, Yondelis® (30 µg/kg), Yondelis® (50 µg/kg), Yondelis® plus DIQ (30 µg/kg and 3 mg/kg respectively),

Yondelis® plus DIQ (50 µg/kg and 3 mg/kg respectively) and DIQ alone (3 mg/kg). Mice were treated on days 1, 4, and 7 with the indicated drugs. For Yondelis® treatments, mice were injected intravenously into the tail vein with 100 µL of Yondelis®-containing PBS. For DIQ treatments, mice were injected intraparenterally with 100 µL of DIQ-containing PBS. For drug combinations, DIQ was injected intraparenterally 1 hour prior to Yondelis® intravenous tail vein injections. Tumor size and mouse body weight were measured every 2-3 days using an electronic caliper and digital gram scale, respectively. As seen in Table 2, treatment of mice with 30 or 50 µg/kg of Yondelis® alone resulted in a significant tumor growth delay (TGD) (12 days and 33 days respectively) and tumor volume inhibition (TVI) (73% or 98% inhibition respectively). The combination of DIQ to either Yondelis® dose regimen enhanced TVI (83% compared to 73% for 30 µg/kg and 99.99% compared to 98% for 50 µg/kg). Further, TGD was also extended in both cases, (13 days versus 15 days for 30 µg/kg and 33 days versus 42 days for 50 µg/kg) although this activity was only statistically significant for the Yondelis® (50 µg/kg) plus DIQ combination. Finally, DIQ alone had slight TVI and TGD, but these activities were not statistically significant and only occurred during treatment, while the anti-tumor effects of Yondelis® continued for approximately 2 weeks following drug administration. Although we have yet to substantiate these data with corroborating cell culture data so as to more readily clarify a mechanism of action of Yondelis® that involves PARP-1, these murine xenograft studies suggest that

the combination of PARP inhibitors with Yondelis® may be useful *in vivo* and warrants further investigation.

Group	T/C on Day 20 (%)	TVI (%)	Tumor Free on Day 40	TGD (Days to 1000 m^3)	T-test compared to Yondelis® alone
Control	100	0	0	0	
Yondelis® (30µg/kg)	27	73	0	12	
Yondelis®(50µg/kg)	2	98	2	33	
Yondelis® (30µg/kg)+DIQ	17	83	1	15	p=0.06
Yondelis® (50µg/kg)+DIQ	0.01	99.9	2	42	p=0.03
DIQ (3mg/kg)	56	44	0	5	

Table 2. Combination therapy of Yondelis® and DIQ in nude mice bearing the human MX-1 mammary carcinoma xenografts. Mice were implanted with tumors 14 days prior to initial drug treatments, which occurred on day 1. Data are presented as the mean tumor volume for each group (%). See *Materials and Methods* for further details. All comparisons were made using a two-tailed student's t-test. p-values less than 0.05 were considered significant.

Cytotoxicity analysis of Zalypsis®, PSL1, and PSL2 in multiple cancer cell lines. To establish the general cytotoxicity profile of Zalypsis®, PSL1, and PSL2, several cancer cell lines were tested for their response to 72 hours of exposure to the drugs (Table 3). Gemcitabine was used as a reference for the pancreatic and ovarian adenocarcinoma cell lines. In almost all cell lines tested, PSL1 was the most cytotoxic agent, while PSL2 elicited a more moderate sensitivity among the cell lines. Zalypsis® was the least cytotoxic agent in all cell lines tested with the exception of the osteosarcoma cells, in which the IC50 values were in the low femtomole range. Despite these differences in potency, all three agents were

significantly more cytotoxic in pancreatic and ovarian adenocarcinoma than gemcitabine.

Cancer	Cell Line	Treatment	IC50 (nM)
Pancreatic Adenocarcinoma	HTB147	Zalypsis®	39
		PSL1	0.5
		PSL2	2.1
		Gemcitabine	600,000
	MIA PaCa-2	Zalypsis®	2.5
		PSL1	0.06
		PSL2	0.4
		Gemcitabine	790
	Panc-1	Zalypsis®	55
		PSL1	0.26
		PSL2	2.3
		Gemcitabine	400,000
	AsPc-1	Zalypsis®	1000
		PSL1	0.38
		PSL2	2.3
		Gemcitabine	3,500
Ovarian Adenocarcinoma	ES-2	Zalypsis®	4.2
		PSL1	0.0032
		PSL2	0.18
		Gemcitabine	5,000
	OVCAR-3	Zalypsis®	10
		PSL1	0.055
		PSL2	4.2
		Gemcitabine	15,000
	SK-OV-3	Zalypsis®	10
		PSL1	0.11
		PSL2	10
		Gemcitabine	60,000
Colon Carcinoma	SW620	Zalypsis®	7
		PSL1	0.05
		PSL2	0.3
	HCT-116 p53 +/+	Zalypsis®	2.5
		PSL1	0.0057
		PSL2	0.3
	HCT-116 p53 -/-	Zalypsis®	2.4
		PSL1	0.0035
		PSL2	0.3
Osteosarcoma	U2-OS	Zalypsis®	0.000002
		PSL1	0.02
		PSL2	0.004
	SaOS-2	Zalypsis®	0.000005
		PSL1	0.24
		PSL2	0.02

Table 3. Cytotoxicity profile of Zalypsis®, PSL1, and PSL2 in various cancer cell lines.

Analysis of Zaypsis®-, PSL1-, and PSL2-mediated PARP-1 catalytic activity. As mentioned in the introduction, we have demonstrated that Zalypsis® can bind DNA while others have indirectly shown the ability of Zalypsis® to induce DNA damage. Thus, we investigated whether PARP-1 is involved in the cellular response to Zalypsis®. The PARP-1 isogenic MEF cells were treated with Zalypsis®, and subjected to subsequent Western blot analysis to establish Zalypsis®-mediated PARylation profiles. PSL1 and PSL2 were also tested to determine what, if any, impact these novel compounds may have on PARP-1 catalytic activity. As shown in Figure 10A, Zalypsis® induced PARylation in a concentration-dependent manner in PARP-1 +/+ MEFs without altering PARP-1 protein expression. PSL1 and PSL2, however, induced more biphasic PARylation profiles and, while PARP-1 protein expression was unaffected by PSL1, greater, potentially non-specific, concentrations of PSL2 inhibited PARP-1 expression (Figure 10C, middle panel). Interestingly, PSL2 exhibited peak PARylation when PARP levels were almost undetectable (Figure 10c, right lane), unlike what has previously been observed wherein extensive PARylation can result in a feedback loop that lead to a reduction in PARylation, and a concomitant reduction of PARP expression levels (71). Such a confounding observation merits deeper analysis of the effects of PSL2 at specific concentrations with respect to its PARylation profile in MEFs. Nevertheless, all three drugs activated PARP-1, and, as expected, neither PARP-1 protein expression nor PARylation was observed in the PARP-1 -/- MEFs upon drug treatment. These experiments were extended into human cancer cells and, as

indicated in Figures 11 and 12, resulted in similar observations, with the exception that Zalypsis® induced a more biphasic PARylation profile in these human cancer cells than in the PARP +/+ MEFs, and that PSL2-induced PARylation had no affect on PARP-1 protein expression. It should be noted, however, that PSL2-mediated PARylation in the human cancer cells required concentrations what were multiple logs lower than those of the other three drugs to reach peak PARylation.

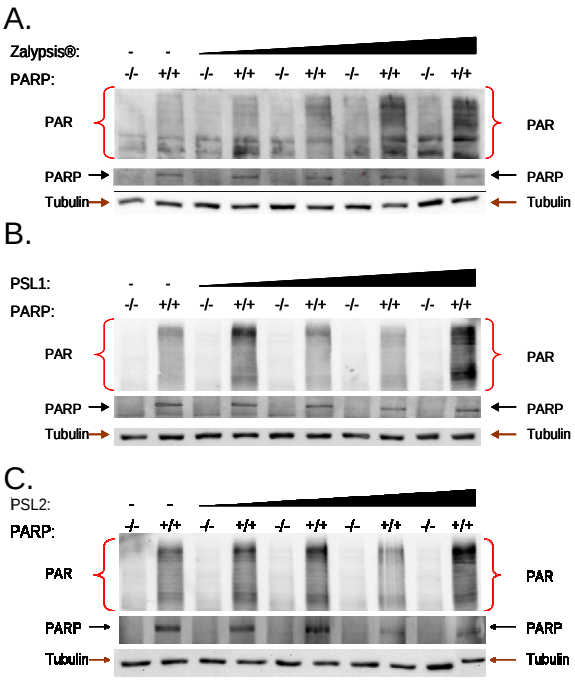

Figure 10. All 3 agents induce PARylation in MEF PARP +/+ cells. Cells were treated with 1-1000 nM of indicated drug for 15 minutes and tested for PARylation via Western blot analysis (red brace). Tubulin was used as a loading control (brown arrow). A. Zalypsis® induced PARylation and did not change the PARP protein expression level (black arrow). B. PSL1 induced PARylation and did not change the PARP protein expression level (black arrow). C. PSL2 induced PARylation while decreasing PARP protein expression levels at greater concentrations treatment points (black arrow).

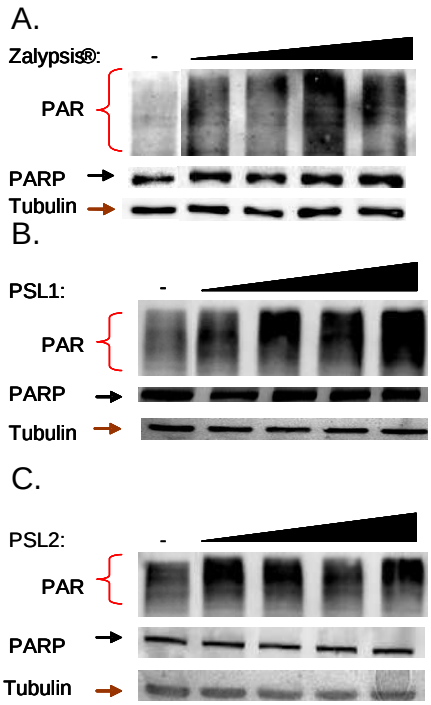

Figure 11. All 3 agents induce PARylation in SaOS-2 cells. Cells were treated with 1-1000 nM of Zalypsis® or PSL1, or 0.001-1 nM of PSL2 for 15 minutes and tested for PARylation via Western blot analysis (red brace). Tubulin was used as a loading control (brown arrow). A. Zalypsis® induced PARylation and did not change the PARP protein expression level (black arrow). B. PSL1 induced PARylation and did not change the PARP protein expression level (black arrow). C. PSL2 induced PARylation and did not change the PARP protein expression level (black arrow).

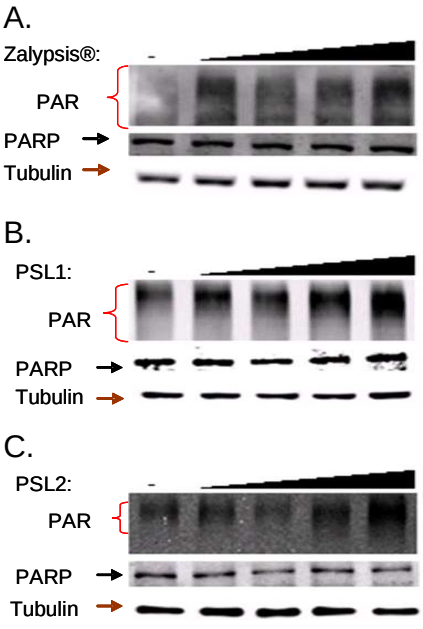

Figure 12. All 3 agents induce PARylation in U2-OS cells. Cells were treated with 1-1000 nM of Zalypsis® or PSL1, or 0.001-1 nM of PSL2 for 15 minutes and tested for PARylation via Western blot analysis (red brace). Tubulin was used as a loading control (brown arrow). A. Zalypsis® induced PARylation and did not change the PARP protein expression level (black arrow). B. PSL1 induced PARylation and did not change the PARP protein expression level (black arrow). C. PSL2 induced PARylation and did not change the PARP protein expression level (black arrow).

Preliminary investigation of potentiation of Zalypsis®, PSL1, and PSL2 using PARP-1 inhibitors.

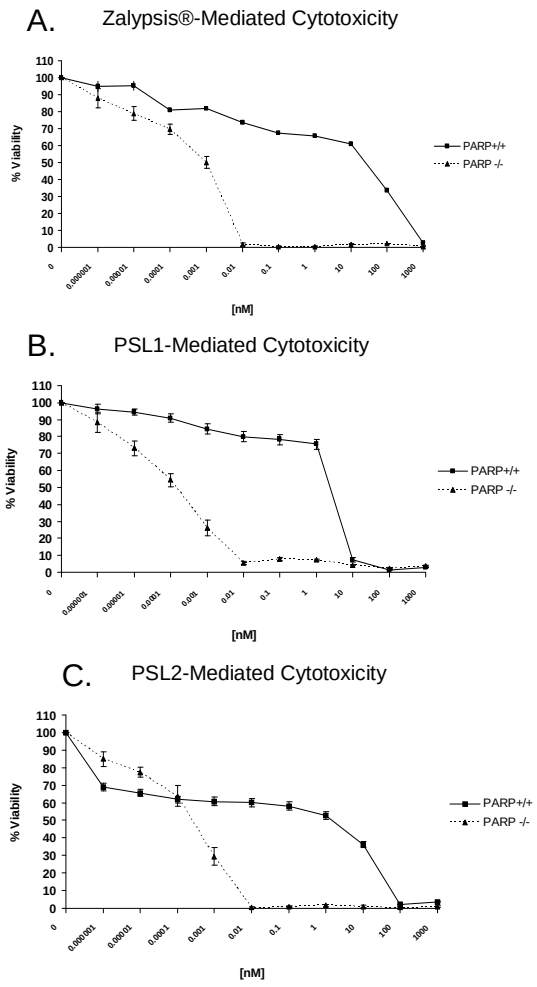

Figure 13. Loss of PARP-1 results in hypersensitivity to Zalypsis®, PSL1, and PSL2 in MEF PARP-1 -/- cells. Cells were assayed via MTS following 72 hours of treatment with indicated drug. A. Zalypsis®-mediated cytotoxicity analysis of MEF PARP-1 isogenic cells. The IC_{50} of PARP-1 null cells was 0.001 nM while that for PARP-1 wild-type cells was 30 nM. B. PSL1-mediated cytotoxicity analysis of MEF PARP-1 isogenic cells. The IC_{50} for PARP-1 null cells was 0.0001 nM while that for PARP-1 wild-type cells was 3 nM. C. PSL2-mediated cytotoxicity analysis of MEF PARP-1 isogenic cells. The IC_{50} for PARP-1 null cells was 0.0003 nM while that for PARP-1 wild-type cells is 2 nM.

Since Zalypsis®, PSL1, and PSL2 activated PARP-1 catalytic activity, we investigated whether the loss of PARP-1 impacted on the cytotoxicity mediated by the three drugs. The cytotoxicity profile of doxorubicin was established as a positive control (Figure 8B). MTS viability assays revealed that, like Yondelis®, all three agents are significantly more potent in PARP-1 -/- MEFs than in PARP-1 +/+ MEFs (Figure 13), exhibiting an approximate 6,000-30,000 fold greater cytotoxicity in the former, which dwarfs the approximate 3-fold sensitivity observed using doxorubicin (Figure 8B).

We next confirmed DIQ's PARylation-inhibition activity in MEF PARP-1 +/+

cells as well as in human cancer cells (Figure 14). Pretreatment with DIQ for 1 hour was sufficient to significantly attenuate both basal and drug-induced PARP-1 catalytic activity, without affecting PARP-1 protein expression. We are currently conducting cytotoxicity assays in the absence or presence of DIQ to ascertain if PARP-1 inhibition will potentiate the cytotoxicity of the 3 drugs.

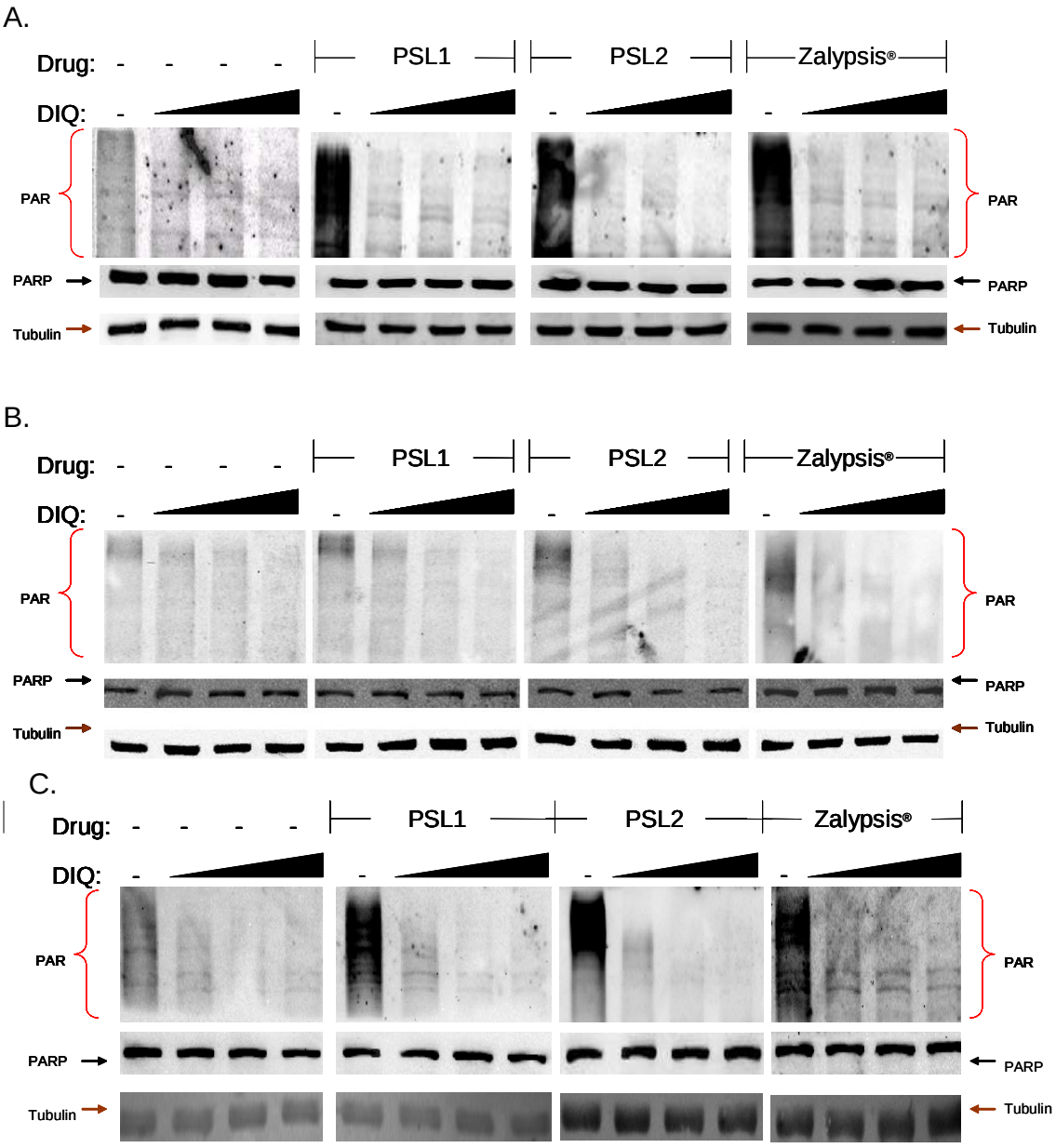

Figure 14. Attenuation of drug-mediated PARylation by DIQ. Cells were treated with 1-100 μM DIQ for 1 hour prior to 15 minutes of treatment with drug-containing media. Western blot analysis for PARylation (red brace) was conducted following cell harvesting. Tubulin was used as a loading control (brown arrow). A. MEF PARP-1 +/+ cells were pretreated with DIQ prior to treatment with either 1000 nM of Zalypsis®, PSL1, or PSL2. B. SaOS-2 osteosarcoma cells were pretreated with DIQ prior to treatment with either 100 nM of Zalypsis®, 1000 nM of PSL1, or 0.01 nM of PSL2. Neither DIQ nor drug treatment affected PARP-1 protein expression (black arrow). C. U2-OS osteosarcoma cells were pretreated with DIQ prior to treatment with either 1000 nM of Zalypsis®, 1000 nM PSL1, or 1 nM of PSL2. Neither DIQ nor drug treatment affected PARP-1 protein expression (black arrow).

Preliminary investigation of the transcriptional impact of Zalypsis®, PSL1, and PSL2 using the MDR1 reporter system as a model.

Our initial studies investigating the effect of Zalypsis® on transcription using the *MDR1* reporter system revealed that TSA-mediated activation of *MDR1* is abrogated in the presence of Zalypsis®, while basal transcription remains unaffected (33). To test whether this transcriptional inhibition by Zalypsis® is limited to the activity of histone deacetylase (HDAC) inhibitors such as TSA, we subjected cells stably expressing the *MDR1* transcriptional reporter system to treatment with another known *MDR1* inducer, TPA, a phorbol ester, in the absence or presence of Zalypsis®. PSL1 and PSL2 were also tested to determine what, if any, impact these novel compounds may have on transcriptional activity. Yondelis® was used as a positive control as we have previously demonstrated that it can inhibit TPA-mediated activation of *MDR1* transcription without altering constitutive expression (data not shown). As shown in Figure 15, Zalypsis®, PSL1, and PSL2 inhibited TPA-mediated activation of the *MDR1* gene, without affecting basal transcription, offering the first evidence of the transcriptional impact of the latter two agents as well as adding to the list of different types of transcriptional inducers, and therefore different types of mechanisms of transcriptional modulation, whose activity Zalypsis® can abrogate. Further, preliminary results from ongoing studies, in which the potential of Zalypsis®, PSL1, and PSL2 to inhibit activation of *MDR1* by TSA, caffeine, and sodium butyrate was determined, strongly suggest that, like Yondelis®, the three drugs can attenuate transcriptional activation by various

different inducers (data not shown). These data will be used to inform a study on the effect of PARP-1 abrogation and/or inhibition on the observed inhibition of transcriptional activation by the four drugs.

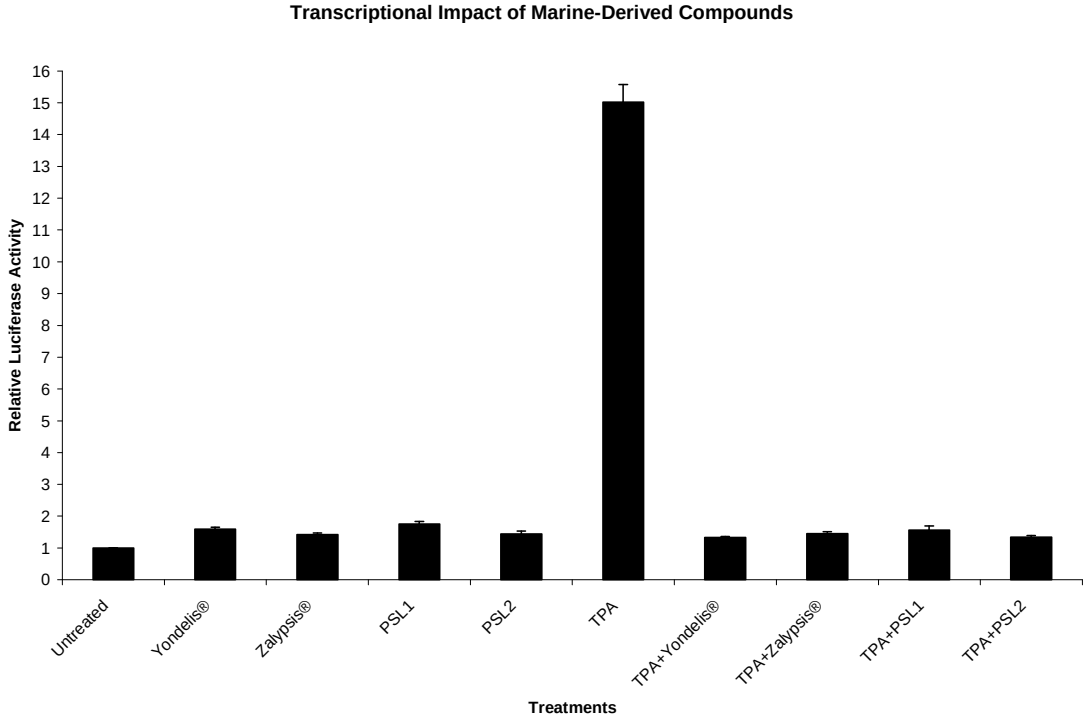

Figure 15. Zalypsis®, PSL1, and PSL2 inhibit TPA-activated but not basal *MDR1* transcription. Cells were treated with 50nM of indicated drug for 24 hours followed by quantification of luciferase activity. Luciferase values were normalized to empty vector and untreated controls, yielding relative luciferase activity.

Chapter 4

Discussion

In this report we have investigated the role of PARP-1, in the cellular response to several marine-derived compounds. Yondelis® and Zalypsis® are highly promising, novel chemotherapeutic agents that are currently in late and early stage clinical trials, respectively, for the treatment of a variety of solid tumors. Considerable attention has been given to determine the mechanism of action of Yondelis®, and two models have seemed to emerge: 1) Yondelis® binds to and damages DNA and 2) it uniquely inhibits activated but not constitutive transcription. Although more preclinical studies are required to clarify its mechanism of action, Zalypsis® seems to display some of the functional characteristics which may ultimately allow it to share in the aforementioned modes of action of Yondelis®. In our effort to reconcile said models, we have pursued a study of the impact that PARP-1 may have in activity of the two drugs. Additionally, to initiate characterization of PSL1 and PSL2, we have also included these compounds in our studies to determine what, if any, cellular response the drugs may elicit in the context of PARP-1 ablation and/or inhibition.

Our data demonstrate PARP-1 to be a promising candidate as a mediator in the cytotoxicity of Yondelis® and Zalypsis®, as well as in the cytotoxicity of the hitherto completely uncharacterized, but related, marine-derived putative anti-cancer agents, PSL1 and PSL2. In deed, Yondelis®, Zalypsis®, PSL1, and

PSL2, activate PARP-1 catalytic activity, and cells lacking this enzyme are exquisitely sensitive to all four drugs. The observed PARylation was determined to be variable over the concentration of and duration of exposure to (data not shown) the drugs, as well as over differing cell types. Given the potential impact that induction of PARP-1 activity may have on cellular defense against the drugs under study, it is of interest to correlate PARylation-inducing concentrations with those that illicit cytotoxicity. However, the bi-phasic PARylation profiles in several of the cell lines tested, as well as the temporal kinetics of the observed PARP-1 activity upon treatment with the drugs (5-30 minutes) indicate that any PARylation observed at the end of the cytotoxicity assays (72 hours) would be likely due to indirect effects of the drugs, such as apoptosis-induced DNA fragmentation. Nevertheless, as PARP-1 plays a protective role in the cellular response to stress and insult, characterizing the dynamics of PARylation profiles of the four drugs over concentration range and time of exposure in various cancer types, may ultimately allow for the establishment of a cancer-specific marker for the activity of the agents.

We have demonstrated *in vivo* that catalytic inhibition of PARP-1, an attenuation that was predicted *in vitro*, sensitizes cells to the cytotoxic effect(s) of Yondelis®. Yet preliminary *in vitro* studies using DIQ to inhibit PARP-1 activity prior to treatment with the drugs have failed to show significant changes in cellular sensitivity. This may be due to the particular PARP-1 inhibitor being used to abrogate PARP-1 catalytic activity, as DIQ is considered to be a member of first-

generation PARP-1 inhibitors with suspected secondary targets *in vivo*, including not only other members of the PARP protein family but also non PARP-like targets that impact on cellular metabolism (72,73). This may imply that the lack of synergism observed in the DIQ and Yondelis® combinatorial *in vivo* studies may be due to off-targets effects of DIQ. We are currently testing the potential of other commercially available, and more target-specific, PARP-1 inhibitors, such as NU-1025, to sensitize PARP-1 +/+ MEFs and human cancer cell lines to Yondelis® as well as to the other three drugs. Another more intriguing explanation for the lack of *in vitro* PARP-1 inhibition-mediated sensitization could be that the dramatic sensitivity of the PARP-1 -/- cells may be dependent less on PARylation, and more on a PARP-1-mediated cytoprotective mechanism that is independent of the protein's catalytic function. In deed, it has been demonstrated that whereas inhibition of PARP-1 function in PARP-1 +/+ MEFs was insufficient to induce genomic instability, as measured by the manifestation of tetraploidy, PARP-1 deficiency-mediated tetraploidy in PARP-1 -/- MEFs was abrogated via reintroduction of PARP-1 cDNA (74). Additionally, it has been reported that in the absence of its activating substrate, NAD^+, PARP-1 interacts with different transcription factors to enhance activator-dependent transcription, while the presence of NAD^+ and consequent PARP activation represses transcription, presumably by PARylation of certain transcription factors (74). Thus, the positive transcriptional effects of PARP-1 in its non-activated state, that impact the expression of cytoprotective genes, may be lost in the PARP-1 -/- MEFs.

It is evident, therefore, that the functions of PARP-1 are not necessarily bound by its enzymatic activity, implying that studies of the impact of PARP-1 on cellular defense against DNA damage warrants a nuanced approach to dissect the role of the protein's PARylation effects versus those of its scaffolding effects. Accordingly, future studies will address rescue of resistance of PARP-1 -/- MEFs to the four drugs via introduction of exogenous wildtype or catalytic-domain-mutated PARP-1 cDNA. Moreover, PARP-1 +/+ MEFs will be subjected to shRNA-mediated knock-down of PARP-1, or to over expression of the N-terminal apoptotic fragment of the protein, which has a dominant negative effect on endogenous PARP-1 activity (75), to test the potential to sensitize the cells to the drugs. These two complimentary experimental systems will help refine our current understanding of the mechanism by which PARP-1 is mediating cytotoxicity. Notwithstanding the limited results of the preliminary PARP-1 inhibition studies, the data thus far offer first evidence of the critical role that PARP-1 plays in cellular defense against the four drugs, and establish PARylation as a putative surrogate marker for the efficacy of the agents.

The effects of DNA damaging agents on PARP-1 activation and DNA repair have been well studied in various pre-clinical models. PARP-1 null mice are more sensitive to the cytotoxic effects of a variety of DNA damaging agents including cisplatin, N-methyl-N'-nitro-N-nitrosoguanidine and doxorubicin (76). In these models, the increased sensitivity of mice, or derived cells, that lack functional

PARP-1 is mediated through the DNA damage repair system. In particular, the base excision repair (BER) machinery was shown to be compromised in its ability to repair DNA damage in these systems, due to either a lack of access to the lesion site, or the lack of efficient detection and signaling of DNA damage in the absence of PARP-1. Indeed, the rate of BER-mediated DNA repair is lower, and the accumulation of DNA damage is higher, in cells lacking functional PARP-1 protein (58,59).

Interestingly, however, in the current study, PARP-1 deficient-dependent sensitivity to Yondelis®, a known DNA alkylator, does not correlate with accumulation of or repair of DNA damage, an observation that seems markedly antithetical to the predominant observations of alkylator-mediated effects on the rate of DNA damage accrual and subsequent repair in PARP-1 -/- MEF cells (77,78). Although this confounds the elucidation of the role of DNA damage in the observed PARP-1-mediated cytotoxicity of Yondelis®, it does imply that DNA damage may not be the primary mechanism of Yondelis®-mediated cytotoxicity but rather a secondary outcome of the drug's interaction with DNA, and that accumulation of DNA strand breaks is a contributory factor to a multifaceted mode of PARP-1-dependent cytotoxicity. In deed, we have observed no Yondelis®-mediated DNA damage prior to 24 hours of exposure, a time point at which we have noted an induction of Yondelis®-mediated early apoptosis (data not shown). Similar data were obtained for Zalypsis®-mediated DNA damage studies (data now shown). Thus, it is not clear if the DNA damage observed at

this late time point is due to a direct effect of the two drugs or is a result of the initiation of apoptosis (79). Yet we have not outright dismissed the possibility that there may be compensatory DNA damage repair pathways at play, which may be abrogating the ostensibly deleterious effects of Yondelis® on DNA integrity (80), as well as the possibility that the transcriptional coupled nucleotide excision repair (TC-NER) pathway may be compromised in the PARP-1 -/- MEFs, as PARP-1 has been reported to be involved in TC-NER (81). This latter point is especially important considering that Yondelis® is less cytotoxic in cells deficient in TC-NER (82). Additionally, given that Yondelis® has been shown to induce significant DNA strand breakage in human cancer cell lines (56-58), it is plausible that the lack of a significant accumulation in DNA strand breaks may be specific to the MEF system under investigation. In deed, Godon *et al*. have demonstrated that, at least in regard to single-strand DNA break repair, PARP-1 -/- MEFs can have similar kinetics to those of PARP-1 +/+ MEFs due to a compensatory mechanism of BER (83). However, as this study was limited to the investigation of reactive oxygen species- and irradiation-mediated DNA strand breakage, it is still unclear whether the putative BER compensatory pathway would be amenable to repair of DNA alkylator-mediated DNA damage. Nevertheless, recent evidence does indicate that the role of PARP-1 in DNA damage repair relative to various other DNA damage repair proteins that also facilitate genomic stability is considered to be both redundant (84) and non-redundant (85). As such, we are currently profiling the breadth of DNA damage repair capacity of the PARP-1 -/- MEFs, so as to ascertain the genome protective

context in which Yondelis imparts its seemingly DNA damage-independent cytotoxicity in these cells.

We have long hypothesized that the unique ability of Yondelis® and Zalypsis®, and now PSL1 and PSL2, to inhibit activated transcription, a characteristic that other DNA binding agents lack, may play an equal, if not more critical, role in the cytotoxicity mediated by the two drugs. Indeed, the inability to correlate DNA damage and repair with increased sensitivity of PARP-1 -/- cells to Yondelis® strongly suggests another molecular target of cytotoxic action. Although DNA damage studies of Zalypsis®, PSL1, and PSL2 are not yet complete, we are considering several models that may explain the role of PARP-1 in the cellular response to the four agents. Interestingly, Yondelis® has been shown to bend DNA *in vitro*, and PARP-1 has been shown to interact with bent DNA (86) and nucleosome bound DNA (87) often with better affinity and kinetics of PARylation that it does to damaged DNA. Thus, the binding of Yondelis® could provide a signal for recruitment of PARP-1. It is also possible that Yondelis® or Zalypsis® itself is disrupting higher-order chromatin structure, which leads to the activation of PARP-1, its subsequent dissociation from DNA, and possibly to the alteration of activated transcription. Along this line, we have preliminary data showing that Yondelis® covalently interacts with one or more nuclear proteins (data now shown). Thus, one possible explanation for the increased sensitivity of the PARP-1 -/- cells to Yondelis® or Zalypsis® is that the modulation of higher-order chromatin structure by PARP-1 in response to stimuli is lost, causing increased

susceptibility of nucleosomes and chromatin to the drugs. Alternatively, the increased sensitivity of PARP-1 -/- cells to the two drugs may be due to the loss of PARP-1-dependent transcription, which may compromise the ability of these cells to respond to the agents; this would predict that genes that are involved in the cellular response to the four drugs are PARP-1-dependent. Consequently, lack of PARP-1 protein would make cells more vulnerable to Yondelis® or Zalypsis®, resulting in an increase in apoptotic cell death. Finally, since the lack of PARP-1 in the null cells leads to genomic instability which, over time, leads to the accumulation of mutations (88-91), the resulting cellular status may not be directly due to the loss of PARP-1 itself but rather a downstream effect of this loss that leads to the hypersensitivity of null cells. In this case it should be noted that we have observed this hypersensitivity in two sets of MEF cells isolated from independently derived mice of different genetic backgrounds (59); if indeed the hypersensitivity is due to an event secondary to the loss of PARP-1, this event does not appear to be random, suggesting that the locus involved is a “hot spot” for genomic instability.

Interestingly, the preferential association of PARP-1 with regions of chromatin that are undergoing transcriptional induction following extracellular stimuli (50) and developmental cues (92) provides a reasonable explanation as to why Yondelis® and Zalypsis® specifically inhibit only activated or induced transcription while leaving basal or constitutive transcription unaffected. It is possible that two drugs activate PARP-1 catalytic activity in a different way than

other compounds. Just as histone acetyltransferases respond differentially to various extracellular stimuli, PARP-1 catalytic activity is differentially regulated in response to different stimuli, and consequently, patterns of PAR attachment to nuclear protein targets are also predicted to differ. This regulated cascade of events could result in very different outcomes with regard to transcriptional regulation and DNA damage repair. Indeed, PARP-1 has already been shown to activate the transcription of genes involved in the immune and inflammatory response systems following exposure to pro-inflammatory agents and cytokines (40),37,39,(45,93). Thus, PARP-1 exhibits the ability to either activate (39,40,42-47,49) or inhibit (48) transcription, in a catalytic domain-dependent (44,94,95), or –independent (39,42,49,92,96,97) manner. If the role of PARP-1 in transcriptional modulation by the four drugs is validated, this would suggest that treatment with the agents should also lead to the activation, rather than inhibition, of some genes, particularly those genes that are normally repressed by PARP-1. In support of this hypothesis, a recent study has shown that Yondelis® does indeed possess the ability to activate transcription of certain promoter constructs, while inhibiting only the activation or induction, of others (98). Using the *MDR1* transcription system, we have already demonstrated that the four compounds inhibit activated transcription without altering constitutive expression. We will explore the effects of PARP-1 silencing or PARP-1 catalytic inhibition on said transcriptional impact of the drugs on the *MDR1* system, allowing for a clarified understanding of the mechanism by which loss of PARP-1 mediated transcription may be responsible for the observed hyper-cytotoxicity of the drugs.

Although more studies are needed before we have a clear picture of how Yondelis®, Zalypsis®, PSL1, and PSL2 are activating PARP-1, and the downstream consequences on transcription and cytotoxicity, it is clear that PARP-1 is serving a protective role during exposure to the agents. Although we have not yet observed DIQ-mediated sensitization of cells in vitro, the data presented here showing that DIQ can sensitize human xenografts to Yondelis® *in vivo* are promising, and warrant further investigation. We are currently testing the proposed models for PARP-1-mediated sensitivity to the four agents, with a long-term goal of identifying and characterizing other genes in this pathway that modulate the cellular response(s) to the four drugs. Given that the exploration of marine-derived anticancer agents in the treatment of human malignancies is still in its infancy, it is imperative that protein markers are identified and validated in order to predict individual patient/tumor response. A better understanding of the mechanism of action and resistance/sensitivity to such putative anti-cancer drugs can guide their future use, and direct pharmacogenetic trials to ensure their greatest efficacy.

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