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New Targeted Molecules for the Therapy of Ovarian Cancer

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LIST OF ABBREVIATIONS

AML: Acute Myelogenous Leukemia
APL: Acute Promyelocytic Leukemia
ATO: Arsenic Trioxide
ATRA: All-trans retinoic acid
BRCA: Breast Related Cancer Antigen
CICs: Cortical Inclusion Cysts
Cyt C: Cytochrome C
DMSO: Dimethyl sulfoxide
DPS: Delayed Primary Surgery
EMT: Epithelial-Mesenchymal Transition
EOC: Epithelial Ovarian Cancer
FAK: Focal Adhesion Kinase
FBDD: Fragment-Based Drug Discovery
FBS: Fetal Bovine Serum
FDA: U.S. Food and Drug Administration
FIGO: International Federation of Gynecology and Obstetrics
GCO: Global Cancer Observatory
H&E: Hematoxylin and Eosin
HGSOC: High-Grade Serous Ovarian Carcinoma
HRD: Homologous Recombination Deficiency
IC50: Half-Maximal Inhibitory Concentration
IHC: Immunohistochemistry
HR: Homologous Recombination
IPS: Immediate Primary Surgery
KD: Knockdown
OC: Ovarian Cancer
OS: Overall Survival
OSE: Ovarian Surface Epithelium
PARP: Poly(ADP-Ribose) Polymerase
PARPi: PARP inhibitors
PBS: Phosphate Buffered Saline
PDAC: Pancreatic Ductal Adenocarcinoma
PFA: Paraformaldehyde
PFS: Progression-Free Survival
PIN1: Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PKM2: M2 Isoform of Pyruvate Kinase
PLD: Pegylated liposomal doxorubicin
PPIases: Peptidyl-prolyl isomerase
RNAi: RNA interference
SET: Solid areas, pseudo-Endometrioid and Transitional cell-like
SGK1: Serum/Glucocorticoid Regulated Kinase 1
STAT3: Signal Transducer and Activator of Transcription 3
STICs: Serous Tubal Intraepithelial Carcinomas
TCF: beta-Catenin/T-cell Transcription Factor
TCGA: The Cancer Genome Atlas
WB: Western Blot
WES: Whole-Exome Sequencing

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ABSTRACT

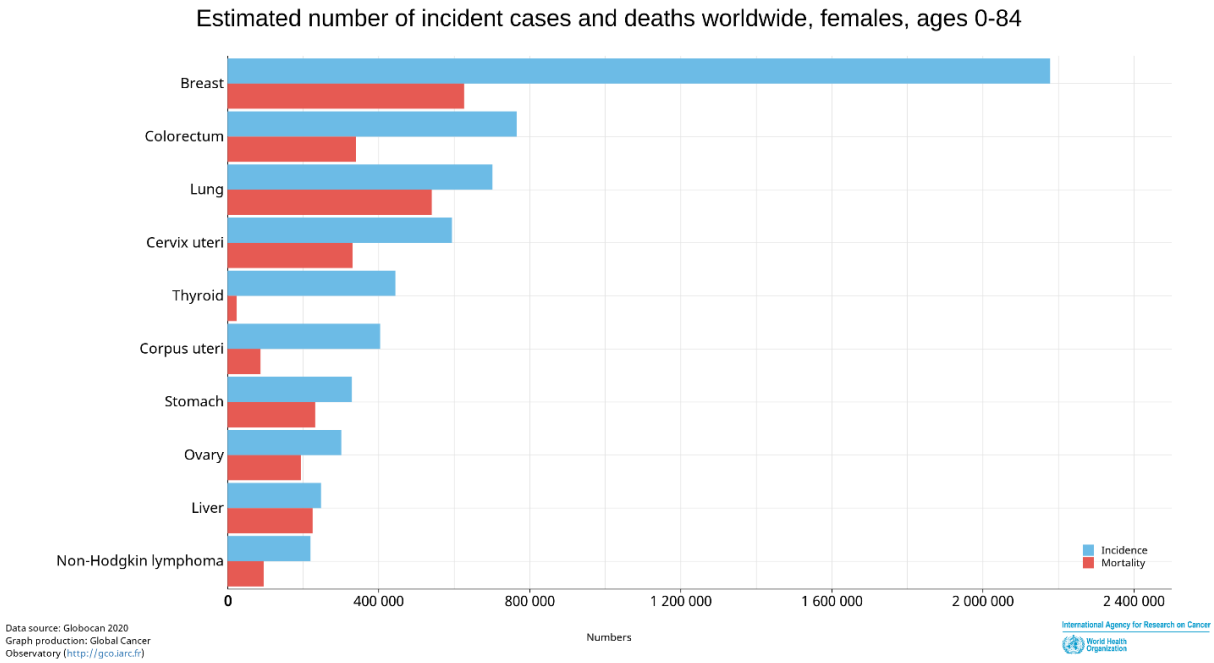
Patients with high-grade serous ovarian cancer (HGSOC), the most aggressive epithelial ovarian cancer (EOC) subtype, have a 5-year survival rate of about 93% when diagnosed at an early stage, but it drops to 30-40% when diagnosed in the advanced stage. HGSOC aggressiveness is mainly caused by the late diagnosis (51% stage III, 29% stage IV) when the tumor has already spread in the peritoneal cavity. PIN1 is a unique peptidyl-prolyl isomerase that targets the phosphorylated Ser/Thr(Pro) motifs to regulate several key proteins in different signaling pathways. Pin1 is overexpressed in several cancer types and it regulates more than 40 oncogenes and 20 tumor suppressors. Many functions are modulated through PIN1-mediated isomerization such as cell cycle progression, cellular proliferation, invasion, migration, and apoptosis. Downregulation of Pin1 decreases tumor progression. Recently, Pin1 was shown to be overexpressed in ovarian cancer (OC) which, together with the high number of interactions with other proteins, makes Pin1 a promising target for HGSOC. The aim of this work is to investigate the effects of the PIN1 inhibitor VS10 on cancer cell lines and to find the molecular signaling pathways in which Pin1 is involved. Migration, mesothelial clearance assay, and the effects on spheroid formation and preformed spheroids were studied to better understand the effects on the metastatic process. Furthermore, in order to clarify the molecular mechanism that triggers the cytotoxicity induced by Pin1 inhibition in several OC cell lines, silencing Pin1 has been demonstrated to be associated with ^{Ser473}pAkt dephosphorylation by Western Blot (WB) analysis. Additionally, cell viability and colony-forming assays showed that Akt overexpression rescued the lethal phenotype due to Pin1 knockdown in OVCAR3 and KURAMOCHI OC cell lines. Among PIN1 inhibitors, All-trans retinoic acid (ATRA), a drug in clinic for the treatment of acute promyelocytic leukemia, has been demonstrated to be active on PIN1. Our group developed many PIN1 inhibitors including VS10, a non-covalent and

selective molecule, which is active in killing cancer cells. ATRA and VS10 have been combined with first- and second-line chemotherapy drugs to treat SKOV3 cell line whether these drug combinations could work synergistically to improve current therapy. This drug combination screening showed that Doxorubicin and Caelyx act in synergy with both VS10 and ATRA. This drug combination was studied in 5 sensible and 2 OC cell lines resistant to cisplatin treatment. These results candidate Pin1 as a promising new molecular target for HGSOC patients' therapy.

1. INTRODUCTION

1.1 OVARIAN CANCER

Figure 1 – The numbers of cancer. Worldwide cases (blue) and mortality (red) by cancer site in



2020 (Sung et al., 2021).

OC is the fourth cause of cancer-associated death in developed countries (Jayson et al., 2014; Reid et al., 2017). According to GCO and reported in Figure 1, there were more than 300˙000 cases and more than 190˙000 deaths from OC worldwide in 2020 (Sung et al., 2021). Since early-stage OC patients have no or nonspecific symptoms, late diagnosis is the main cause of high OC mortality: in fact, more than 75% of cases are diagnosed at advanced stage when cancer already invaded tissues beyond the ovaries and the peritoneal cavity (Charkhchi et al., 2020; Lheureux et al., 2019). The early stage of this disease usually shows no symptoms and late-stage symptoms are nonspecific (Doubeni et al., 2016). The risk factors can be either genetic or nongenetic. The main genetic risk factor is the mutation in the BRCA1 and BRCA2 tumor suppressors which account for about 10% of OC cases (Doubeni et al., 2016).

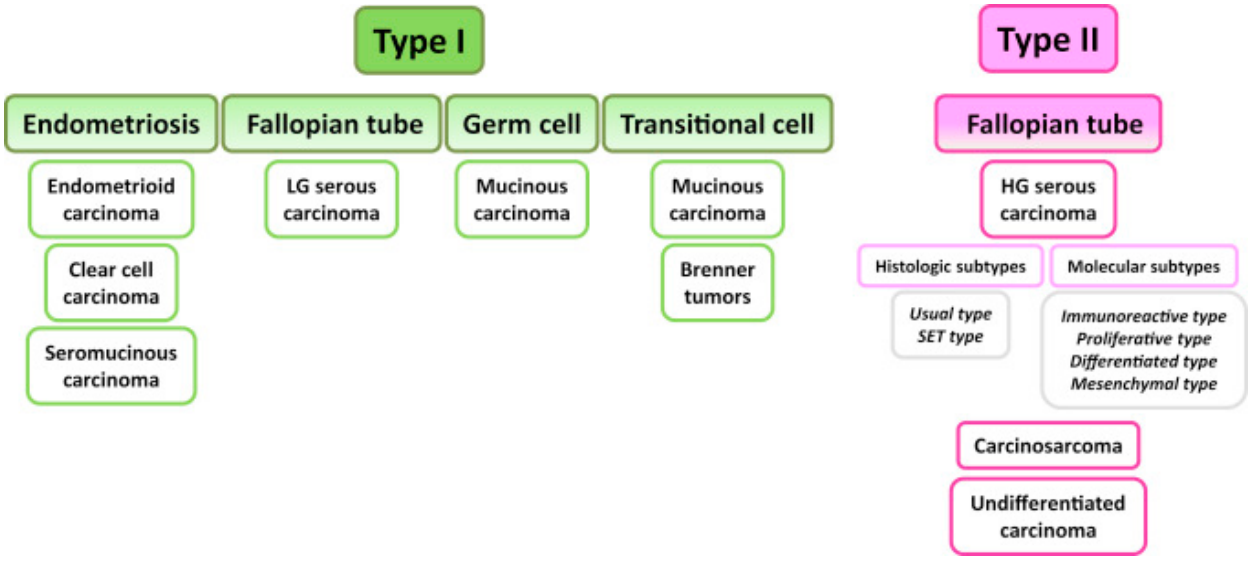


Figure 2 – OC classification by types *(Kurman & Shih, 2016)*.

Mutations in these tumor suppressors are responsible for the hereditary progression of breast and OC (Pruthi et al., 2010). Hence, nongenetic risk factors include ageing, obesity or weight gain, and postmenopausal hormone therapy (Holschneider & Berek, 2000; Hunn & Rodriguez, 2012). OC is classified into two subtypes: non-epithelial OC (e.g. sex cord-stromal, germ cell, and non-specified OC) and EOC (e.g. transitional cell, mucinous, clear cells, and serous OC) (van Zyl et al., 2018). In 2016 Kurman and Shih proposed a new dualistic model for classifying EOC integrating the current histopathologic classification and integrating it with the new molecular genetic finding to obtain a more accurate model. In this context, EOC is sub-classified as Type 1 and Type 2 (Figure 2). Type 1 EOC are low-grade tumors that present K-RAS, BRAF, and PTEN mutations, characterized by stability, slow cancer progression and that usually have a good prognosis after surgical resection (Kurman & Shih, 2016; van Zyl et al., 2018; Zhang et al., 2016). Instead, the Type 2 EOC is rather invasive and aggressive presenting mutations in the TP53 gene and somatic molecular alterations in BRCA1/2 genes of 20-40% of HGSOC-EOCs (Figure 3).

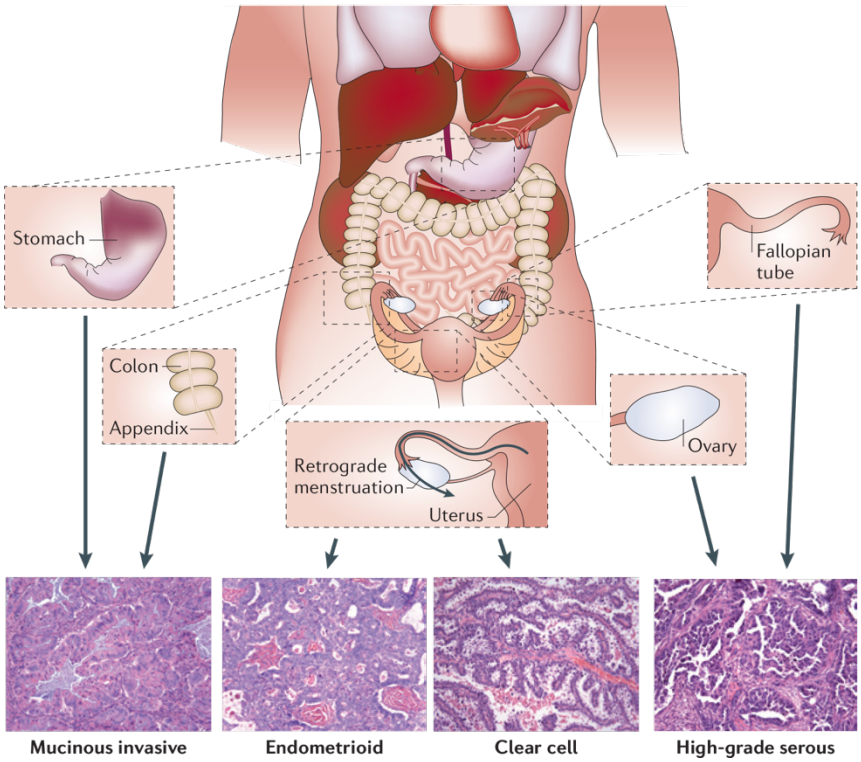


Figure 3 - The origins of OC (Vaughan et al., 2011).

The most aggressive Type 2 EOC is HGSOC which accounts for 67% of all OCs and 70-80% of deaths among all forms of OC (Kurman & Shih, 2016; Lisio et al., 2019; Zhang et al., 2016). According to 2014 WHO classification of OC, Type I OCs include endometrioid, clear cell, seromucinous, low-grade serous, mucinous, and Brenner carcinomas which are classified as low-grade and account for only 10% of OC deaths. Instead, type II OCs include carcinosarcomas, high-grade serous, and undifferentiated carcinomas which are classified as high-grade and are characterized by high aggressiveness and rapid development. Cytoreductive surgery followed by chemotherapy is the common strategy to treat these patients, but although OS is very modest, most of them die (Kurman & Shih, 2016). Moreover, the origin of Type I and Type II is extra-ovarian, specifically clear cell and mucinous EOC are supposed to originate from endometrial stem cells (associated with endometriosis), while high-grade serous carcinoma originates from fallopian tube epithelium (Chen et al., 2020; Scott et al., 2013).

1.2 HIGH-GRADE SEROUS OVARIAN CANCER

HGSOC is the most aggressive EOC subtype, accounting for 70-80% of OC deaths (Bowtell et al., 2015). The aggressive nature of HGSOC is reflected in its late diagnosis (51% stage III, 29% stage IV) when the tumor has already spread to the peritoneal cavity (Torre et al., 2018). The 5-year survival rate is about 30-40% but increases to 93% with early detection (Bowtell et al., 2015; Torre et al., 2018).

In 2011 TCGA provided a milestone in understanding the genetics of HGSOC through whole-exome sequencing (WES) results. This type of tumor is characterized by genomic instability presenting a low prevalence of recurrently mutated genes, except for TP53, which is mutated in 96% of cases. Therefore, homologous recombination (HR) defects are suggested to be present in up to 50% of HGSOC at the time of diagnosis. The genes that are thought to be involved are PTEN, EMSY, RAD51, and the DNA sensing genes ATR and ATM. According to TCGA, which studied nearly 500 HGSOC samples, the most significant mutated genes are TP53, BRCA1, BRCA2, FAT3, NF1, CSMD3, CDK12, and GABRA6. Mutations in TP53 were detected in almost all samples in which BRCA1/2 were found (21% of samples) combining somatic and germline mutations. Instead, the other 7 genes are mutated in 2-6% of the cases. Furthermore, the TCGA identified amplification of CCNE1, MYC, and MECOM in more than 20% of tumors and 50 focal deletions such as the tumor suppressors PTEN, RB1, and NF1 were located in regions of homozygous deletions in about 2% of cases (Bell et al., 2011).

Hematoxylin and eosin staining (H&E) staining show that HGSOC is characterized by solid cell masses with intermediate-sized slit-like windows that have prominent eosinophilic nucleoli and hyperchromatic and pleomorphic giant nuclei (Kurman & Shih, 2016; Lisio et al., 2019; Prat, 2012). These tumor cells stained with Ki67 showed a high mitotic index with partially atypical mitoses.

Several markers are used to diagnose HGSOC by Immunohistochemistry (IHC) which are described in Table 1.

Table 1 - IHC markers used for HGSOC diagnosis

Gene	Description	References
P53	It is involved in cell cycle progression and proliferation, apoptosis and genomic stability and its mutation reaches 96% in HGSOC. Missense mutations of p53 are reflected in strong positive staining of HGSOC cells. Hence, aberrant protein is produced and it is not recognized by the proteasome leading to accumulation in cells. Instead, nonsense mutations in TP53 are also observed where protein staining in tumors is almost totally negative.	(Kurman & Shih, 2016; Lisio et al., 2019; Matulonis et al., 2016)
CK7	Expressed in both healthy and tumor cells of epithelial origin. It is used as epithelial marker.	(Chu et al., 2000)
WT1	It is overexpressed in OC and other malignancies. WT1 is involved as a transcription factor in the development of the urogenital system.	(Kurman & Shih, 2016; Manocha & Jain, 2019)
PAX8	PAX8 is highly expressed in OC and other cells of Müllerian origin. It is involved in the regulation of WT1 expression and the embryological development of many tissues (e.g. Müllerian systems)	(Li & Beihua, 2013; Nonaka et al., 2008)
P16	The p16 protein shows diffuse staining in approximately 60-80% of HGSOC. The tumor suppressor gene CDKN2A encodes this protein. Its role in the cell cycle is to slow down cell progression from G1 to the S phase.	(O'Neill et al., 2007; Sallum et al., 2018; Yoon et al., 2016)
P63	P63 encodes different transcripts which lead to p53 activation and apoptosis. It is expressed in serous carcinomas of the ovary whose progression is malignant.	(Cai et al., 2014; Mirsadraei et al., 2017)

The ovarian surface epithelium (OSE) has been thought to be the tissue precursor of all epithelial ovarian tumors. It was believed that at the base of HGSOC there was a constant cycle of repairs and regeneration of OSE due to ovulation. This process forms cortical inclusion cysts (CICs) by creating invaginations in OSE. Hence, this hypothesis suggests that CICs could be precancerous lesions in the pro-inflammatory and pro-oxidative environment (Ahmed et al., 2013). In 2001, Piek and colleagues discovered that women with

BRCA1 or BRCA2 mutations have a small area of dyplastic lesions in the distal fallopian tubes (Piek et al., 2001). This discovery led to the new hypothesis that the early lesions called serous tubal intraepithelial carcinomas (STICs) are the origin of HGSOC (Bowtell et al., 2015; Howitt et al., 2015; Piek et al., 2001, 2003). Therefore, STICs are not present in all HGSOC cases but are identified in approximately 80% of early-stage HGSOC and up to 50% of advanced-stage HGSOC (Matulonis et al., 2016).

Cell lines are the main model used to study cancer, Domcke et al. analyzed 47 OC cell lines and identified those with the highest genetic similarity to ovarian tumors. They verified that some of the cell lines in the panel likely originated from other non-HGSOC as they differ from the HGSOC tumor samples. They classified the 47 OC cell lines into three categories based on an empirical score: likely, possibly, and unlikely HGSOC. The correlation between cell line and HGSOC tumor sample was the copy-number profile, the presence of mutations in “non-HGSOC” genes that were commonly altered in other OC subtypes, the presence of TP53 mutations, and the frequency of non-synonymous mutations in protein-coding genes considered to classify the cell lines. Their study showed that KURAMOCHI and OVSAHO are the cell lines that better mimic HGSOC (Domcke et al., 2013). Instead, OVCAR3, SKOV3, and A2780 are the cell lines with the highest Pubmed citations and are classified as possibly HGSOC (OVCAR3) and unlikely HGSOC (SKOV3 and A2780) (Figure 4).

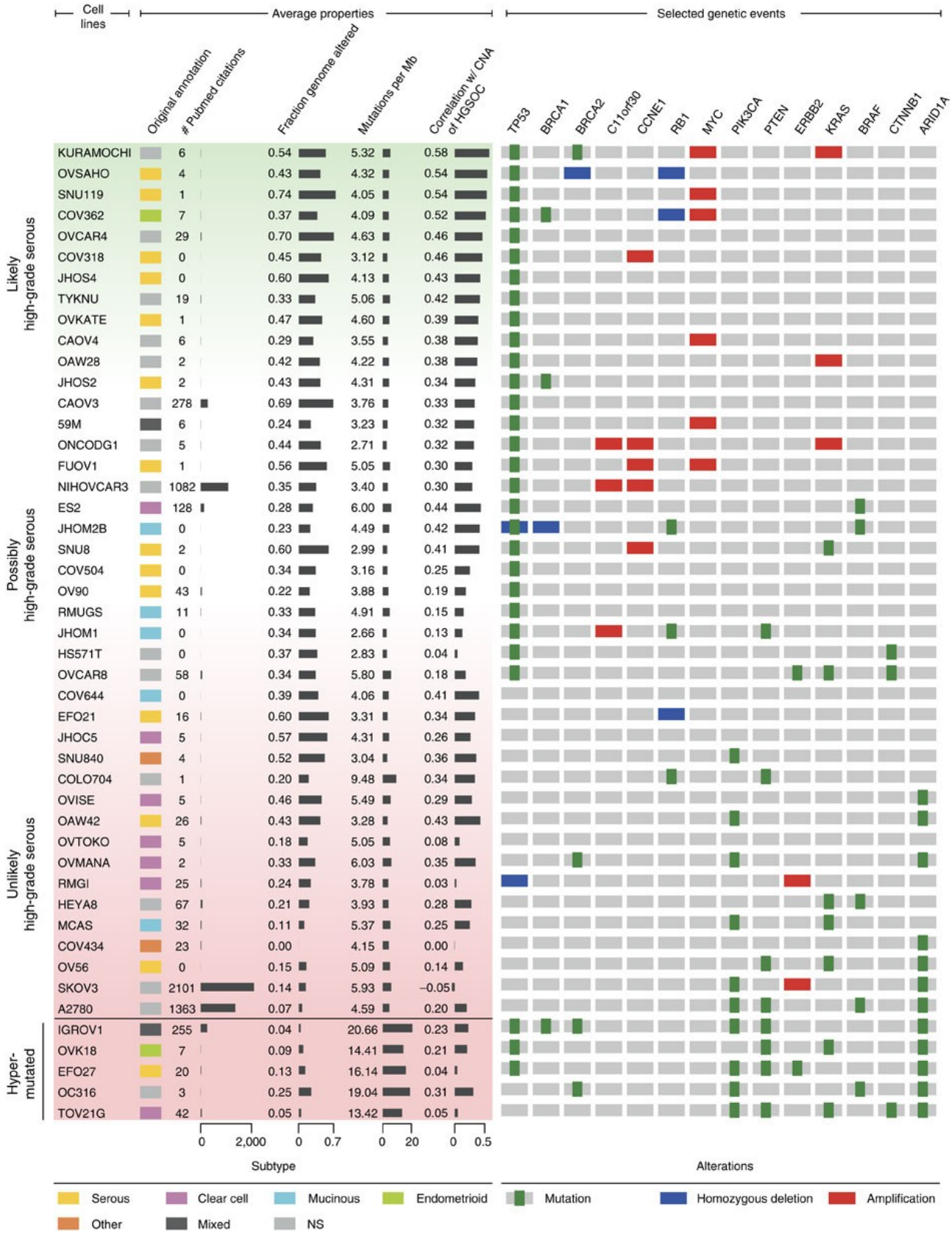


Figure 4 - Ranking OC cell lines by suitability as HGSOC models (Domcke et al., 2013).

1.3 CURRENT THERAPY FOR HGSOC

OC is a lethal disease that is the fourth leading cause of cancer-related death in women worldwide (Bray et al., 2018). Diagnoses in FIGO (International Federation of Gynecology and Obstetrics) stage III/IV and recurrent disease within 3 years of their primary therapy are the main causes of its lethality (Jayson et al., 2014; Mutch & Prat, 2014; Reid et al., 2017). Standard therapy usually consists of immediate primary surgery (IPS) followed by adjuvant platinum-based chemotherapy. Conversely, in women with advanced-stage disease diagnosis delayed primary surgery (DPS) is performed after completing 3-4 cycles of platinum-based neoadjuvant chemotherapy to increase patient OS (Kehoe et al., 2015; Querleu et al., 2017; Vergote et al., 2010). EOC first-line chemotherapy usually consists of 3-weekly platinum and 3-weekly paclitaxel chemotherapy, despite poor long-term results showing 70-80% recurrence rates within the first 2 years. (Koh et al., 2019; Ledermann et al., 2013). In the last two decades, alternatives to the carboplatin-paclitaxel have been researched, but no chemotherapeutic regimen achieved greater efficacy. The advances in target therapy led to the approval of Bevacizumab for use in first-line chemotherapy regimens in 2011. Bevacizumab is a humanized antibody to VEGF-A and together with the carboplatin-paclitaxel combination appears to enhance progression-free survival (PFS), as demonstrated by two different clinical studies: GOG218 and ICON7 (Burger et al., 2011; Perren et al., 2011). Bevacizumab is used in combination with carboplatin-paclitaxel, for treatment of OC stage III and IV, in combination with carboplatin-gemcitabine for platinum-sensitive recurrent cancer not previously treated with anti-angiogenic therapies, and in combination with topotecan, paclitaxel, or pegylated liposomal doxorubicin (PLD) in patients with relapsed platinum-resistant cancer who have not previously been treated with anti-angiogenic therapies (Gadducci et al., 2019). Hence, relapsed OC is curable only in a few patients and the "platinum-free interval" (PFI) indicates the best choice of chemotherapy for recurrent OC. The categorization of OC based on PFI defines “platinum-sensible” with PFI

higher than 12 months, “partially platinum-sensitive” with PFI between 6 and 12 months, “platinum-resistant” with PFI within 6 months and “platinum-refractory” is defined when the disease progresses during platinum-based therapy (Friedlander et al., 2011; Stuart et al., 2011). Hence, CA125 is a widely used glycoprotein used to diagnose EOC and track the patient over time to identify recurrence (Guo & Peng, 2017).

Table 2 - Current indications for PARPi in HGSOC (Voutsadakis, 2020)

Indication	*Olaparib*	*Niraparib*	*Rucaparib*
Maintenance after chemotherapy response		+	
Maintenance after chemotherapy response HRD+	+ (with bevacizumab)		
Recurrent after chemotherapy response	+	+	+
Recurrent BRCA mutant	+		+

Indeed, second-line treatments aim to delay symptomatic disease progression, prolong survival and improve the quality of life. Hence, patients sensitive or partially sensitive to platinum are treated with platinum-based combination therapy where trabectedin plus PLD increased PFS and OS in platinum- resistant and platinum-refractory patients (Gadducci et al., 2019). Few second-line chemotherapeutics are available to overcome platinum resistance but their outcomes could be improved with the introduction of targeted therapies. The introduction of PARP (poly-ADP-ribose polymerase) inhibitors (PARPi) represented the first target therapy in HGSOC after the recognition of the synthetic lethality of BRCA1 and BRCA2 mutations and Homologous Recombination Deficiency (HRD)-producing lesion (Matsumoto et al., 2019). The proteins encoded by the BRCA1 and BRCA2 genes are involved in the DNA damage repair to maintain genomic integrity. OC commonly promote the loss of function of these genes making cancer cells more dependent on other DNA repairing process such as single-strand DNA repair. The role of PARP in the single-strand DNA repair is crucial, and cancer cells with deficient BRCA functions are vulnerable to cytotoxic agents when PARP is inhibited, the so-called synthetic lethality (Drew, 2015;

Kaelin, 2005). Hence, PARPi (e.g. olaparib, niraparib, rucaparib) have been used for the treatment of recurrent OC according to chemotherapy response, and olaparib and rucabarib are also used in germline BRCA-mutated OC after chemotherapy progression (Ison et al., 2018; Kim et al., 2015). Therefore, niraparib is used for maintenance therapy after the success of first-line chemotherapy while the combination of olaparib with bevacizumab is used for maintenance therapy in BRCA mutated or HRD positive patients (Table *2*). More PARPi are currently available (e.g. talazoparib and veliparib) but they have not yet received regulatory approval for OC (Boussios et al., 2020).

1.4 PEPTIDYL-PROLYL CIS-TRANS ISOMERASE NIMA-INTERACTING 1 (PIN1)

A large number of oncogenes and tumor suppressors are directly controlled by Pro-directed phosphorylation and/or participate in signaling cascades that involve this phosphorylation. In particular, the same kinases and phosphatases often act on both oncogenes and tumor suppressors. In fact, until recently it was unclear how these phosphorylation events are coordinated to promote or inhibit tumorigenesis (Zhou & Lu, 2016). Proline only adopts cis and trans conformations, a process catalyzed by peptidyl-prolyl isomerase (PPIases). Although PPIases are able to control the interconversion kinetics of cis/trans isomerization, a non-essential cellular role has been ascribed to them. Therefore, the discovery of Pin1 underscores the importance of this enzymatic activity as a key regulatory mechanism in human physiology and pathology. In fact, Pro-directed kinases and phosphatases are conformation-specific and only work on the trans conformation increasing the importance of PIN1 as it specifically isomerizes pSer/Thr-Pro motifs (Zhou et al., 2000). Furthermore, local structural changes are induced by the Pro-directed phosphorylation, making them amenable to further modifications. Thus, PIN1 is a unique peptidyl-prolyl isomerase that regulates key proteins in many signaling pathways through phosphorylation of proline (Pro)-Ser/Thr motifs (Balastik et al., 2007; Shaw, 2007).

The PIN1 structure consists of two domains; the WW domain which specifically binds pSer/Thr-Pro motifs, and the PPIase domain which catalyzes cis-trans isomerization to regulate the structure and function of its substrate. The WW domain (named after two invariant Trp residues) is located at the N-terminal while the C-terminal represents the PPIase domain (Figure 5a). The interactions of these two domains with the specific pSer/Thr-Pro motifs were demonstrated by an early structure-function analysis that revealed the “double-check” mechanism (Pei-Jung et al., 1999; Ping Lu et al., 1996; Ranganathan et

al., 1997). Specifically, phosphorylation sites in PIN1 substrates are often regulated at the specific pSer/Thr-Pro motifs bound by the WW domain (Lu & Zhou, 2007; Wulf et al., 2001). Instead, protein function is regulated through conformational control generated from isomerization of the PPIase domain of pSer/Thr-Pro motifs (Figure 5c). The phosphorylation-dependent interaction was confirmed by complex PIN1 structures with their phosphopeptides bond (Verdecia et al., 2000; Wintjens et al., 2001). Conversely, the specificity of PIN1 to bind pSer/Thr-Pro motifs and the coordination of its domains to act on PIN1 substrates remains unclear. The sequence-specific dynamics are thought to be important for PIN1 substrate specificity and the crucial sequence resides in the WW domain. Once the ligand is bound to the intrinsically flexible loop, the flexibility changes (Peng et al., 2007).

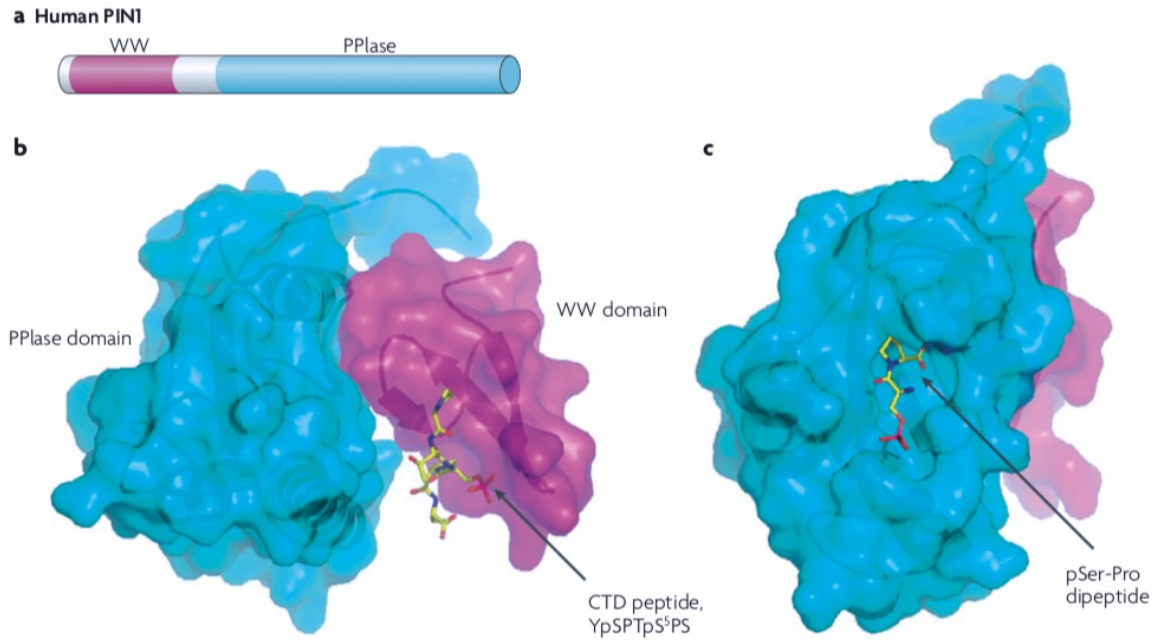


Figure 5 - PIN1 protein structure. a) PIN1 contains an N-terminal WW domain, that mediates binding to specific pSer/Thr-Pro motifs, and a C-terminal peptidyl-prolyl cis/trans isomerase (PPIase) domain that catalyzes the isomerization of specific pSer/Thr-Pro motifs in the substrate. b) X-ray structures of PIN1 in a complex with a C-terminal domain (CTD) peptide (YpSPTpS5PS) in the WW domain. c) X-ray structure of Pin1 in complex with a pSer-Pro dipeptide modeled in the PPIase domain *(Lu & Zhou, 2007)*.

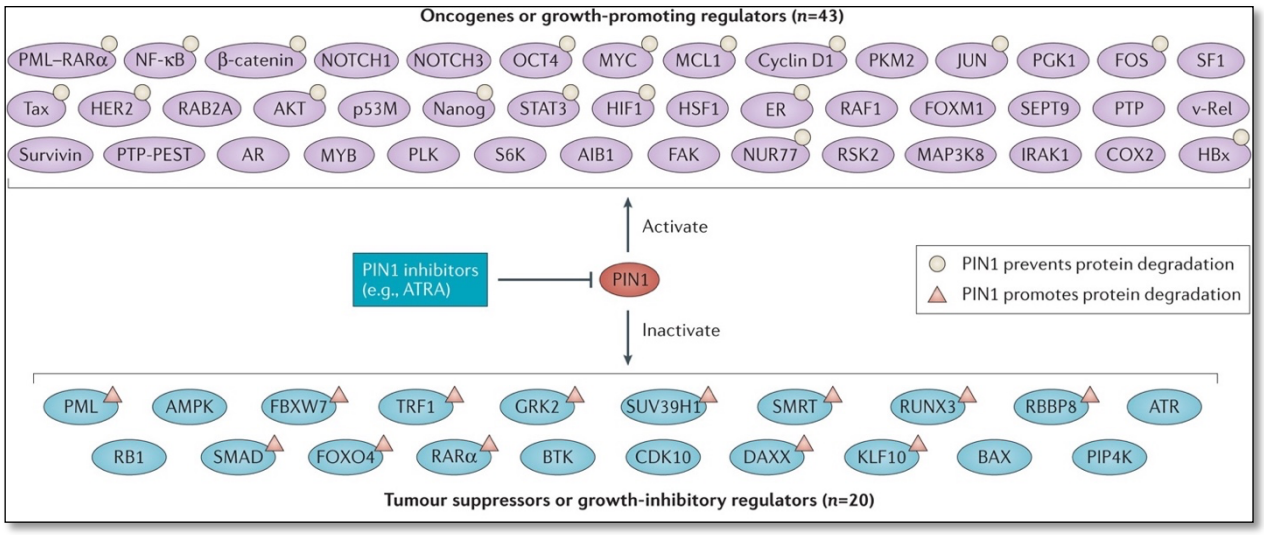


Figure 6 – Pin1 regulates more than 40 oncogenes or growth-promoting regulators and 20 tumor suppressors *(Zhou & Lu, 2016)*.

The role of Pin1 in cell cycle control was first identified in the mitotic phase. It modulates various cell cycle regulators in G1/S phases, such as RB, p27, p53, cyclin E, and cyclin D1. Indeed, cyclin D1 gene expression can be increased by Pin1 through multiple mechanisms, like the activation of nuclear factor (NF)-kB transcription factors and -Jun/c-Fos, beta-catenin/T-cell transcription factor (TCF) (Ryo et al., 2003; Wulf et al., 2001). Different cell functions are affected by Pin1 inhibition depending on cellular contexts such as epithelial-mesenchymal transition, cellular proliferation, new angiogenesis, apoptosis, migration, and invasion. In fact, cell cycle progression networks are supposed to be regulated like a molecular target from Pin1 (Lin et al., 2015). Pin1 has been shown to be overexpressed in many cancers and to act as a modulator of multiple cancer-driving signaling pathways playing a crucial role in the oncogenesis process. Indeed, PIN1 has been shown to regulate oncogenesis by inactivating more than 20 tumor suppressors and growth inhibitors and activating more than 40 oncogenes and growth enhancers (Figure 7). Pin1 regulates many critical cancer-driving receptors and intracellular signaling regulators that play critical roles in determining the outcome and duration of multiple signaling pathways.

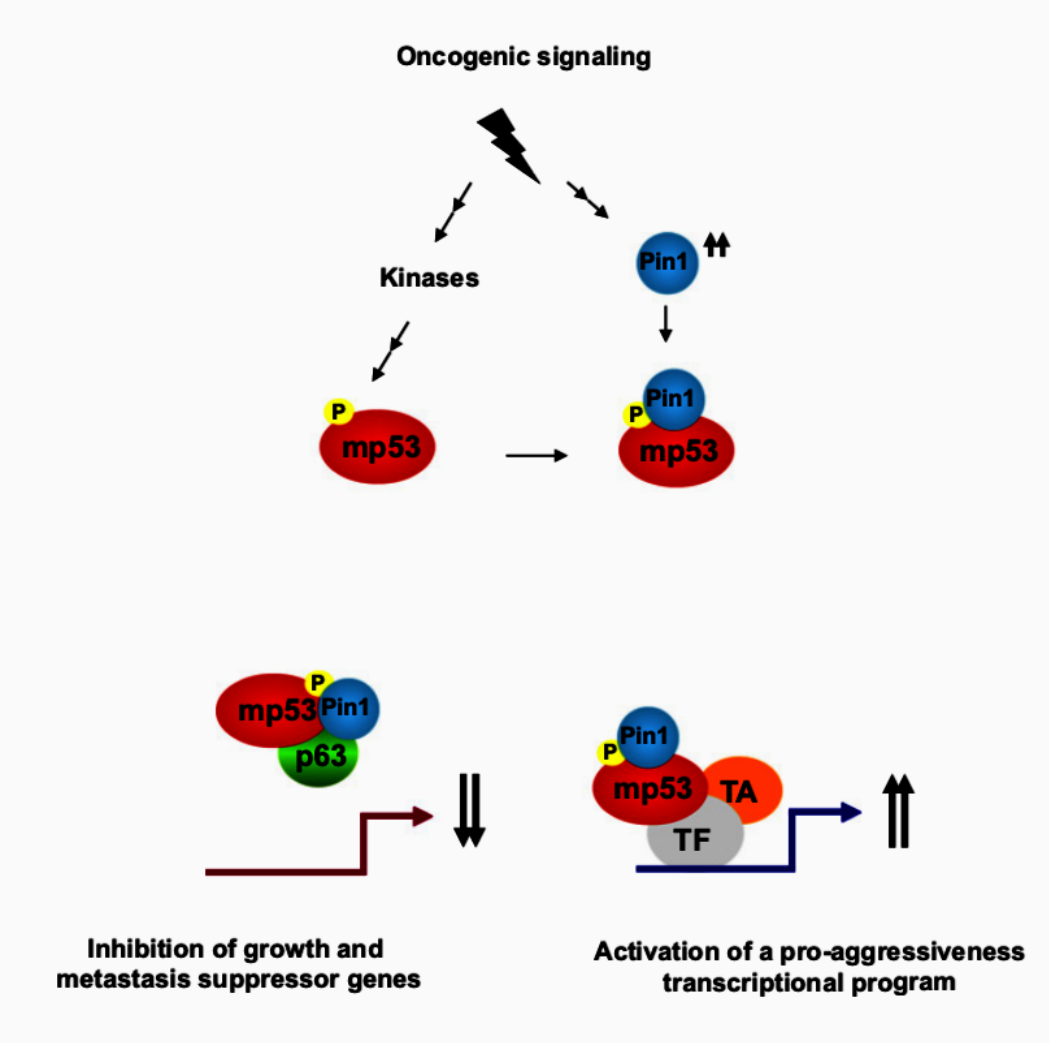


Figure 7 – Mutant p53 gain of function amplified by Pin1 (Girardini et al., 2011).

For instance, PIN1 interacts with NOTCH1, WNT/β-catenin, and c-MYC to inhibit several tumor suppressors (Zhou & Lu, 2016). PIN1-mediated intracellular signaling regulators take in AKT, MYC, focal adhesion kinase (FAK), serum/glucocorticoid regulated kinase 1 (SGK1), RAF1, SMAD2 and SMAD3, M2 isoform of pyruvate kinase (PKM2), and signal transducer and activator of transcription 3 (STAT3).

The interaction and cooperation between Pin1 and the mutant p53 (mp53) were studied in breast cancer due to the importance of the latter for cell cycle progression or apoptosis in response to different stimuli. Regulation of p53 transcriptional activities and stability was due to post-translational modifications of p53 and the association with other proteins (Girardini et al., 2011; Zacchi et al., 2002). Furthermore, the interaction between p53 and PIN1 also

acts on DNA damage by regulating many proteins involved in the cell cycle and apoptosis through p53 phosphorylation (Figure 6). The interaction between PIN1 and mp53 occurs for the phosphorylation of the latter at specific residues in the presence of oncogenic signaling. The complex obtained is able to inhibit some tumor suppressor genes and activate a pro-aggressiveness transcriptional program by inhibiting p63 (Girardini et al., 2011).

Pin1 expression is generally associated with cell proliferative potential in human tissues and its overexpression is commonly observed in human cancers leading to poor clinical outcomes for cancer patients. Hence, PIN1 acts in multiple oncogenic pathways such as mammary epithelial cell transformation in human breast cancer (Bao et al., 2004; Blume-Jensen & Hunter, 2001; Lu, 2003; Lu et al., 2006; Wulf et al., 2001). Many functions during cell cycle progression are modulated through PIN1-mediated isomerization. Indeed, the PIN1 expression level is important in regulating cell cycle progression. In fact, its overexpression causes tumorigenesis by promoting cell proliferation and malignant cell transformation through the alteration of cell cycle coordination (Cheng et al., 2013; R. Pang et al., 2007; R. W. Pang et al., 2006). Furthermore, several kinds of human cancers (e.g. breast, colon, liver, brain, etc.) have shown a correlation between PIN1 expression and tumor progression (Ayala et al., 2003; Bao et al., 2004; R. Pang et al., 2004; Wulf et al., 2001).

In conclusion, PIN1 activate more than 40 oncogenes and inactivate 20 tumor suppressor through *cis-trans* isomerizations. Overexpression and/or over-activation of PIN1 is associated with a poor clinical prognosis and its role makes PIN1 an interesting target for cancer treatment (la Montagna et al., 2013; Lucchetti et al., 2013; Rizzolio et al., 2012, 2013; Zhou et al., 2000).

1.5 PIN1 INHIBITORS

Cancer cells use many signaling pathways to proliferate and downregulate apoptotic signals such as the PIN1 target serine/threonine-proline motifs (Pawson & Scott, 2005). Cancer cells and cancer stem cells present the upregulation of PIN1 while normal tissues show low levels of expression (Singh & Settleman, 2010). Thus, drug resistance can be reversed, cancer cells targeted, and chemotherapies can be sensitized through PIN1 inhibition (Ding et al., 2008; Rustighi et al., 2014).

Since 1998, following the identification of **Juglone** (Figure 8A), the first PIN1 inhibitor, different classes of PIN1 inhibitors have been developed and discovered. PPIase assays, structural similarity, mechanism-based high-throughput screening, phenotypic association with PIN1 inhibition, substrate-mimicking design, and binding assays have been used to design and discover both covalent and non-covalent PIN1 inhibitors. Indeed, Juglone was identified by low-throughput PPIase screens which inhibit parvulin- and PIN1-type PPIases. It was demonstrated that Juglone suppresses cell proliferation and inhibits PIN1 activity in many types of cancers (e.g. HCC, prostate cancer, and glioblastoma) (Kanaoka et al., 2015; Lee et al., 2009; Wang et al., 2017; Yu et al., 2020). Hence, it irreversibly binds the PPIase domain and the dosage of 10-20 μM can reduce PIN1 protein expression (Hennig et al., 1998; Wang et al., 2017). Furthermore, Juglone inhibits prostate cancer tumor growth in a mouse model by intraperitoneal injection. The limitation of Juglone for its application in cancer treatment is the lack of specificity to PIN1, in fact, it covalently alters the active site Cys in various enzymes (Kanaoka et al., 2015).

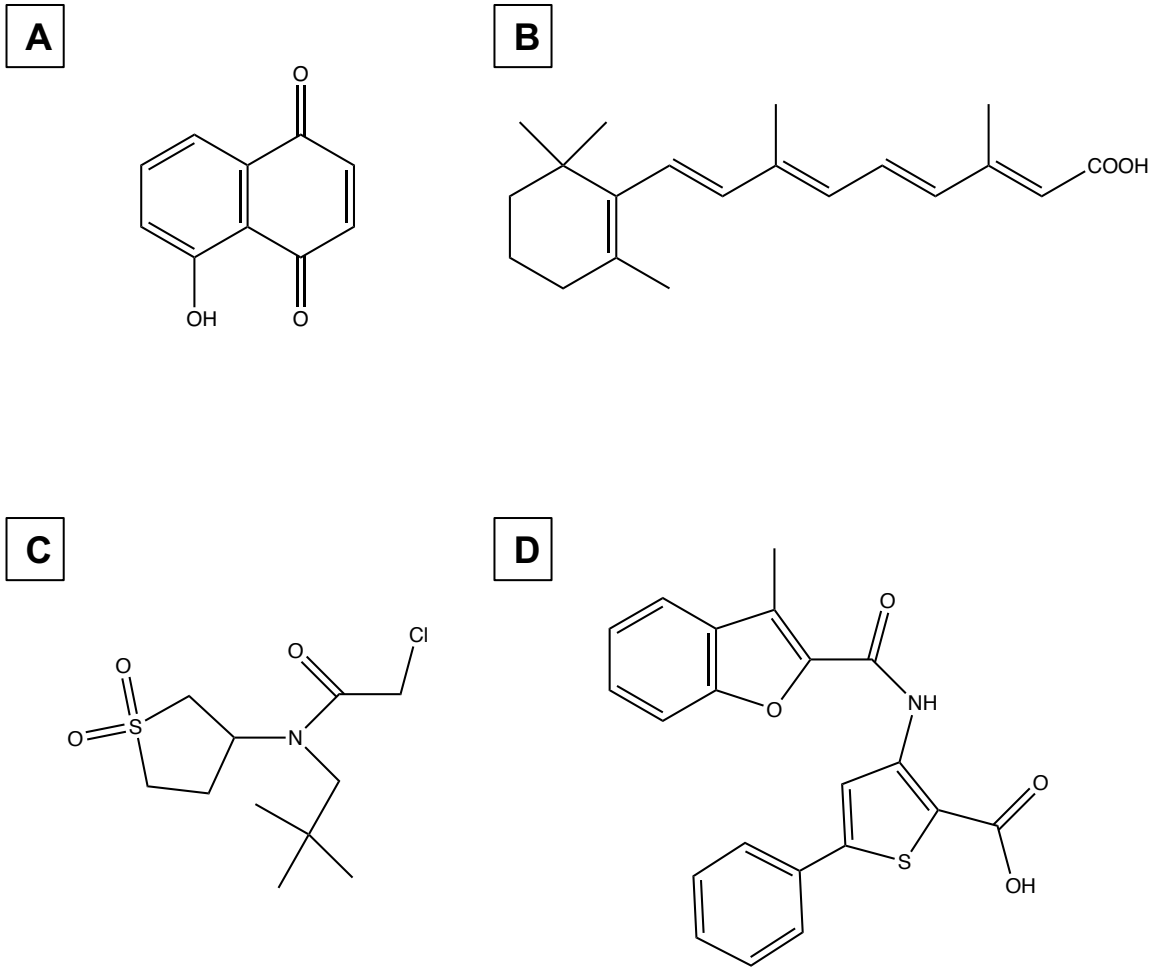


Figure 8 - Structure of PIN1 inhibitors: A) Juglone B) ATRA C) Sulfopin and D) VS10.

All-trans retinoic acid (**ATRA**) (Figure 8B) is an FDA-approved PIN1 inhibitor for the treatment of acute promyelocytic leukemia (APL) caused by an aberrant promyelocytic leukemia-retinoic acid receptor α (PML- RARα). ATRA binds the PPIase catalytic domain of PIN1 and inhibits PIN1 oncogenic functions by reducing cyclin D1 expression and blocking PIN1-induced centrosome amplification and inducing PIN1 protein degradation. PIN1 constitutively interacts with PML-RARalpha and stabilizes it in AML. Increased sensitivity to ATRA is achieved through the suppression of PIN1 which destabilizes RARalpha and PML-RARalpha (Liao et al., 2017; Wei et al., 2015). However, ATRA has a short half-life and is not specific to PIN1 (Notario et al., 2003; Ochoa et al., 2003; Schenk et al., 2014).

PIN1 inhibitors such as the previously mentioned Juglone and ATRA, and others like arsenic trioxide (ATO) and KPT-6566 have shown anticancer activity but lack specificity and/or cell

permeability, making them unsuitable for pharmacological inhibition of PIN1 *in vivo* (Campaner et al., 2017; Hennig et al., 1998; Kozono et al., 2018; Moore & Potter, 2013; Wei et al., 2015). Hence, covalent fragment-based drug discovery (FBDD) screening for compounds that target PIN1 Cys113 led to the development of **Sulfopin** (Figure 8C), a double-digit nanomolar, highly selective PIN1 inhibitor that engages PIN1 in cells and *in vivo*. PIN1 inhibition with Sulfopin resulted in modest effects on cancer cell viability and downregulation of Myc-dependent target genes. MYCN-driven zebrafish, murine models of neuroblastoma and murine pancreatic ductal adenocarcinoma (PDAC) treated with Sulfopin significantly reduced tumor progression and increased the OS. Furthermore, Sulfopin showed high selectivity for PIN1 making it suitable for *in vivo* applications (Dubiella et al., 2021).

In 2019, **VS10** (Figure 8D), another PIN1 inhibitor, has been discovered through consensus docking which presents micromolar efficacy and represents a potential new therapeutic agent in Pin1-overexpressing tumors. Hence, PIN1 degradation by the proteasome and reduced PIN1 levels downstream targets ^{Ser473}pAkt, β-catenin and cyclin D1 are achieved in VS10-treated OC cells (Russo Spena et al., 2019).

2. AIM OF THE WORK

Patients with advanced-stage HGSOC showed poor improvement in OS, despite good short-term responses to standard treatments consisting of cytoreductive surgery and platinum-based chemotherapy. Most patients with HGSOC are diagnosed at advanced stage of disease when the tumor has already spread to the peritoneum and invaded tissues beyond the ovaries. Unlike other cancer types, the therapeutic approaches to OC did not change in the past 20 years even with the development of target therapy (Lisio et al., 2019; Matulonis et al., 2016). Molecular targeted therapy aims to decrease toxicity and be a more effective strategy for OC. PARPi and anti-VEGF monoclonal antibodies are two of the most effective and approved targeted OC drugs. Cancer therapy is constantly researching for new challenges such as reducing cancer progression by simultaneously targeting different deregulated pathways (Zannini et al., 2019). Furthermore, the inadequacy of efficacy, systemic toxicity, drug resistance, and the inability to increase the drug administration indefinitely suggest the need to find an alternative to mono-chemotherapy. Therefore, combination therapy has begun to gain increasing attention to reduce the dose of the drug and achieve greater therapeutic efficacy (Feng et al., 2014). Furthermore, novel drug combination targets could improve current therapy as they may exhibit different resistance mechanisms than traditional therapy leading to greater chemotherapy success. PIN1 seems to be a candidate for this challenge since it is a unique peptidyl-prolyl isomerase that interacts with many signaling pathways through phosphorylation of proline (Pro)-Ser/Thr motifs (Balastik et al., 2007; Shaw, 2007). PIN1 is hyperactivated or overexpressed in many cancer types and it is possible to block tumor growth through its loss or inactivation (Zannini et al., 2019).

This thesis aims to clarify the molecular pathways and cellular functions where Pin1 is involved. These results are achieved through Pin1 pharmacological and lentiviral

downregulation. Furthermore, the role of Pin1 as a therapeutic enhancer to overcome drug resistance and toxicity was investigated. The main objectives to clarify the role of Pin1 in OC are the following:

1. Assess the effects of the PIN1 inhibitor VS10 on spheroids
    - Effects VS10 on the spheroid formation and preformed spheroid
    - Mesothelial Clearance Assay
    - Inhibition of spheroid migration
2. Assess the apoptosis activation through Cytochrome c release
3. Determinate PIN1 interaction with other molecular pathways
4. Evaluate PIN1/Mutant p53 breast axis in OC models
5. Evaluate the drug combination between PIN1 inhibitors and first- and second-line chemotherapeutics.

By addressing these goals, this project will advance the understanding of the role of PIN1 in HGSOC and highlight PIN1 as a promising target for the treatment of OC.

3. MATERIALS AND METHODS

3.1 CELL CULTURE

KURAMOCHI, OVCAR3, A2780, SKOV3, and OVSAHO cells were cultured in T75 flasks in RPMI 1640 Medium with L-Glutamine (Gibco, 21875-034), supplemented with 10% FBS (Microgerm, RM10432) and 100 U/ml Penicillin and 100 μg/ml Streptomycin (Euroclone, ECB3001D).

A2780cis and SKOV3cis cisplatin-resistant cells were cultured in T75 flasks in RPMI 1640 Medium with L-Glutamine (Gibco, 21875-034), supplemented with 10% FBS (Microgerm, RM10432), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Euroclone, ECB3001D), and cisplatin at the concentration of 0.6 mg/ml and 0.15 mg/ml, respectively.

MET5A were cultured in T75 flask in Medium 199 containing 1.5 g/L sodium bicarbonate, with 10% FBS (Microgerm, RM10432), 100 U/ml Pencillin and 100 μg/ml Streptomicin (Euroclone, ECB3001D) 3.3 nM epidermal growth factor (EGF) (PeproTech, af-100-15), 400 nM hydrocortisone (Sigma-Aldrich, H4001), 870 nM human insulin solution (Sigma-Aldrich, I9278), 20 mM HEPES (Euroclone, ECM0180), and the trace elements 0.3869 mg/L Selenious acid (Sigma-Aldrich, 211176), 0.0198 mg/L Manganese chloride (Sigma-Aldrich, 63535), 14.2100 mg/L Sodium metasilicate (Sigma-Aldrich, S4392), 0.1236 mg/L Ammonium molybdate (Sigma-Aldrich, M1019), 0.0585 mg/L Ammonium metavanadate (Sigma-Aldrich, 398128), 0.0131 mg/L Nickel(II) sulfate (Sigma-Aldrich, 227676) and 0.0113 mg/L Tin(II) chloride (Sigma-Aldrich, 96527).

All the cells were cultured using the following incubator condition: 95% humidity, 5% CO2, and 37°C.

Upon reaching 85-90% confluence, the cells were washed twice with PBS (Gibco, 14190144) and detached using 0.25% trypsin-EDTA (Sigma-Aldrich, T4049). Hence, cells were resuspended in the appropriate media to inactivate the trypsin, then they were

collected in a 15 ml tube and centrifuged for 5 minutes at 1000 RPM. The obtained cellular pellet was resuspended in fresh medium and cells were counted using the Bürker chamber. All the used cells were cryopreserved for long-time storage in FBS containing 10% DMSO in liquid nitrogen after one week in a Mr. Frosty at -80°C. Mycoplasm contamination was checked every month using MycoAlert® PLUS Mycoplasma Detection Kit (Lonza, LT07-705).

OVCAR3 and KURAMOCHI overexpressing Akt were obtained by infecting them with lentiviruses. Briefly, 5 x 10^5 cells were plated in a 10 mm petri dish and the day after 1 ml of lentivirus was added together with μg/ml of hexabromide (Sigma-Aldrich, 107689).

OVCAR3 and KURAMOCHI with Pin1 knockdown and empty vector (shCt, sh1, and sh2) were obtained by infecting them with lentiviral vector (LV). Briefly, 5 x 10^5 cells were plated in a 10 mm petri dish and the day after 1 ml of LV was added together with μg/ml of hexabromide (Sigma-Aldrich, 107689).

3.2 RESCUE AKT – CELL VIABILITY

OVCAR3 and KURAMOCHI infected with Ct, sh1, AktOE, and Akt-sh1 were used to evaluate the rescue of phenotype. Once obtained Akt-overexpressing cell lines, they were infected with Pin1 knockdown LV (sh1) as previously described in Section 3.1. After three days, 10^3 cells per condition were plated in four 96-well plates in quintuplicate. Cell viability was measured at four time points (0, 24, 48, and 120 hours) by using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7571).

3.3 RESCUE AKT – COLONY-FORMING ASSAY

OVCAR3 and KURAMOCHI infected with Ct, sh1, AktOE, and Akt-sh1 were plated in 6-well plates, in triplicate, at the concentration of 10^4 cells per well. After 20 days, the medium was removed, cells were washed with PBS, fixed with 4% PFA (Sigma-Aldrich, 158127), and then stained with Crystal Violet 0.05% (Sigma-Aldrich, C0775). Finally, the samples were destained with ddH_2O and images acquired.

3.4 WESTERN BLOT ANALYSIS

The cells were collected using a scraper, washed two times with PBS to remove traces of medium, and kept on ice. Samples were then lysed using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with Protease Inhibitor Cocktail (Roche, 04693116001), sodium orthovanadate (Sigma-Aldrich, S6508) and sodium fluoride (Sigma-Aldrich, S6776) or phosphorylated proteins lysis buffer (10mM Tris-HCl, 2mM EDTA, 1% SDS) supplemented with Protease Inhibitor Cocktail (Roche, 04693116001), sodium orthovanadate (Sigma-Aldrich, S6508) and sodium fluoride (Sigma-Aldrich, S6776) according to the final target. Protein concentration was estimated by Bradford assay by using Bio-Rad Protein Assay Dye (Bio-Rad, 5000006) (Bradford, 1976). Samples were prepared using equal amounts of protein (15 – 20 μg), 5X Sample Buffer (GenScript, MB01015) and diluted to a final volume of 20 μL with ddH_2O. Samples were heated at 100°C for 10 minutes and proteins were separated by 4-20% SDS-polyacrylamide gel electrophoresis (GenScript, M42012) and transferred to nitrocellulose membrane (GE Healthcare, 10600002) using Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, 1658004). The protein signal was detected with LiteAblot® EXTEND Chemiluminescent Substrate (Euroclone, EMP013001) using VWR® Imager CHEMI Premium (VWR, 730-1469P) and images were analyzed using ImageJ software (Schneider

et al., 2012). The secondary antibodies used are Goat anti-Mouse IgG (Invitrogen, 31430), Goat anti-Rabbit IgG (Invitrogen, 32460), and Rabbit anti-Goat IgG (Invitrogen, 31402). Primary antibodies are reported in Table 3.

Table 3 - List of antibodies used in WB including producer and catalog number.

Antibody	Producer	Cat. number
14-3-3z	Signalway Antibody	21188
Phospho-14-3-3z (Ser58)	Signalway Antibody	11181
Akt	Cell Signaling	4691
Phospho-Akt (Ser473)	Cell Signaling	4060
ATM	Bethyl	A300-299A
Bad	Cell Signaling	9239
Phospho-BAD (Ser136)	Cell Signaling	4366
Phospho-BAD (Ser155)	Cell Signaling	9297
Bax	Santa Cruz	sc-7480
Bcl-2	Cell Signaling	15071
Phospho-Bcl2 (Ser70)	Cell Signaling	2827
Bub1	Bethyl	A300-373A
c-Myc	Abcam	ab32072
Caspase-9	Santa Cruz	sc-56073
CDK1	Santa Cruz	sc-54
CDK2	Santa Cruz	sc-163
CDK4	Proteintech	11026-1-AP
Cyclin A2	Proteintech	18202-1-AP
Cyclin B1	Santa Cruz	sc-245
Cyclin D1	BD Biosciences	556470
Phospho-Cyclin D1 (Thr286)	Cell Signaling	3300
E-Cadherin	Genetex	GTX100443
EGFR	Santa Cruz	sc-03
FAK	Santa Cruz	sc-1688
Phospho-FAK (Tyr397)	Cell Signaling	3283
FOXM1	Cell Signaling	5436
FoxO1	Cell Signaling	14952
Phospho-FoxO1 (Ser319)	Cell Signaling	9464
FoxO3a	Cell Signaling	12829
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	Cell Signaling	9464
GSK-3β	Cell Signaling	12456
Phospho-GSK-3β (Ser9)	Cell Signaling	5558
Phospho-Histone H2A.X (Ser139)	Millipore	05-636
Phospho-Histone H3 (Ser10)	Abcam	ab5176
KRAS	Proteintech	12063-1-AP
MEK1/2	Cell Signaling	9122
Phospho-MEK1/2 (Ser217/221)	Cell Signaling	9121
Stathmin	BD Biosciences	611146

mTOR	Cell Signaling	2983S
NOTCH3	Abcam	ab23426
p38 MAPK	Bethyl	A300-707A
Phospho-p38 (Thr180/Tyr182)	Cell Signaling	4511
p44/42 MAPK (Erk1/2)	Cell Signaling	9102
Phospho-p42/44 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling	9101
p53	Proteintech	21891-1-AP
Phospho-p53 (Ser15)	Cell Signaling	9286
p70 S6 Kinase	Cell Signaling	9202
Phospho-p70 S6 Kinase (Ser371)	Cell Signaling	9208
Phospho-p130 (Ser672)	Novus Biotechnologies	nb100-80046
PD-L1	Cell Signaling	13684
PIN1	Santa Cruz	sc-46660
PTEN	Santa Cruz	sc-7974
Rb	Santa Cruz	sc-102
Phospho-Rb (Ser780)	Cell Signaling	9307
S6 Ribosomal Protein	Cell Signaling	2217S
Phospho-S6 Ribosomal Protein (Ser235/236)	Cell Signaling	4858S
Tuberin	Cell Signaling	4308S
Phospho-Tuberin (Thr1462)	Cell Signaling	3617
Wee1	Santa Cruz	sc-5285
Phospho-Wee1 (Ser642)	Cell Signaling	4910

3.5 AKT/PKB PHOSPHO ANTIBODY ARRAY

OVCAR3-Ct and OVCAR3-sh1 were collected and the pellet was used to perform AKT/PKB Phospho Antibody Array in Tebu-Bio laboratories. The following total and phosphorylated proteins had been analyzed:

14-3-3 theta/tau (Ab-232), 14-3-3 theta/tau (Phospho-Ser232), 14-3-3 zeta (Ab-58), 14-3-3 zeta (Phospho-Ser58), 14-3-3 zeta/delta (Ab-232), 14-3-3 zeta/delta (Phospho-Thr232), 6-Phosphofructo-2-Kinase (PFKFB2) (inter), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) (Ab-483), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) (Phospho-Ser483), AKT (Ab-308), AKT (Ab-326), AKT (Ab-473), AKT (N-term), AKT (Phospho-Ser473), AKT (Phospho-Thr308), AKT (Phospho-Tyr326), AKT1 (Ab-124), AKT1 (Ab-246), AKT1 (Ab-450), AKT1 (Ab-474), AKT1 (Ab-72), AKT1 (Phospho-Ser124), AKT1 (Phospho-Ser246), AKT1 (Phospho-Thr450), AKT1 (Phospho-Thr72), AKT1 (Phospho-Tyr474), AKT1/2/3 (Ab-315), AKT1S1 (Ab-246), AKT1S1 (Phospho-Thr246), AKT2 (Ab-474), AKT2 (Phospho-Ser474), BAD (Ab-112), BAD (Ab-134),BAD (Ab-136), BAD (Ab-155), BAD (Ab-91/128), BAD (Phospho-Ser112), BAD (Phospho-Ser134), BAD (Phospho-Ser136), BAD (Phospho-Ser155), BAD (Phospho-Ser91/128), BAX (Ab-167),Bax (N-term), BCL-2 (Ab-56), BCL-2 (Ab-69), BCL-2 (Ab-70), BCL-2 (Phospho-Ser70), BCL-2 (Phospho-Ser87), BCL-2 (Phospho-Thr56), BCL-2 (Phospho-Thr69), BIM (Ab-69/65), BIM (Phospho-Ser69/65), Cyclin D1 (Ab-286), Cyclin D1 (Phospho-Thr286), eNOS (Ab-1177), eNOS (Ab-495), eNOS (Ab-615), eNOS (Phospho-Ser1177), eNOS (Phospho-Ser615), eNOS (Phospho-Thr495), FAK (Ab-397), FAK (Ab-407), FAK (Ab-576), FAK (Ab-861), FAK (Ab-910), FAK (Ab-925), FAK (Phospho-Ser910), FAK (Phospho-Tyr397), FAK (Phospho-Tyr407), FAK (Phospho-Tyr576), FAK (Phospho-Tyr861), FAK (Phospho-Tyr925), FKHR (Ab-256), FKHR (Ab-319), FKHR (Phospho-Ser256), FKHR (Phospho-Ser319), FOXO1/3/4-PAN (Ab-24/32), FOXO1/3/4-PAN (Phospho-Thr24/32), FOXO1A (Ab-329), FOXO1A (Phospho-Ser329), FOXO1A/3A

(Phospho-Ser322/325), Gab1 (Ab-627), Gab1 (Ab-659), Gab1 (Phospho-Tyr627), Gab1 (Phospho-Tyr659), Gab2 (Ab-623), Gab2 (Phospho-Tyr643), GABA B receptor (C-term), GABA-RB (Ab-434), GABA-RB (Phospho-Ser434), GSK3a-b (Ab-216/279), GSK3a-b (Phospho-Tyr216/279), GSK3α (Ab-21), GSK3α (Phospho-Ser21), GSK3β (Ab-9), GSK3β (Phospho-Ser9), IKK α (Ab-23), IKK α (Phospho-Thr23), IKKa/b (Ab-180/181), IKKa/b (Phospho-Ser180/181), IRS-1 (Ab-307), IRS-1 (Ab-312), IRS-1 (Ab-323), IRS-1 (Ab-636), IRS-1 (Ab-639), IRS-1 (Ab-794), IRS-1 (Phospho-Ser1101), IRS-1 (Phospho-Ser307), IRS-1 (Phospho-Ser312), IRS-1 (Phospho-Ser323), IRS-1 (Phospho-Ser612), IRS-1 (Phospho-Ser636), IRS-1 (Phospho-Ser639), IRS-1 (Phospho-Ser794), JAK1 (Ab-1022), JAK1 (Phospho-Tyr1022), LYN (Ab-507), LYN (Phospho-Tyr507), MDM2 (Ab-166), MDM2 (Phospho-Ser166), mTOR (Ab-2446), mTOR (Ab-2448), mTOR (Ab-2481), mTOR (Phospho-Ser2448), mTOR (Phospho-Ser2481), mTOR (Phospho-Thr2446), MYT1 (Ab-83), p21Cip1 (Ab-145), p21Cip1 (Phospho-Thr145), p27Kip1 (Ab-10), p27Kip1 (Ab-187), p27Kip1 (Phospho-Ser10), p27Kip1 (Phospho-Thr187), p53 (Ab-15), p53 (Ab-18) p53 (Ab-20), p53 (Ab-315), p53 (Ab-33), p53 (Ab-37), p53 (Ab-376), p53 (Ab-378), p53 (Ab-387), p53 (Ab-392), p53 (Ab-46)p53 (Ab-6) p53 (Ab-9) p53 (Phospho-Ser15) p53 (Phospho-Ser20)p53 (Phospho-Ser315), p53 (Phospho-Ser33), p53 (Phospho-Ser366), p53 (Phospho-Ser37), p53 (Phospho-Ser378), p53 (Phospho-Ser392), p53 (Phospho-Ser46), p53 (Phospho-Ser6), p53 (Phospho-Ser9), p53 (Phospho-Thr18), p53 (Phospho-Thr81), p70S6K (Ab-229), p70S6K (Ab-371), p70S6K (Ab-411), p70S6K (Ab-418), p70S6K (Ab-421), p70S6K (Ab-424), p70S6K (Ab-427), p70S6K (Phospho-Ser371), p70S6K (Phospho-Ser411), p70S6K (Phospho-Ser418), p70S6K (Phospho-Ser424), p70S6K (Phospho-Thr229), p70S6K (Phospho-Thr389), p70S6K (Phospho-Thr421), p70S6K-beta (Ab-423), p70S6K-beta (Phospho-Ser423), Paxillin (Ab-118), Paxillin (Ab-31) Paxillin (Phospho-Tyr118), Paxillin (Phospho-Tyr31), PDK1 (Ab-241), PDK1 (Phospho-Ser241), PI3-kinase p85-alpha (Phospho-Tyr607), PI3-kinase p85-subunit alpha/gamma (Ab-467/199), PI3-

kinase p85-subunit alpha/gamma (Phospho-Tyr467/Tyr199), PIP5K (inter), PIP5K (Phospho-Ser307), PP2A-a (Ab-307), PP2A-a (Phospho-Tyr307), PTEN (Ab-370), PTEN (Ab-380), PTEN (Ab-380/382/383), PTEN (Phospho-Ser370), PTEN (Phospho-Ser380), PTEN (Phospho-Ser380/Thr382/Thr383), RapGEF1 (Phospho-Tyr504), S6 Ribosomal Protein (Ab-235), S6 Ribosomal Protein (Phospho-Ser235) , S6K (inter), S6K-α6 (inter), SYK (Ab-348), SYK (Ab-525), SYK (inter), SYK (Phospho-Tyr323), SYK (Phospho-Tyr348), SYK (Phospho-Tyr525), SYN1-Synapsin1 (Ab-62), SYN1-Synapsin1 (Phospho-Ser62), Tuberin (Ab-981), Tuberin/TSC2 (Ab-1462), Tuberin/TSC2 (Ab-939), Tuberin/TSC2 (Phospho-Ser939), Tuberin/TSC2 (Phospho-Thr1462), WEE1 (Ab-53), WEE1 (Phospho-Ser642), XIAP(Ab-87), XIAP (Phospho-Ser87).

3.6 IMMUNOFLUORESCENCE ANALYSIS

Coverslips were functionalized with Poly-D-Lysine 1 µg/mL on 6-MW plates. After 5 minutes, coverslips were washed and 4 x 10^5 SKOV3 and KURAMOCHI cells were seeded in five wells per time point for a total of four time points (6, 24, 48, 72 hours). The cells were then treated using PIN1 inhibitors VS10 35 µM, VS10 70 µM, cisplatin 10 µM and two samples as controls. After 72 hours, the medium was removed, and cells were washed and fixed using PFA 4% for 20 minutes. Then, PFA was removed, cells were rinsed three times with PBS and the specimen was permeabilized by adding 1X PBS/0.3% Triton X-100 solution and incubated for 15 minutes. The specimen was then washed three times with PBS and incubated with 8% BSA solution in PBS (Sigma-Aldrich, 05479) for 1 hour at room temperature. Samples were incubated with Cytochrome c primary antibody (Cell Signaling, 12963) diluted 1:100 in 1X PBS/1% BSA solution overnight at 4°C. After three times washes with PBS, Goat anti-Mouse IgG Alexa Fluor® Plus 488 secondary antibody (Invitrogen, A32723) were diluted 1:1˙000 in 1X PBS/1% BSA solution, added to samples and incubated for 1 hour at room temperature in the dark. Samples were then washed three times with PBS and counterstained with 0.1 µg/mL DAPI solution in PBS for one minute. Finally, DAPI was removed, and samples were washed with PBS and mounted on microscope slides. Cells were analyzed and images were acquired by fluorescence microscope (Leica, DM5500 B) setting up the fluorescence background intensity on the negative control sample (where only the secondary antibody was incubated to avoid overexposure).

3.7 MESOTHELIAL CLEARANCE ASSAY

MET5A and SKOV3 cell lines were cultured as described in Section 3.1. Petri dish was coated with 95% Ethanol/20 mg/ml Poly-HEMA solution and then 3 x 10^5 SKOV3 cells were seeded. SKOV3 formed spheroids after 48 hours and they were pretreated for 24 hours with VS10 35 µM and VS10 70 µM. MET5A cells were stained using the green fluorescent dye DiO (Invitrogen, D275) and 500˙000 cells were plated in each well of a 6-well plate to obtain a mesothelial monolayer. The day after, spheroids were transferred to the 6-well plate containing MET5A cells and the treatment with VS10 35 µM and VS10 70 µM was performed again. Brightfield and fluorescent pictures were taken after 1, 4 and 8 hours using a fluorescent microscope (Nikon, ECLIPSE Ti2).

3.8 QRT-PCR

OVCAR3 and KURAMOCHI cell lines encoding PIN1 knockdown and empty vector were detached after three days of using 0.25% trypsin-EDTA, collected and washed three times with PBS. RNA was obtained using InviTrap® Spin Universal RNA Mini Kit (Invitek Molecular, 1060100200) and cDNA synthesis was obtained using GoScript™ Reverse Transcriptase (Promega, A5001). qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega, A6001), QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, A28567) and the following primers: PIN1, Bub1, C21orf45, CCNE, CENPA, hCPSF6, DEPDC1, EPB41L4B, FAM64, NCAPH, PARP3, and βACTIN. Data were collected using QuantStudio 3 and 5 Real-Time PCR System Software and analyzed using the $2^{-\Delta\Delta CT}$ method on Excel.

3.9 DRUG COMBINATION

KURAMOCHI, OVCAR3, A2780, A2780cis, SKOV3, SKOV3cis and OVSAHO cell lines were cultured according to cell culture protocol. Cells were seeded (10^3 each well) in 96-well transparent plates (Falcon, 353072) and the day after was treated with 12 concentrations of the selected drugs using 1:2 serial dilution. Second-line chemotherapeutics Paclitaxel, Abraxane, Carboplatin, Olaparib, Nirapalib, Gemcitabine, Topotecan, Trabectidin, Doxorubicin and Caelyx and PIN1 inhibitors ATRA and VS10 and the combination between PIN1 inhibitors and second-line chemotherapeutics were used to treat KURAMOCHI and SKOV3 as screening. Doxorubicin, Caelyx, ATRA and VS10 were used for all other cell lines. Synergism was evaluated using the Bliss additivity model and results were plotted using GraphPad Prism (Bliss, 1939).

3.10 STATISTICAL ANALYSIS

All the experiments were performed in triplicate and the p-value was calculated using a two-tailed Student's t-test using GraphPad Prism. P values are expressed as follows:

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

4. RESULTS

4.1 TREATMENT WITH PIN1 INHIBITOR VS10 INDUCES OVARIAN CANCER CELL DEATH

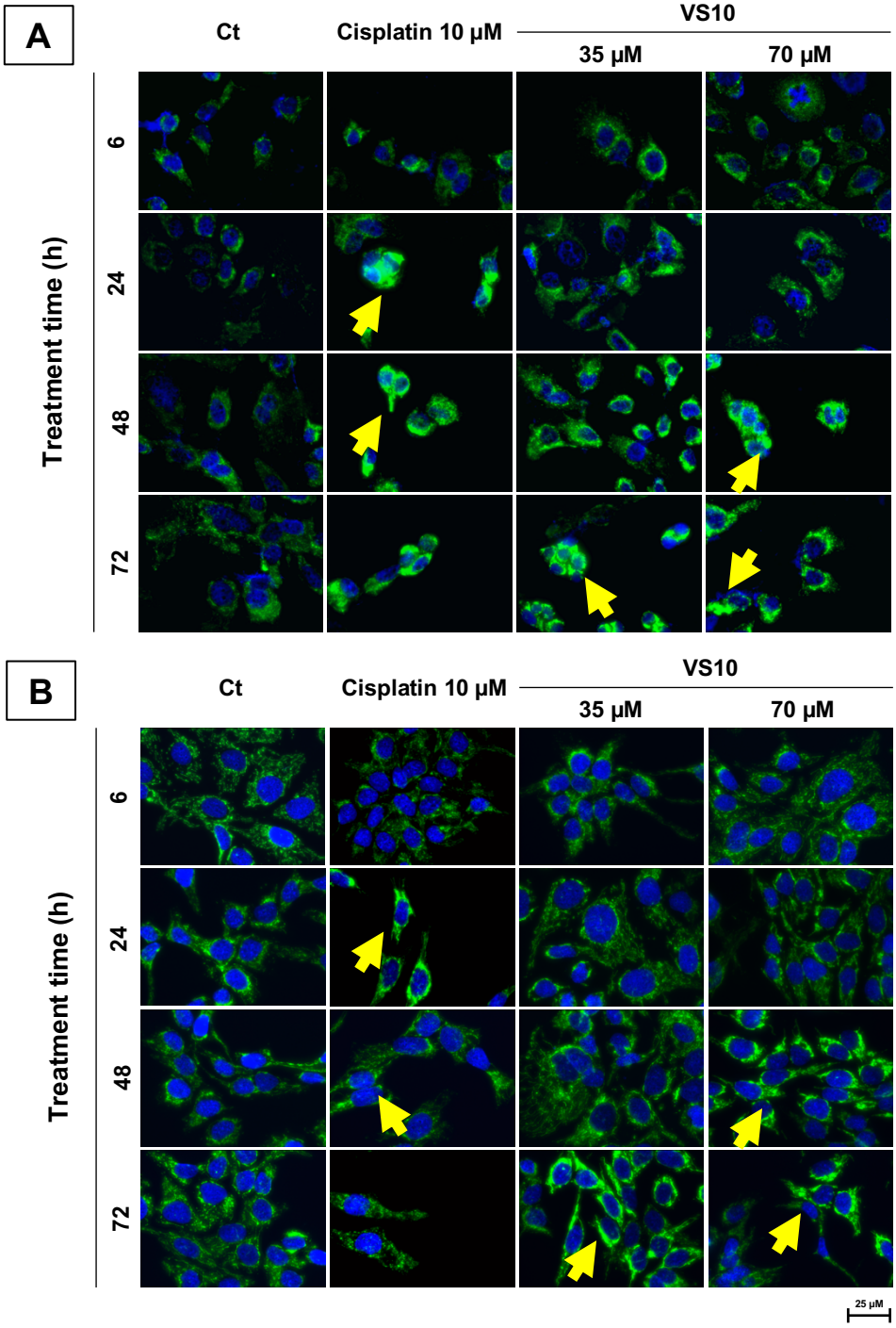


Figure 9 - KURAMOCHI and SKOV3 Cyt C immunofluorescence release. A) Immunofluorescence analysis of Cyt C release in VS10-treated Kuramochi and B) SKOV3 cell lines after 6, 24, 48 and 72 hours of treatment with VS10 35 µM and 70 µM. Cisplatin 10 µM was used as positive control. Nuclei were counterstained using DAPI 0.1 µg/mL solution. Yellow arrows show Cyt C release. Magnification 63X.

Our previous experiments showed that PIN1 inhibitor VS10 exhibited strong cytotoxicity on several OC cell lines such as SKOV3, OVCAR3, OVCAR5 and A2780 with the half-maximal inhibitory concentration (IC50) of VS10 ranging from 53.9 to 76.4 μM (Russo Spena et al., 2019). According to the genetic profile of HGSOC provided by whole-exome sequencing (WES) of TCGA, KURAMOCHI cell line has been reported to better reproduce the molecular characteristics of HGSOC disease. As shown in Table 4, estimation of the IC50-value of VS10 on KURAMOCHI cell line of 76.6 μM confirmed the strong inhibitory activity of this selective inhibitor of Pin1. Besides the molecular profile, invasiveness of the peritoneal cavity in the advanced stage remains the main cause of the high lethality of this disease (Torre et al., 2018). Spheroids are thought to play a key role in OC metastatic spread. In this regard, SKOV3 is a widely used OC cell line to reproduce spheroids. On the basis of these observations, the two cell lines were chosen as the main cellular models to study the molecular mechanisms that selectively trigger VS10 cytotoxicity through PIN1 inhibition.

Table 4 - Newly updated IC50 of VS10 in OC cell lines, values are mean (SD)

Cancer cell lines	***IC50 (μM)***
SKOV3	98.3 (2.5)
KURAMOCHI	76.6 (10.8)

Cytochrome c (Cyt C) is a widely used mitochondria protein for the detection of the early stage of apoptosis. In order to study the mechanism that mediates VS10 cytotoxicity, Cyt C release in KURAMOCHI and SKOV3 cell lines treated with PIN1 inhibitor VS10 was evaluated by immunofluorescence analysis. According to IC50 of VS10 (Table 4), KURAMOCHI and SKOV3 cell lines were exposed to VS10 35 and 70 μM. Cisplatin 10 μM was used as positive control. As it appears evident in Figure 9, the release of Cyt C is dose-dependent. Although Cyt C release started already after 24h of treatment, a higher concentration of VS10 (70 μM) induced an intense release of Cyt C in both cell lines at 48

hours of exposure. Instead, when used at lower concentration of VS10 35 μM, Cyt C release becomes prominent after 72 hours. These results suggest the involvement of apoptosis in VS10-induced cytotoxicity via mitochondrial pathway.

4.2 ACTIVITY OF PIN1 INHIBITOR VS10 ON EOC DISSEMINATION

4.2.1 EFFECTS OF PIN1 INHIBITION BY VS10 ON SPHEROID FORMATION

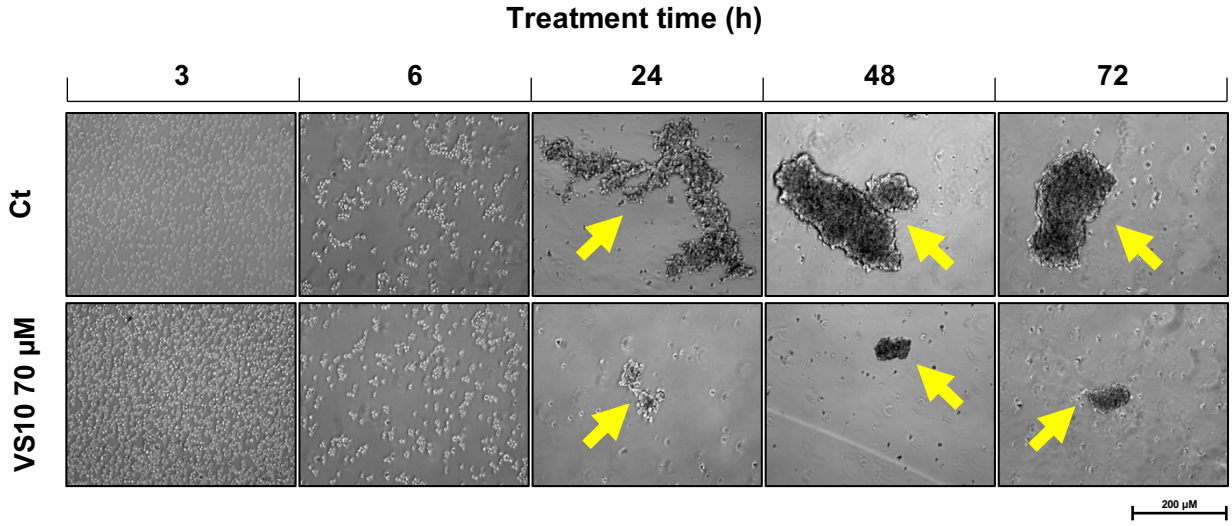


Figure 10 – Effect of VS10 on spheroid formation. SKOV3 cells were seeded in a polyHEMA coated plate and treated with VS10 70 µM. Images were acquired after 3, 6, 24, 48 and 72 hours of treatment. Yellow arrows indicate the differences between treated and untreated spheroids. Magnification 20X.

The unusual mechanism of EOC dissemination, which characteristically involves local invasion of pelvic and abdominal organs, has been supposed as the main reason for the failure of EOC treatment. Recently, aggregates of malignant cells, commonly referred to as spheroids found in malignant ascites, represent a chemoresistant mechanism to effectively treat advanced EOC.

Since current chemotherapeutic agents do not address the anchorage-independent growth conditions, associated with the formation and growth of 3D structures typical of EOC in the late stage, it may be assumed that spheroid formation represents a key component of platinum/taxane-sensitive recurrence. The efficacy of PIN1 inhibitor VS10 to interfere with the spheroid formation and thus with the potential to block EOC dissemination has been investigated. As shown in Figure 10, the inhibition of PIN1 with VS10 70 µM interfered with SKOV3 self-organization into spheroids. Indeed, after 24 hours from seeding in a poly-HEMA coated 6-well plate, untreated SKOV3 cells formed spheroids, while VS10-treated

SKOV3 cells were not able to form spheroids but only to irregularly aggregate in disorganized cellular mass. This data demonstrates that the selective inhibition of PIN1 by VS10 could also reduce the viability of metastatic cells, limiting the dissemination and the potential EOC recurrence.

4.2.2 EFFECTS OF PIN1 INHIBITOR VS10 ON PREFORMED SPHEROIDS

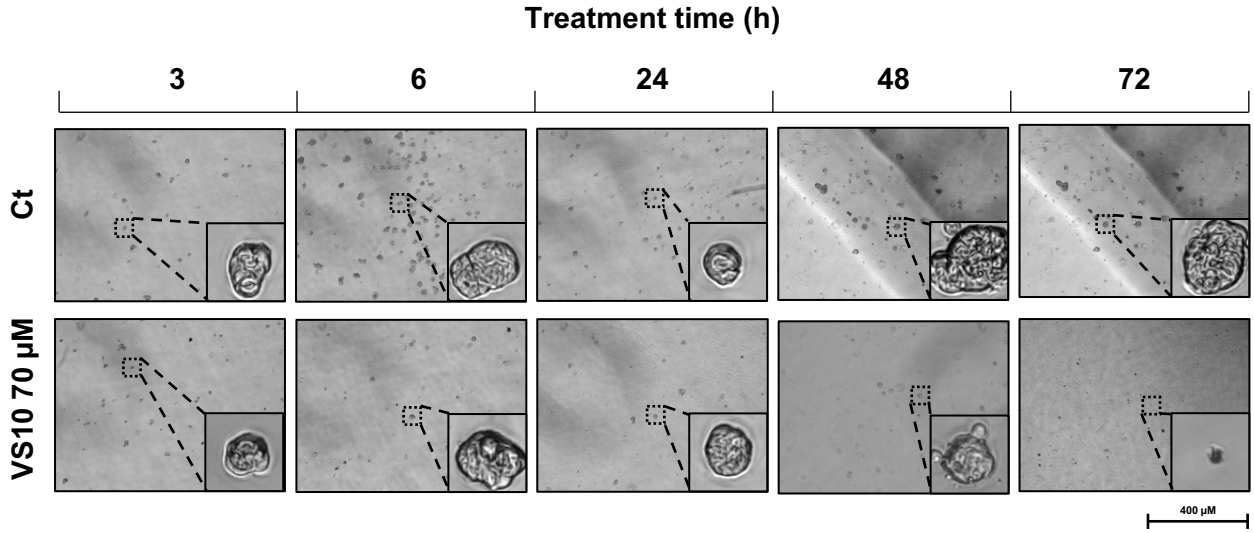


Figure 11 – Effects of VS10 on preformed spheroids. Preformed spheroids were seeded and treated with VS10 70 µM. Pictures were taken after 3, 6, 24, 48 and 72 hours. Magnification 10X.

In order to assess whether inhibition of PIN1 with VS10 could reveal to control tumor growth in different OC phases, VS10-induced cytotoxicity was evaluated on preformed spheroids. Treating preformed spheroids represent a challenge since spheroids are 3D structures characterized by an external layer of proliferative cells and an internal core of necrotic cells. Cells, cultured in a poly-HEMA coated 6-well plate, formed spheroids after 24 hours. Treatment with VS10 for 24 hours led spheroids to lose their structure, reducing their viability and forming aggregates. After 48 hours of VS10 exposure, the numbers of aggregates and spheroids were slightly decreased, losing their cell viability after 72 hours (Figure 11). These results underlined the potentiality of VS10 to control tumor growth through selective PIN1 inhibition in the advanced stage of EOC.

4.2.3 MESOTHELIAL CLEARANCE ASSAY

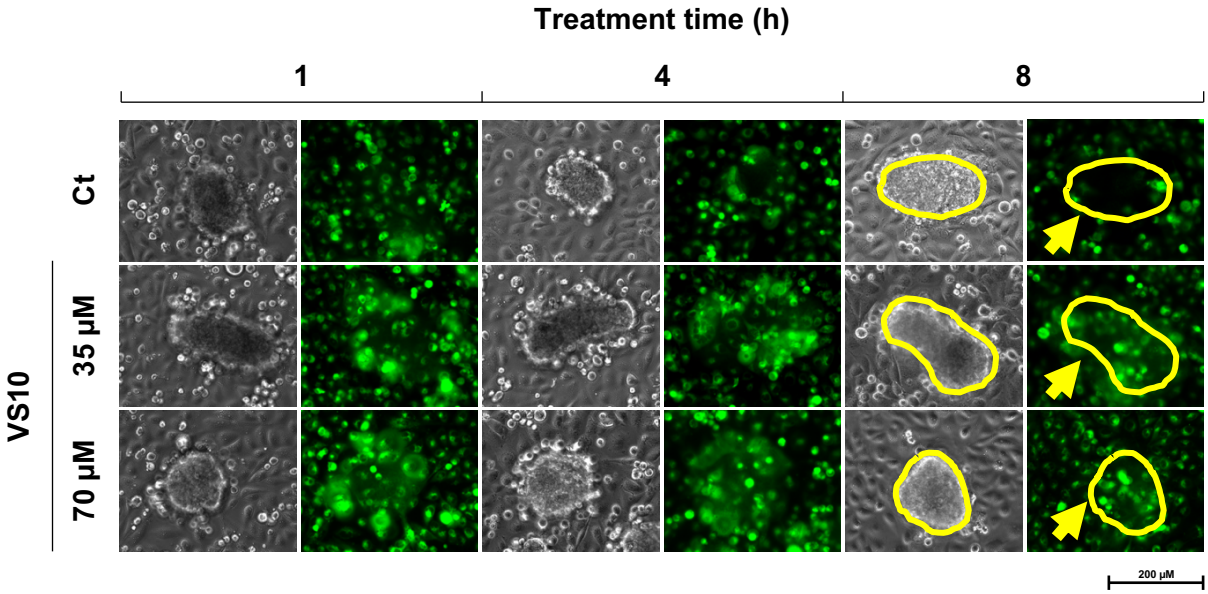


Figure 12 – Mesothelial Clearance Assay. MeT-5A were fluorescent-labeled with green fluorescent dye DiO and seeded. SKOV3 preformed spheroids were treated for 24 hours with VS10 35 µM and 70 µM and seeded over MeT-5A layer and treated again for 1, 4 and 8 hours. Images were acquired at each time point using brightfield and FITC at 40X Magnification. The area covered by SKOV3 spheroids and MET5A is shown in yellow.

OC metastasis arises when tumor cells detach from the primary tumor site and spread throughout the peritoneal cavity as multicellular clusters or spheroids. A single, continuous, layer of mesothelial cells cover all the organs within the peritoneal cavity and it's absent from the underneath peritoneal tumor masses. In this experiment, mesothelial cells MeT-5A were labeled using the fluorescent dye DiO and used to mimic the mesothelial layer. SKOV3 preformed spheroids were pretreated with VS10 35 and 70 µM, and then transferred on the MET5A monolayer plate. SKOV3 spheroids were monitored for 8 hours to evaluate if VS10 treatment, and thus the PIN1 inhibition, affects their ability to invade the MeT-5A monolayer. As shown in Figure 12, it appears evident that SKOV3 untreated spheroids invaded the MET5A monolayer after 8 hours, since the fluorescent cells were shifted from the spheroid infiltration. Conversely, VS10-treated spheroids were unable to shift mesothelial cells since the green fluorescent cells were still present under the spheroids. These results could represent an important step to limit tumor spread through metastasis.

4.2.4 EFFECTS OF VS10 ON SPHEROID MIGRATION

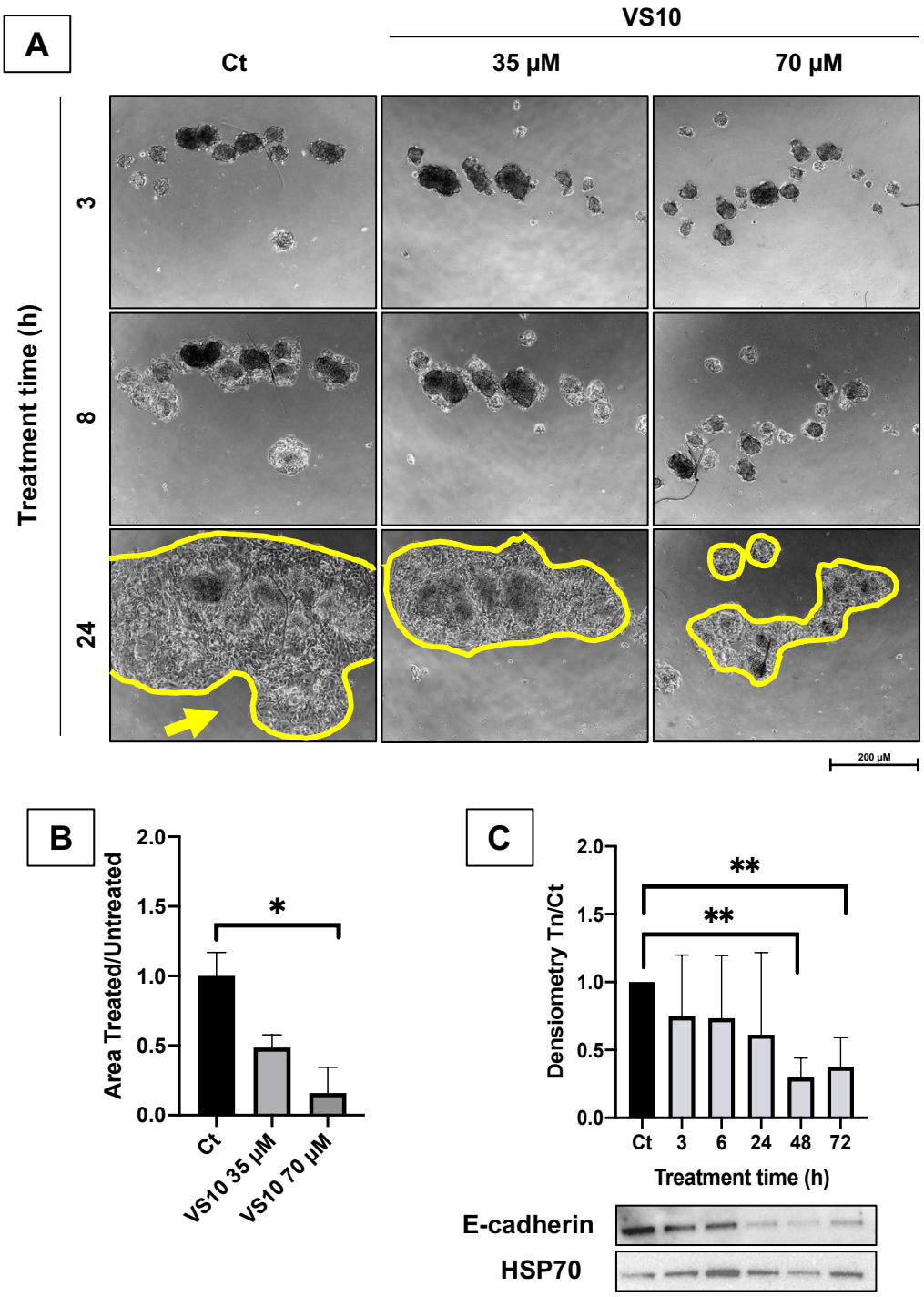


Figure 13 – Effects of VS10 treatment on SKOV3 spheroid migration. A) spheroids were treated with 35 µM and 70 µM of VS10 and pictures were collected after 3, 8 and 24 hours. Magnification 20X. The area covered by cells after the migration is shown in yellow. B) Quantification of the covered area treated/untreated ratio. C) Expression of E-cadherin of SKOV3 spheroids during the treatment with VS10 70 µM up to 72 hours.

Spheroids migration is one of the main metastasis mechanisms in OC patients. SKOV3 spheroids were grown for 24 hours in a poly-HEMA coated plate and pretreated with respectively VS10 35 and 70 μM. Hence, they were transferred to an uncoated plate and their migration ability was monitored over 24 hours. After 24 hours, when attachment of spheroids was completed, treated samples weren't able to spread if compared to the untreated spheroids. In fact, the area covered by VS10-treated spheroids was reduced by 52% and 84% respectively for VS10 35 and 70 μM exposure (Figure 13B). Moreover, since E-cadherin is an important mediator for cell-cell adhesion which is fundamental for the spheroid formation and a key regulator of tumor migration (Shimazui et al., 2004), its expression was evaluated over 72 hours of treatment with VS10 70 μM WB analysis showed that treatment with the PIN1 inhibitor leads to a statistically significant decrease of E-cadherin expression in spheroids treated with VS10 for 48 and 72 hours. Therefore, taken together, these results identify the PIN1 inhibitor VS10 as a potent candidate for controlling OC growth, progression and metastasis.

4.3 SILENCING OF PIN1 AND ITS ROLE IN SIGNALING TRASDUCTION IN OVARIAN CANCER CELLS

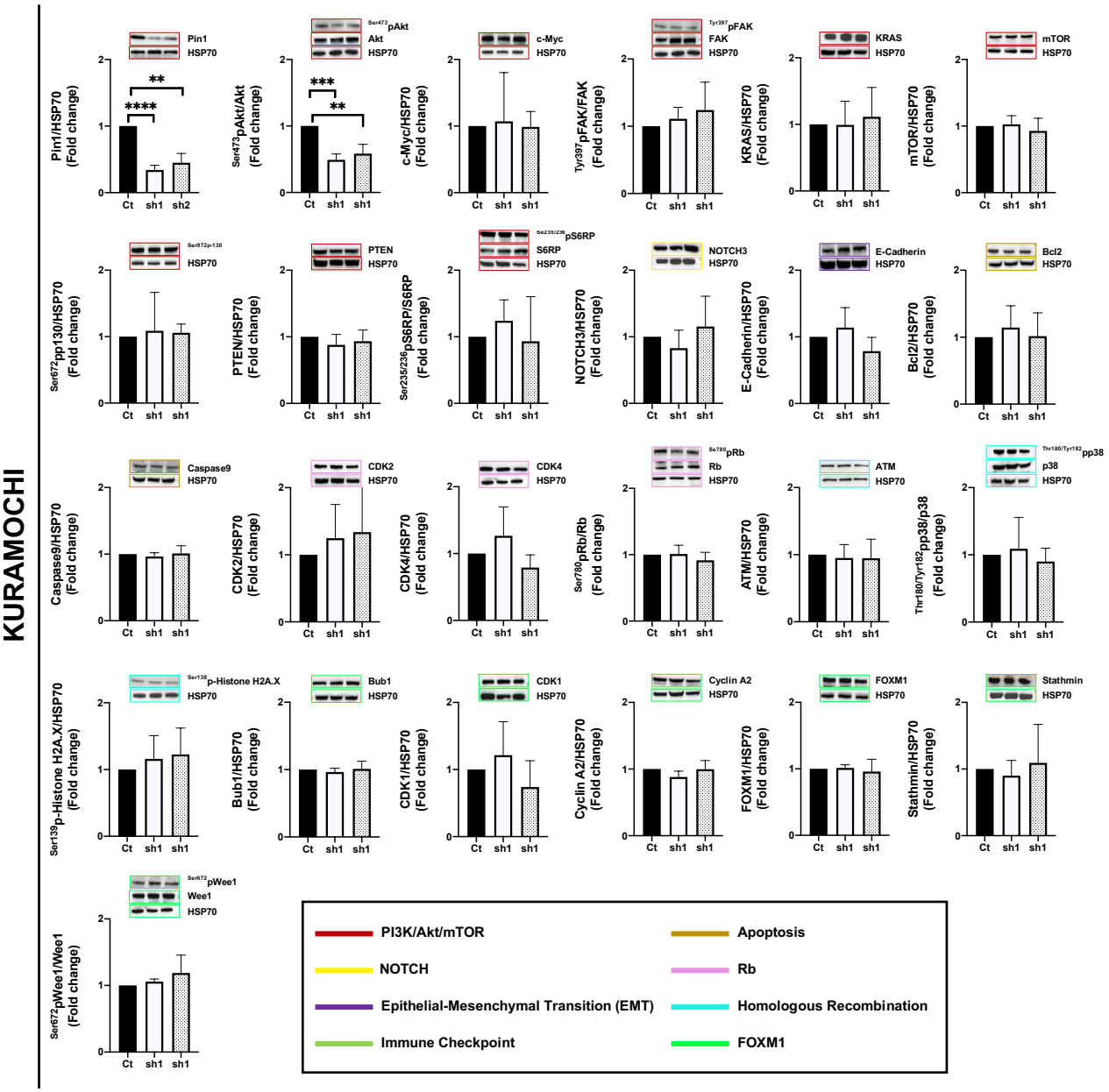


Figure 14 – Effects of Pin1 knockdown in different pathways in KURAMOCHI cell line. KURAMOCHI were infected with shRNA, analyzed by WB and quantified using ImageJ.

The pharmacologic inhibition of PIN1 with VS10 and subsequent cytotoxic activity on several OC cell lines (Table 4), led us to suppose PIN1 as a promising candidate target for future personalized OC therapy. However, although many studies on the role of Pin1 in the signaling transduction in cancer cells have been extensively studied, the molecular pathways that trigger OC cells cytotoxicity through Pin1 ablation have never been analyzed. Therefore, besides the pharmacological blockage, Pin1 has been silenced with RNA interference (RNAi) and its involvement in cell signaling transduction was investigated.

The knockdown (KD) on Pin1 OVCAR3, one of the most used OC cell lines, and KURAMOCHI was performed using two different short-hairpins RNA, sh1 and sh2. OVCAR3 and KURAMOCHI cells infected with an empty vector were used as control. The protein expression of members of the main deregulated pathways in OC reported by TCGA analysis was evaluated: PI3K/AKT/mTOR, Rb, NOTCH, epithelial-mesenchymal transition (EMT), Immune Checkpoint, Homologous Recombination, Apoptosis and FoxM1 pathways. In the 2011 TCGA study, it was suggested that half of the analyzed tumors present homologous recombination defection and that homologous recombination is defective in about half of the tumors analyzed, and that NOTCH and FOXM1 signaling are involved in serous OC pathophysiology.

Results are reported in Figure 14 and Figure 15. This screening showed that Pin1 KD afflicts the expression of ^{Ser473}pAkt involved in the PI3K/Akt/mTOR pathway that regulates different cell processes including protein synthesis, cell survival, migration, proliferation, glucose metabolism and others.

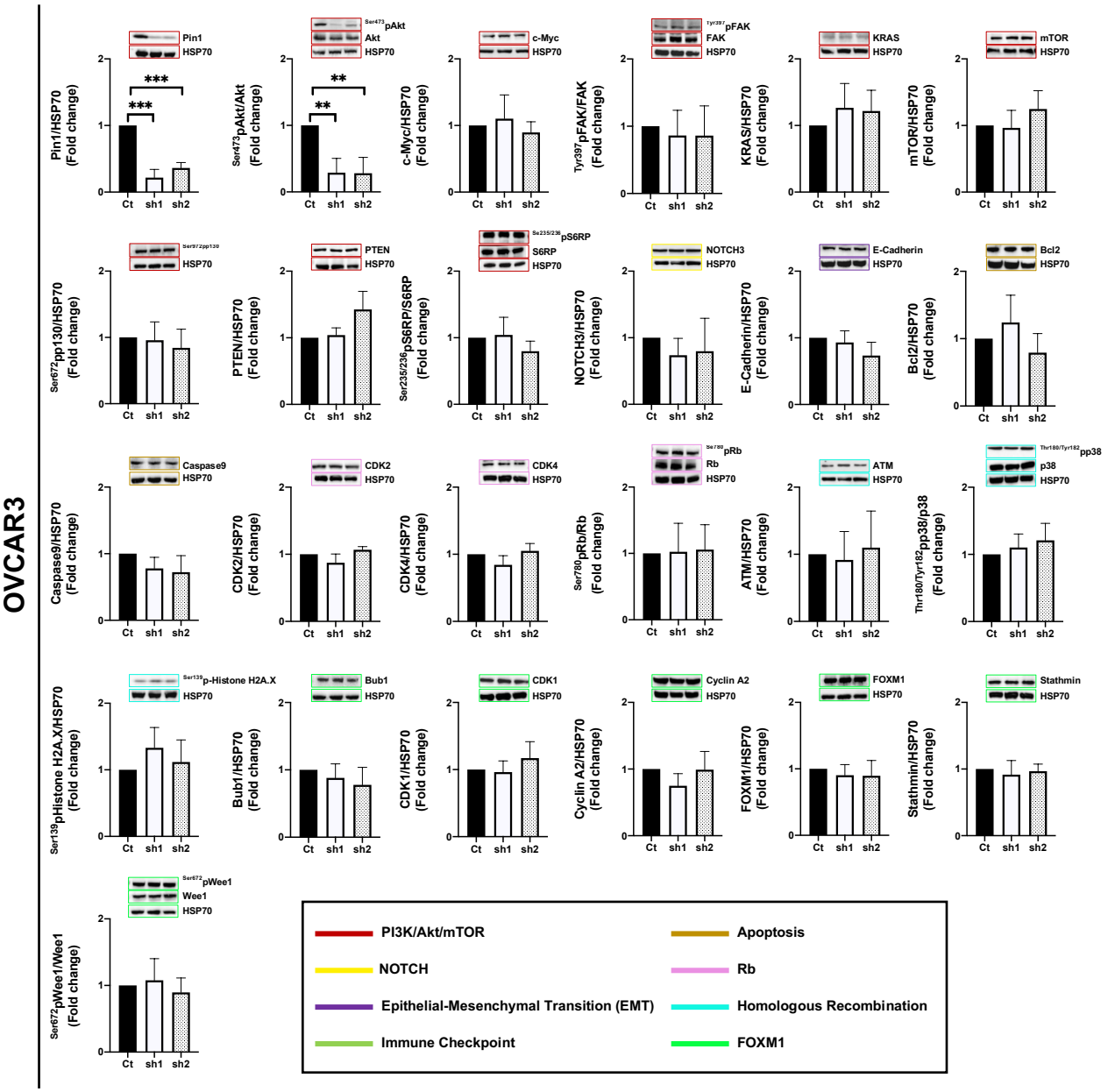


Figure 15 – Effects of Pin1 knockdown in different pathways in OVCAR3 cell line. OVCAR3 were infected with shRNA, analyzed by WB and quantified using ImageJ.

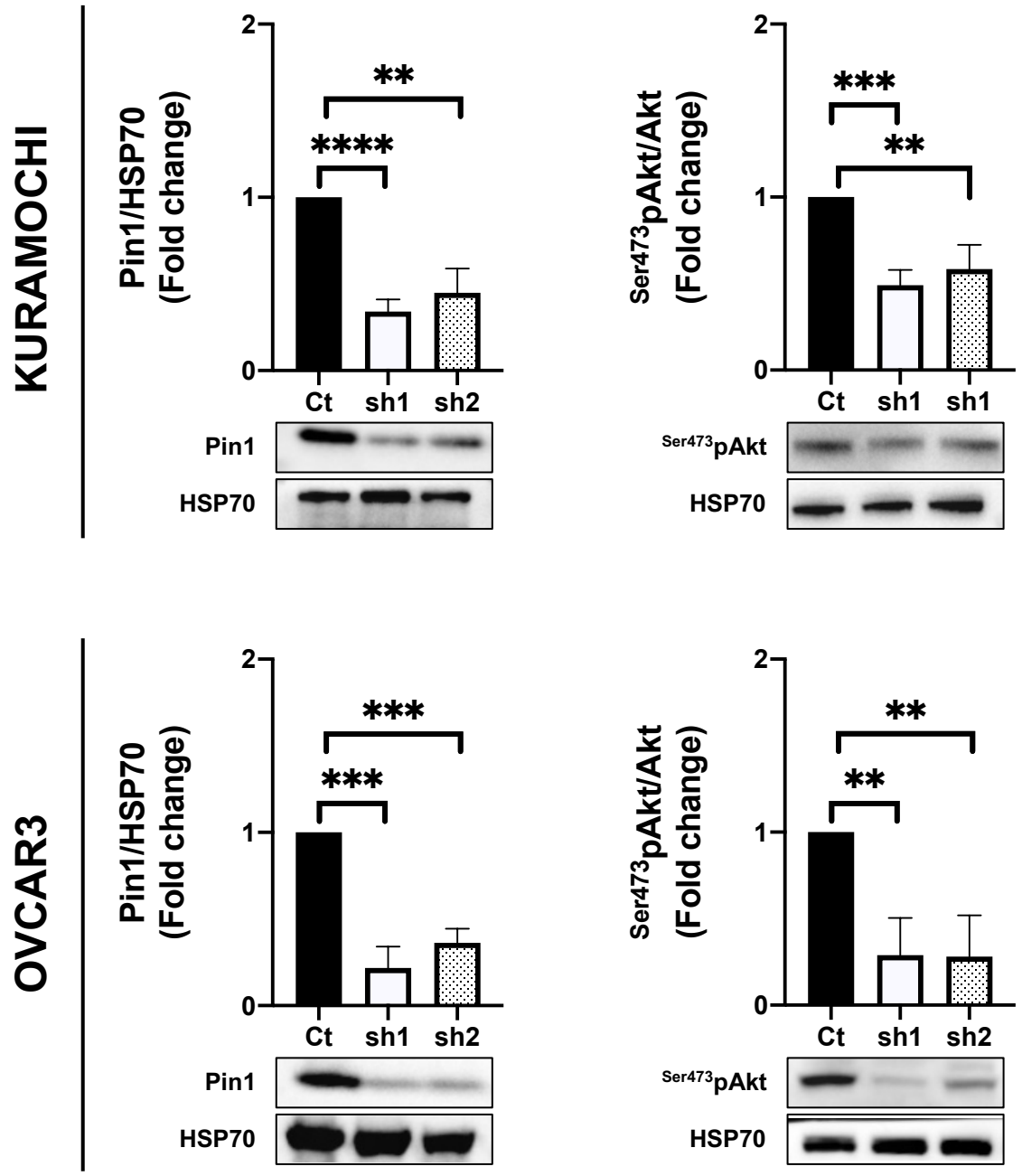


Figure 16 – Pin1 knockdown modifies ^{Ser473}pAkt expression. KURAMOCHI and OVCAR3 were infected with shRNA, analyzed by WB and quantified using ImageJ.

Specifically, PIN1 expression was reduced by 61% and 51% in KURAMOCHI cell line and by 77% and 63% in OVCAR3 cell line confirming the efficiency of gene silencing respectively for sh1 and sh2 (

Figure 16). Interestingly, knockdown of Pin1 induced a prominent reduction of ^{Ser473}pAkt by 54% and 43% in KURAMOCHI-sh1 KD and KURAMOCHI-sh2 KD, respectively (

Figure 16). Similarly, the strong silencing of Pin1 in OVCAR3 led to a significant reduction of ^{Ser473}pAkt by 71% and 69% in OVCAR3-sh1 KD and OVCAR3-sh2 KD. In order to clarify the signaling pathway triggers by PIN1 KD and subsequent dephosphorylation of ^{Ser473}pAkt, the AKT/PKB Phospho Antibody Array was performed using the most responsive cell line to Pin1 KD, OVCAR3-sh1 and OVCAR3-Ct as control. This array included 216 site-specific and phospho-specific antibodies reported in the Material and Methods section. The expression of ^{Ser473}pAkt and Pin1 were previously evaluated by WB. The ^{Ser473}pAkt value obtained from the AKT/PKB Phospho Antibody Array was considered as KD threshold and 1 + (1 - phospho-Akt (Ser473) Value) as overexpression threshold. Cell cycle, protein synthesis and apoptosis seem to be the main cell processes in which PIN1 is involved and this would confirm the deadly phenotype due to PIN1 modulation.

4.3 THE OVEREXPRESSION OF AKT RESCUE THE PHENOTYPE OF PIN1 INHIBITION

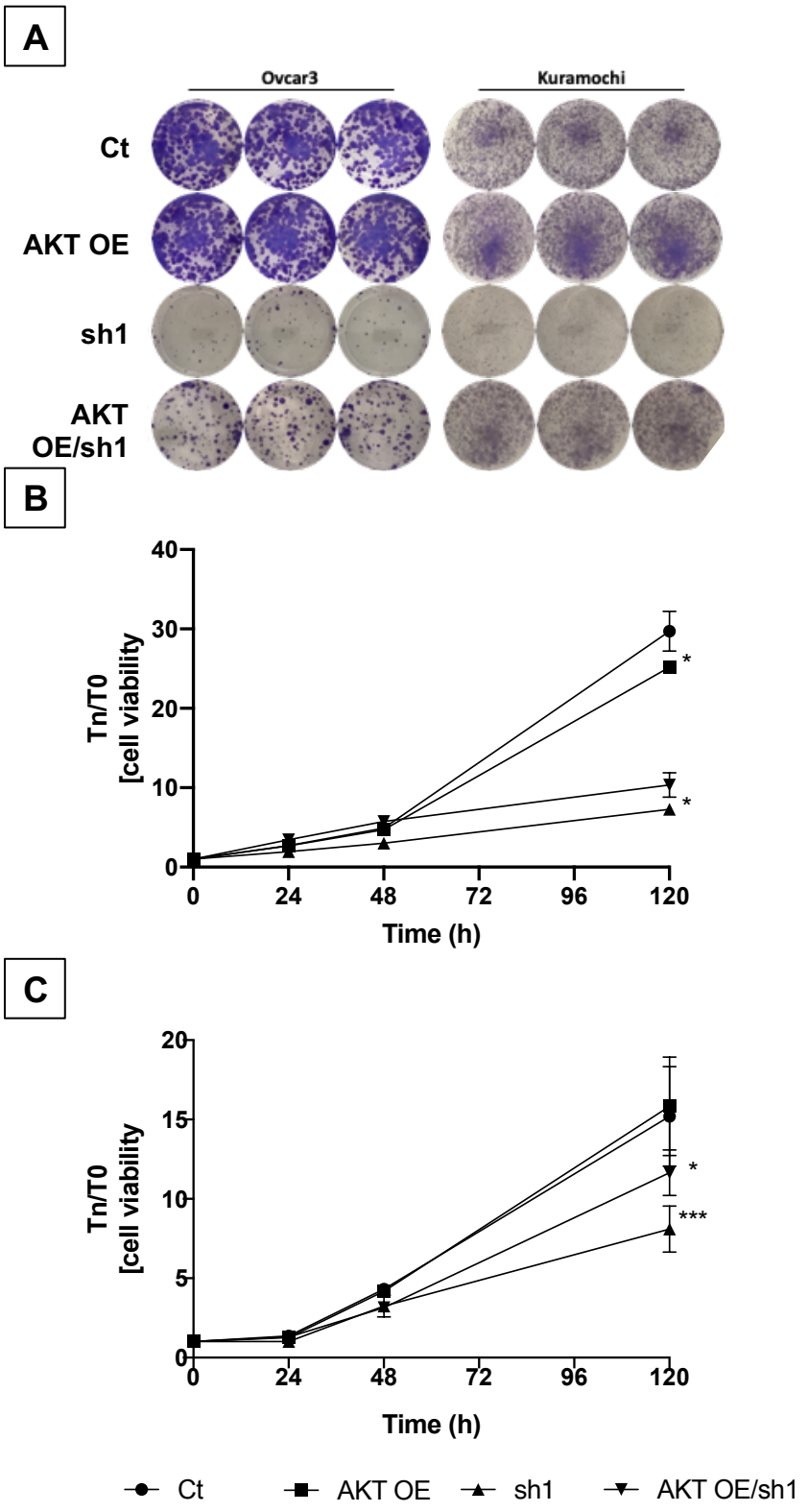


Figure 17 – Rescue of phenotype by overexpressing Akt in OVCAR3 and KURAMOCHI cell lines. A) Colony-forming assay after 20 days. B) OVCAR3 and C) KURAMOCHI cell viability after 0, 24, 48 and 120 hours normalized on T0 value.

On the basis of these results, the relevance of Pin1 in AKT signaling transduction was evaluated by the rescue of phenotype through the colony-forming assay. Silencing of Pin1 with sh1 and sh2 completely abolished the ability of both OVACAR3 and KURAMOCHI cell lines to form colonies with respect to their respective control, evidencing how Pin1 silencing controls the clonogenic potential of OC cells (Figure 17B). On the other hand, overexpression of Akt (AktOE) in these cell lines led to a phenotype similar to control cells. However, when stable AktOE- OVCAR3 and -KURAMOCHI cells were infected with sh1, the downstream pathway triggered by Akt overexpression overcame Pin1 blockage, rescuing the phenotype. Cell viability at 96 hours under these conditions was further evaluated in both cell lines. Indeed, PIN1 silencing dramatically affected the cell viability of OVCAR3 and KURAMOCHI cell lines, with a significant reduction of 27% and 47% at 120 hours with their respectively control transfected with an empty vector.

The results confirmed that Pin1 is an upstream regulator of Akt pathway, opening a novel scenario in the application of Pin1 inhibitors in combination with potential modulators of Akt signaling in OC target therapy.

4.4 EVALUATION OF THE ACTIVATION OF PRO-AGGRESSIVENESS TRANSCRIPTIONAL PROGRAM THROUGH PIN1/P53 AXIS

Although HGSOC is characterized by genomic instability, the TP53 gene is mutated in 96% of cases. The mutation of p53 protein and its interaction with Pin1 has been extensively studied by Del Sal's group (Girardini et al., 2011). As OC cell models, both OVCAR3 and KURAMOCHI are characterized by TP53 mutation. Since Del Sal's group demonstrated that a number of genes are downregulated or overexpressed in PIN1/Mutant p53 breast model, the effect of Pin1 silencing on the same genes was also evaluated in our models of mutant TP53- OVCAR3 and KURAMOCHI Pin1 KD.

Results are reported in Figure 18. Figure 18 showed that knockdown of Pin1 did not deregulate the expression of selected genes with two different shRNA in both TP53-mutant OC cell lines. Even though mutation of TP53 represented a key event in HGSOC oncogenesis, the data here reported indicating a TP53-mutant independent pathway triggered by Pin1 in OC.

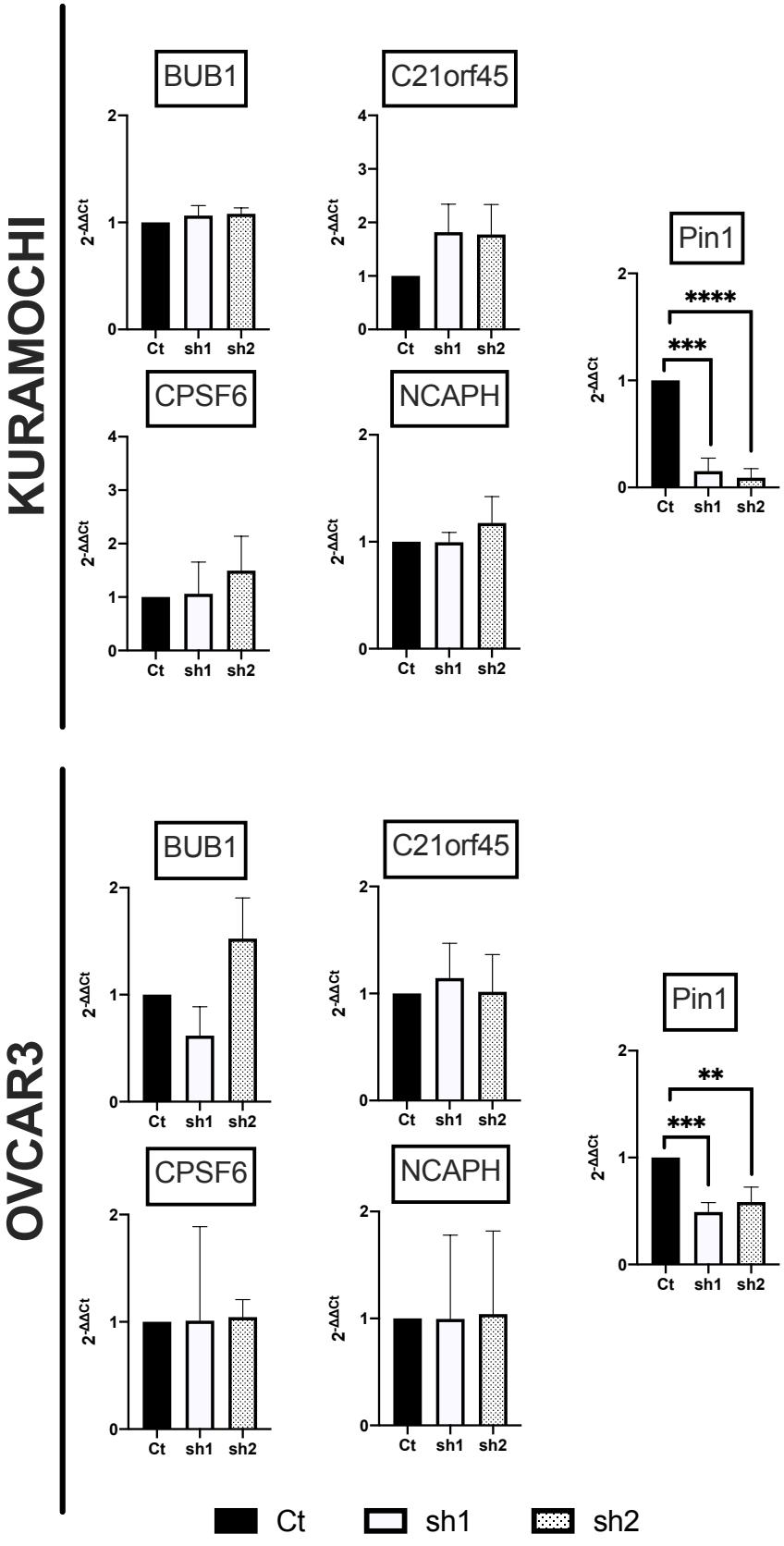


Figure 18 – qRT-PCR of P53 target genes on OVCAR3 and KURAMOCHI cell lines.

4.5 DRUG COMBINATION

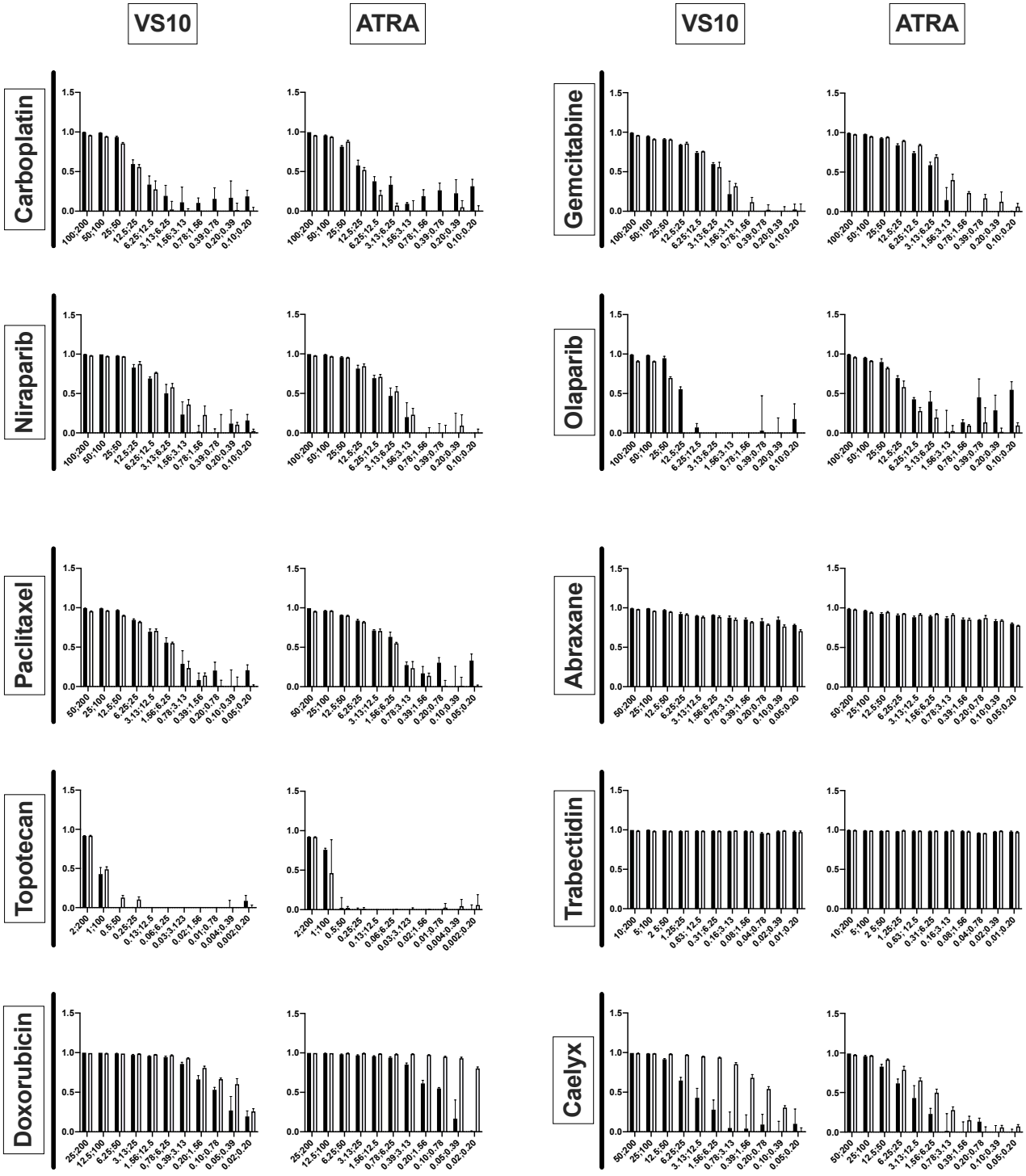


Figure 19 – Drug combination screening on SKOV3 cell line. Carboplatin, Gemcitabine, Niraparib, Olaparib, Paclitaxel, Abraxane, Topotecan, Trabectidin, Doxorubicin and Caelyx were tested alone and in combination with the PIN1 inhibitors ATRA and VS10. Concentrations are reported as C_{DRUG} [μM];$C_{VS10/ATRA}$ [μM]. Topotecan concentrations are expressed as $C_{Topotecan}$ [nM];$C_{VS10/ATRA}$ [μM]. Expected values are reported in black; Observed values are reported in white.

The improvement of the current therapy is one of the main goals in cancer research. OC patients usually present recurrence after platinum-based first-line chemotherapy. Hence, Carboplatin, Gemcitabine, Niraparib, Olaparib, Paclitaxel, Abraxane, Topotecan, Trabectidin, Doxorubicin and Caelyx were screened on SKOV3 cell line to evaluate a possible synergism between PIN1 inhibitors and these chemotherapeutics (Figure 19). Results showed that Doxorubicin and Caelyx have a synergistic behavior with PIN1 inhibitors, so the synergism was evaluated also in A2780, A2780cis, SKOV3cis, OVCAR3, and OVSAHO cell lines (Figure 20).

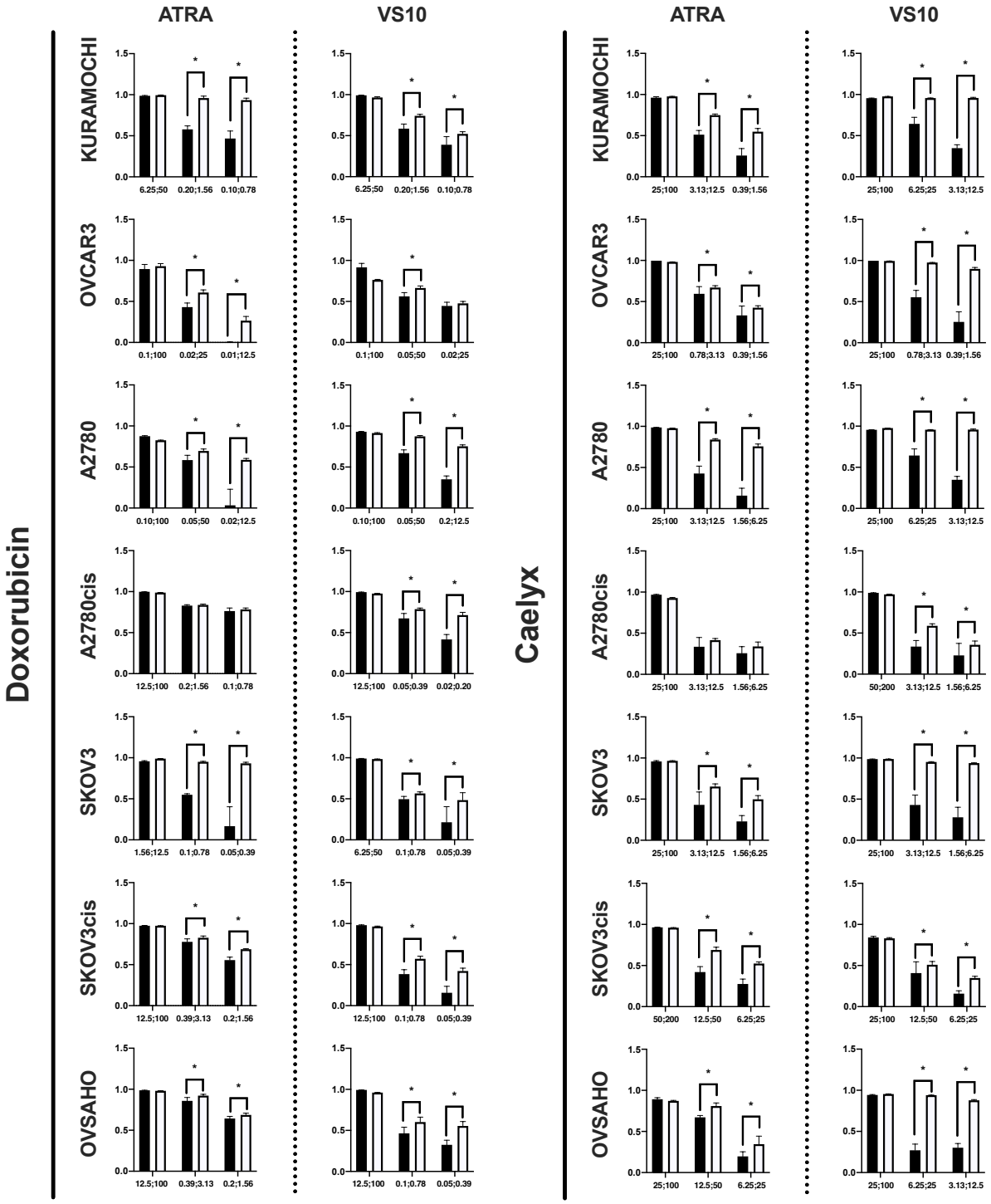


Figure 20 – PIN1 inhibitors enhance Doxorubicin and Caelyx efficacy. OC cell lines were used to study the synergism between PIN1 inhibitors and Doxorubicin. Concentrations are reported as $C_{Doxorubicin/Caelyx}$ [μM];$C_{VS10/ATRA}$ [μM]. Expected values are reported in black; Observed values are reported in white.

5. DISCUSSION

OC represents the fourth leading cause of cancer-associated death in developed countries. HGSOC is characterized by a much more aggressive disease behavior than other OC subtypes which have a very distinct metastatic pattern: cancer cells mostly arise in the fallopian tube after the transformation of fallopian tube epithelial cells and then metastasize first to the ovary, then to the omentum (Lusk et al., 2021). The discovery of new drug targets could represent a breakthrough in the treatment of OC and improve patients' OS. The prolyl-isomerase PIN1 interacts with more than 20 tumor suppressors and more than 40 oncogenes and its inhibition results in the alteration of many molecular pathways. PIN1 overexpression in HGSOC and a large number of interactions of PIN1 with other proteins make it an attractive drug target for the development of new treatments for OC (Russo Spena et al., 2018; Zhou & Lu, 2016). Many PIN1 inhibitors with both covalent and non-covalent mechanisms of action have been discovered in recent years, but lack specificity and/or potency, or exhibit slow cellular uptake to inhibit PIN1 function *in vivo*. ATRA showed activity in cells and mouse models but it has a short half-life and is not specific for PIN1. Instead, Sulfopin is a recent covalent inhibitor which showed high selectivity for PIN1. However, a non-covalent inhibitor could represent a better choice for *in vivo* applications (Dubiella et al., 2021; Wei et al., 2015). PIN1 inhibitor VS10 was discovered through consensus docking in 2019 and preliminary studies suggested it as a new potential therapeutic agent (Russo Spena et al., 2019). This investigation demonstrated that VS10 could be a great candidate to reduce HGSOC metastasis by reducing the spheroid formation (Figure 10), proliferation (Figure 11), invasion (Figure 12) and migration (Figure 13). Those results also confirmed the importance of Pin1 on spheroid formation as reported for Sulfopin and BJP-06–005-3 Pin1 inhibitors for which reduced cell viability was recorded after the spheroid treatment (Dubiella et al., 2021; Pinch et al., 2020). These effects on spheroids

could be explained by the reduction of E-cadherin (Figure 13C) which is an indispensable protein for due to its role as a mediator for cell-cell adhesion (Smyrek et al., 2019).

Cyt C is a peripheral protein located on the inner membrane of mitochondria which functions on the respiratory chain are fundamental for life. Cyt C is released into the cytosol as a result of apoptotic stimuli and, in the presence of ATP, apoptosome is generated through the Cyt C-mediated the allosteric activation and hepta-oligomerization of the adapter molecule of the apoptosis-protease activation factor 1 (Apaf-1). Hence, Cyt C is released at the early stages of apoptosis (Garrido et al., 2006). OC cell lines KURAMOCHI and SKOV3 treated with 35 and 70 μM VS10 showed the Cyt C release after 72 hours and 48 hours of treatment, respectively (Figure 9). These results show that VS10 induces apoptosis through Cyt C release.

The 2011 TCGA study identified altered pathways in OC and focused on different molecular pathways in which shRNA-mediated Pin1 inhibition could alter some protein expression (Bell et al., 2011). WB analysis of both OVCAR3 and KURAMOCHI Pin1 KD of 30 total and phosphorylated proteins highlighted how Pin1 inhibition led to ^{Ser473}pAkt downregulation in both cell lines where other proteins expression are not changing (Figure 14, Figure 15). The interaction between PIN1 and Akt was studied deeply through colony formation assay in which the knockdown of Pin1 reduced cell growth and the overexpression of Akt led to the rescue of phenotype on Pin1 KD cell lines (Figure 17A). Additionally, cell viability assay performed on KURAMOCHI and OVCAR3 cell lines confirmed the recovery of phenotype on AktOE-sh1 cell lines after 120 hours of growth (Figure 17B, C). AKT/PKB Phospho Antibody Array was performed to study the expression of 216 phosphorylated and total proteins on Pin1-Ct and sh1 OVCAR3 cell line. This array highlighted some proteins involved in apoptosis, cell cycle and protein synthesis whose expression changed after the knocking down of Pin1. These results highlighted the wide interaction between PIN1 and other proteins to sustain tumor growth and other key processes. Hence, qRT-PCR of 12 p53 target

genes on Pin1 wild type and knocked down with sh1 and sh2 in both OVCAR3 and KURAMOCHI cell lines did not show any significant alteration since many of them are not confirmed from both knockdown samples and/or from both cell lines (Figure 18).

Drug repositioning is a strategy to identify new uses for approved drugs that fall outside the scope of the original medical indication (Ashburn & Thor, 2004). Current cancer therapy includes different strategies, which may involve just one kind of treatment or a combination of treatments. The drug combination is employed to overcome monotherapy problems and achieve far superior effects compared to those expected from the additive effects, thereby reducing toxicity and allowing for dosage reduction (Duarte & Vale, 2022). In this study, the drug combination between first- and second-line chemotherapeutics agents and PIN1 inhibitors was screened on the SKOV3 cell line. This screening included Carboplatin, Gemcitabine, Niraparib, Olaparib, Paclitaxel, Abraxane, Topotecan, Trabectedin, Doxorubicin and Caelyx. Results showed that Doxorubicin and Caelyx exhibit synergy with both PIN1 inhibitors at different treatment concentrations (Figure 19). Additionally, this synergism was also investigated and confirmed in A2780, A2780cis, SKOV3cis, OVCAR3, and OVSAHO cell lines (Figure 20). Repositioning ATRA as a therapy enhancer in OC could overcome numerous side effects of chemotherapy drugs through drug dose reduction, highlighting the importance of PIN1 in cancer therapy. Hence, the discovery of the new potent PIN1 inhibitor VS10 which accounts for a higher synergism with both Doxorubicin and Caelyx on different cell lines could represent an alternative drug for OC patients.

6. CONCLUSION

HGSOC is a lethal disease that is usually diagnosed in the advanced stage and characterized by recurrence and development of chemoresistance (Gadducci et al., 2019). The discovery of novel drug targets that improve patient therapy outcomes and OS and overcome drug resistance is a key goal for OC therapy. In this thesis, we demonstrated the interaction between ^{Ser473}pAkt and PIN1 through WB, colony-forming assay, cell viability and AKT/PKB Phospho Antibody Array. Therefore, we showed that VS10 pharmacological treatment on SKOV3 spheroids is able to reduce spheroid formation, proliferation, invasion and migration. Hence, VS10 treatment induces apoptosis in KURAMOCHI and SKOV3 cell lines. These data highlight the role of PIN1 as a promising gene since its inhibition affect many cancer cell functions. Eventually, we investigated drug combinations between first- and second-line chemotherapy agents and PIN1 inhibitors to reduce drug dose and consequently side effects. Hence, results showed synergism between Doxorubicin/Caelyx and Pin1 inhibitors placing PIN1 as a good second-line chemotherapy enhancer.

7. FUTURE PERSPECTIVE

This work explained several PIN1 functions and the importance of PIN1 as a therapy enhancer. Other data are necessary to better understand the role of this protein such as the confirmation through WB of the results obtained from the AKT/PKB Phospho Antibody Array. A deeper study of these data will allow us to identify the molecular pathway in which Pin1 is involved and discover other molecular functions that are regulated from Pin1. Hence, *in vivo* experiments are necessary to confirm the synergy between Doxorubicin/Caelyx and the Pin1 inhibitors ATRA and VS10. The aim is to enhance patient chemotherapy through this discovery by reducing the drug dosage and side effects. Eventually, the study of the role of Pin1 in chemoresistance could be a promising new aim since the wide interaction between Pin1 and other oncogenes and tumor suppressors.

8. REFERENCES

Ahmed, N., Abubaker, K., Findlay, J., & Quinn, M. (2013). Cancerous ovarian stem cells: Obscure targets for therapy but relevant to chemoresistance. *Journal of Cellular Biochemistry*, *114*(1), 21–34. https://doi.org/https://doi.org/10.1002/jcb.24317

Ashburn, T. T., & Thor, K. B. (2004). Drug repositioning: identifying and developing new uses for existing drugs. *Nature Reviews Drug Discovery*, *3*(8), 673–683. https://doi.org/10.1038/nrd1468

Ayala, G., Wang, D., Wulf, G., Frolov, A., Li, R., Sowadski, J., Wheeler, T. M., Lu, K. P., & Bao, L. (2003). The Prolyl Isomerase Pin1 Is a Novel Prognostic Marker in Human Prostate Cancer. *Cancer Research*, *63*(19), 6244–6251.

Balastik, M., Lim, J., Pastorino, L., & Lu, K. P. (2007). Pin1 in Alzheimer's disease: Multiple substrates, one regulatory mechanism? *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1772*(4), 422–429. https://doi.org/https://doi.org/10.1016/j.bbadis.2007.01.006

Bao, L., Kimzey, A., Sauter, G., Sowadski, J. M., Lu, K. P., & Wang, D.-G. (2004). Prevalent Overexpression of Prolyl Isomerase Pin1 in Human Cancers. *The American Journal of Pathology*, *164*(5), 1727–1737. https://doi.org/https://doi.org/10.1016/S0002-9440(10)63731-5

Bell, D., Berchuck, A., Birrer, M., Chien, J., Cramer, D. W., Dao, F., Dhir, R., DiSaia, P., Gabra, H., Glenn, P., Godwin, A. K., Gross, J., Hartmann, L., Huang, M., Huntsman, D. G., Iacocca, M., Imielinski, M., Kalloger, S., Karlan, B. Y., … centre, D. coordination. (2011). Integrated genomic analyses of ovarian carcinoma. *Nature*, *474*(7353), 609–615. https://doi.org/10.1038/nature10166

Bliss, C. I. (1939). The Toxicity of Poisons Applied Jointly. *Annals of Applied Biology*, *26*(3), 585–615. https://doi.org/https://doi.org/10.1111/j.1744-7348.1939.tb06990.x

Blume-Jensen, P., & Hunter, T. (2001). Oncogenic kinase signalling. *Nature*, *411*(6835), 355–365. https://doi.org/10.1038/35077225

Boussios, S., Abson, C., Moschetta, M., Rassy, E., Karathanasi, A., Bhat, T., Ghumman, F., Sheriff, M., & Pavlidis, N. (2020). Poly (ADP-Ribose) Polymerase Inhibitors: Talazoparib in Ovarian Cancer and Beyond. *Drugs in R&D*, *20*(2), 55–73. https://doi.org/10.1007/s40268-020-00301-8

Bowtell, D. D., Böhm, S., Ahmed, A. A., Aspuria, P.-J., Bast, R. C., Beral, V., Berek, J. S., Birrer, M. J., Blagden, S., Bookman, M. A., Brenton, J. D., Chiappinelli, K. B., Martins, F. C., Coukos, G., Drapkin, R., Edmondson, R., Fotopoulou, C., Gabra, H., Galon, J., … Balkwill, F. R. (2015). Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. *Nature Reviews Cancer*, *15*(11), 668–679. https://doi.org/10.1038/nrc4019

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254. https://doi.org/https://doi.org/10.1016/0003-2697(76)90527-3

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), 394–424. https://doi.org/https://doi.org/10.3322/caac.21492

Burger, R. A., Brady, M. F., Bookman, M. A., Fleming, G. F., Monk, B. J., Huang, H., Mannel, R. S., Homesley, H. D., Fowler, J., Greer, B. E., Boente, M., Birrer, M. J., & Liang, S. X. (2011). Incorporation of Bevacizumab in the Primary Treatment of Ovarian Cancer. *New England Journal of Medicine*, *365*(26), 2473–2483. https://doi.org/10.1056/NEJMoa1104390

Cai, Y., Zhai, J., Feng, B., Duan, X., & He, X. (2014). Expression of glucose transporter protein 1 and p63 in serous ovarian tumor. *Journal of Obstetrics and Gynaecology Research*, *40*(7), 1925–1930. https://doi.org/https://doi.org/10.1111/jog.12447

Campaner, E., Rustighi, A., Zannini, A., Cristiani, A., Piazza, S., Ciani, Y., Kalid, O., Golan, G., Baloglu, E., Shacham, S., Valsasina, B., Cucchi, U., Pippione, A. C., Lolli, M. L., Giabbai, B., Storici, P., Carloni, P., Rossetti, G., Benvenuti, F., … del Sal, G. (2017). A covalent PIN1 inhibitor selectively targets cancer cells by a dual mechanism of action. *Nature Communications*, *8*(1), 15772. https://doi.org/10.1038/ncomms15772

Charkhchi, P., Cybulski, C., Gronwald, J., Wong, F. O., Narod, S. A., & Akbari, M. R. (2020). CA125 and Ovarian Cancer: A Comprehensive Review. *Cancers*, *12*(12), 3730. https://doi.org/10.3390/cancers12123730

Chen, K., Niu, Y., Wang, S., Fu, Z., Lin, H., Lu, J., Meng, X., Yang, B., Zhang, H., Wu, Y., Xia, D., & Lu, W. (2020). Identification of a Novel Prognostic Classification Model in Epithelial Ovarian Cancer by Cluster Analysis. *Cancer Management and Research*, *12*, 6251–6259. https://doi.org/10.2147/CMAR.S251882

Cheng, C.-W., Chow, A. K. M., Pang, R., Fok, E. W. S., Kwong, Y.-L., & Tse, E. (2013). PIN1 Inhibits Apoptosis in Hepatocellular Carcinoma through Modulation of the Antiapoptotic Function of Survivin. *The American Journal of Pathology*, *182*(3), 765–775. https://doi.org/https://doi.org/10.1016/j.ajpath.2012.11.034

Chu, P., Wu, E., & Weiss, L. M. (2000). Cytokeratin 7 and Cytokeratin 20 Expression in Epithelial Neoplasms: A Survey of 435 Cases. *Modern Pathology*, *13*(9), 962–972. https://doi.org/10.1038/modpathol.3880175

Ding, Q., Huo, L., Yang, J.-Y., Xia, W., Wei, Y., Liao, Y., Chang, C.-J., Yang, Y., Lai, C.-C., Lee, D.-F., Yen, C.-J., Chen, Y.-J. R., Hsu, J.-M., Kuo, H.-P., Lin, C.-Y., Tsai, F.-J., Li, L.-Y., Tsai, C.-H., & Hung, M.-C. (2008). Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. *Cancer Research*, *68*(15), 6109–6117. https://doi.org/10.1158/0008-5472.CAN-08-0579

Domcke, S., Sinha, R., Levine, D. A., Sander, C., & Schultz, N. (2013). Evaluating cell lines as tumour models by comparison of genomic profiles. *Nature Communications*, *4*(1), 2126. https://doi.org/10.1038/ncomms3126

Doubeni, C. A., Doubeni, A. R. B., & Myers, A. E. (2016). *Diagnosis and Management of Ovarian Cancer* (Vol. 93, Issue 11). www.aafp.org/afp

Drew, Y. (2015). The development of PARP inhibitors in ovarian cancer: from bench to bedside. *British Journal of Cancer*, *113 Suppl 1*(Suppl 1), S3–S9. https://doi.org/10.1038/bjc.2015.394

Duarte, D., & Vale, N. (2022). Evaluation of synergism in drug combinations and reference models for future orientations in oncology. *Current Research in Pharmacology and Drug Discovery*, *3*, 100110. https://doi.org/https://doi.org/10.1016/j.crphar.2022.100110

Dubiella, C., Pinch, B. J., Koikawa, K., Zaidman, D., Poon, E., Manz, T. D., Nabet, B., He, S., Resnick, E., Rogel, A., Langer, E. M., Daniel, C. J., Seo, H.-S., Chen, Y., Adelmant, G., Sharifzadeh, S., Ficarro, S. B., Jamin, Y., Martins da Costa, B., … London, N. (2021). Sulfopin is a covalent inhibitor of Pin1 that blocks Myc-driven tumors in vivo. *Nature Chemical Biology*, *17*(9), 954–963. https://doi.org/10.1038/s41589-021-00786-7

Feng, L.-X., Li, M., Liu, Y.-J., Yang, S.-M., & Zhang, N. (2014). Synergistic enhancement of cancer therapy using a combination of ceramide and docetaxel. *International Journal of Molecular Sciences*, *15*(3), 4201–4220. https://doi.org/10.3390/ijms15034201

Friedlander, M., Trimble, E., Tinker, A., Alberts, D., Avall-Lundqvist, E., Brady, M., Harter, P., Pignata, S., Pujade- Lauraine, E., Sehouli, J., Vergote, I., Beale, P., Bekkers, R., Calvert, P., Copeland, L., Glasspool, R., Gonzalez-Martin, A., Katsaros, D., Kim, J. W., … Stuart, G. C. E. (2011). Clinical Trials in Recurrent Ovarian Cancer. *International Journal of Gynecologic Cancer*, *21*(4), 771. https://doi.org/10.1097/IGC.0b013e31821bb8aa

Gadducci, A., Guarneri, V., Peccatori, F. A., Ronzino, G., Scandurra, G., Zamagni, C., Zola, P., & Salutari, V. (2019). Current strategies for the targeted treatment of high-grade serous epithelial ovarian cancer and relevance of BRCA mutational status. *Journal of Ovarian Research*, *12*(1), 9. https://doi.org/10.1186/s13048-019-0484-6

Garrido, C., Galluzzi, L., Brunet, M., Puig, P. E., Didelot, C., & Kroemer, G. (2006). Mechanisms of cytochrome c release from mitochondria. *Cell Death & Differentiation*, *13*(9), 1423–1433. https://doi.org/10.1038/sj.cdd.4401950

Girardini, J. E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A., Mano, M., Rosato, A., Crook, T., Scanziani, E., Means, A. R., Lozano, G., Schneider, C., & Del Sal, G. (2011). A Pin1/Mutant p53 Axis Promotes Aggressiveness in Breast Cancer. *Cancer Cell*, *20*(1), 79–91. https://doi.org/https://doi.org/10.1016/j.ccr.2011.06.004

Guo, N., & Peng, Z. (2017). Does serum CA125 have clinical value for follow-up monitoring of postoperative patients with epithelial ovarian cancer? Results of a 12-year study. *Journal of Ovarian Research*, *10*(1), 14. https://doi.org/10.1186/s13048-017-0310-y

Hennig, L., Christner, C., Kipping, M., Schelbert, B., Rücknagel, K. P., Grabley, S., Küllertz, G., & Fischer, G. (1998). Selective Inactivation of Parvulin-Like Peptidyl-Prolyl cis/trans Isomerases by Juglone. *Biochemistry*, *37*(17), 5953–5960. https://doi.org/10.1021/bi973162p

Holschneider, C. H., & Berek, J. S. (2000). Ovarian cancer: Epidemiology, biology, and prognostic factors. *Seminars in Surgical Oncology*, *19*(1), 3–10. https://doi.org/https://doi.org/10.1002/1098-2388(200007/08)19:1<3::AID-SSU2>3.0.CO;2-S

Howitt, B. E., Hanamornroongruang, S., Lin, D. I., Conner, J. E., Schulte, S., Horowitz, N., Crum, C. P., & Meserve, E. E. (2015). *Evidence for a Dualistic Model of High-grade Serous Carcinoma BRCA Mutation Status, Histology, and Tubal Intraepithelial Carcinoma*. www.ajsp.com

Hunn, J., & Rodriguez, G. C. (2012). Ovarian Cancer: Etiology, Risk Factors, and Epidemiology. *Clinical Obstetrics and Gynecology*, *55*(1). https://journals.lww.com/clinicalobgyn/Fulltext/2012/03000/Ovarian_Cancer__Etiology,_Risk_Factors,_and.3.aspx

Ison, G., Howie, L. J., Amiri-Kordestani, L., Zhang, L., Tang, S., Sridhara, R., Pierre, V., Charlab, R., Ramamoorthy, A., Song, P., Li, F., Yu, J., Manheng, W., Palmby, T. R., Ghosh, S., Horne, H. N., Lee, E. Y., Philip, R., Dave, K., … Pazdur, R. (2018). FDA Approval Summary: Niraparib for the Maintenance Treatment of Patients with Recurrent Ovarian Cancer in Response to Platinum-Based Chemotherapy. *Clinical Cancer Research*, *24*(17), 4066–4071. https://doi.org/10.1158/1078-0432.CCR-18-0042

Jayson, G. C., Kohn, E. C., Kitchener, H. C., & Ledermann, J. A. (2014). Ovarian cancer. *The Lancet*, *384*(9951), 1376–1388. https://doi.org/https://doi.org/10.1016/S0140-6736(13)62146-7

Kaelin, W. G. (2005). The Concept of Synthetic Lethality in the Context of Anticancer Therapy. *Nature Reviews Cancer*, *5*(9), 689–698. https://doi.org/10.1038/nrc1691

Kanaoka, R., Kushiyama, A., Seno, Y., Nakatsu, Y., Matsunaga, Y., Fukushima, T., Tsuchiya, Y., Sakoda, H., Fujishiro, M., Yamamotoya, T., Kamata, H., Matsubara, A., & Asano, T. (2015). Pin1 Inhibitor Juglone Exerts Anti-Oncogenic Effects on LNCaP and DU145 Cells despite the Patterns of Gene Regulation by Pin1 Differing between These Cell Lines. *PloS One*, *10*(6), e0127467–e0127467. https://doi.org/10.1371/journal.pone.0127467

Kehoe, S., Hook, J., Nankivell, M., Jayson, G. C., Kitchener, H., Lopes, T., Luesley, D., Perren, T., Bannoo, S., Mascarenhas, M., Dobbs, S., Essapen, S., Twigg, J., Herod, J., McCluggage, G., Parmar, M., & Swart, A.-M. (2015). Primary chemotherapy versus primary surgery for newly diagnosed advanced ovarian cancer (CHORUS): an open-label, randomised, controlled, non-inferiority trial. *The Lancet*, *386*(9990), 249–257. https://doi.org/https://doi.org/10.1016/S0140-6736(14)62223-6

Kim, G., Ison, G., McKee, A. E., Zhang, H., Tang, S., Gwise, T., Sridhara, R., Lee, E., Tzou, A., Philip, R., Chiu, H.-J., Ricks, T. K., Palmby, T., Russell, A. M., Ladouceur, G., Pfuma, E., Li, H., Zhao, L., Liu, Q., … Pazdur, R. (2015). FDA Approval Summary: Olaparib Monotherapy in Patients with Deleterious Germline BRCA-Mutated Advanced Ovarian Cancer Treated with Three or More Lines of Chemotherapy. *Clinical Cancer Research*, *21*(19), 4257–4261. https://doi.org/10.1158/1078-0432.CCR-15-0887

Koh, W.-J., Abu-Rustum, N. R., Bean, S., Bradley, K., Campos, S. M., Cho, K. R., Chon, H. S., Chu, C., Clark, R., Cohn, D., Crispens, M. A., Damast, S., Dorigo, O., Eifel, P. J., Fisher, C. M., Frederick, P., Gaffney, D. K., Han, E., Huh, W. K., … Scavone, J. L. (2019). Cervical Cancer, Version 3.2019, NCCN Clinical Practice Guidelines in Oncology. *Journal of the National Comprehensive Cancer Network J Natl Compr Canc Netw*, *17*(1), 64–84. https://doi.org/10.6004/jnccn.2019.0001

Kozono, S., Lin, Y.-M., Seo, H.-S., Pinch, B., Lian, X., Qiu, C., Herbert, M. K., Chen, C.-H., Tan, L., Gao, Z. J., Massefski, W., Doctor, Z. M., Jackson, B. P., Chen, Y., Dhe-Paganon, S., Lu, K. P., & Zhou, X. Z. (2018). Arsenic targets Pin1 and cooperates with retinoic acid to inhibit cancer-driving pathways and tumor-initiating cells. *Nature Communications*, *9*(1), 3069. https://doi.org/10.1038/s41467-018-05402-2

Kurman, R. J., & Shih, I.-M. (2016). The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. *The American Journal of Pathology*, *186*(4), 733–747. https://doi.org/https://doi.org/10.1016/j.ajpath.2015.11.011

la Montagna, R., Caligiuri, I., Giordano, A., & Rizzolio, F. (2013). Pin1 and Nuclear Receptors: A New Language? *Journal of Cellular Physiology*, *228*(9), 1799–1801. https://doi.org/https://doi.org/10.1002/jcp.24316

Ledermann, J. A., Raja, F. A., Fotopoulou, C., Gonzalez-Martin, A., Colombo, N., & Sessa, C. (2013). Newly diagnosed and relapsed epithelial ovarian carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. *Annals of Oncology*, *24*, vi24–vi32. https://doi.org/https://doi.org/10.1093/annonc/mdt333

Lee, N. Y., Choi, H.-K., Shim, J.-H., Kang, K.-W., Dong, Z., & Choi, H. S. (2009). The prolyl isomerase Pin1 interacts with a ribosomal protein S6 kinase to enhance insulin-induced AP-1 activity and cellular transformation. *Carcinogenesis*, *30*(4), 671–681. https://doi.org/10.1093/carcin/bgp027

Lheureux, S., Braunstein, M., & Oza, A. M. (2019). Epithelial ovarian cancer: Evolution of management in the era of precision medicine. *CA: A Cancer Journal for Clinicians*, *69*(4), 280–304. https://doi.org/https://doi.org/10.3322/caac.21559

Li, X., & Beihua, K. (2013). PAX8 is a novel marker for differentiating between various types of tumor, particularly ovarian epithelial carcinomas (review). In *Oncology Letters* (Vol. 5, Issue 3, pp. 735–738). https://doi.org/10.3892/ol.2013.1121

Liao, X.-H., Zhang, A. L., Zheng, M., Li, M.-Q., Chen, C. P., Xu, H., Chu, Q.-S., Yang, D., Lu, W., Tsai, T.-F., Liu, H., Zhou, X. Z., & Lu, K. P. (2017). Chemical or genetic Pin1 inhibition exerts potent anticancer activity against hepatocellular carcinoma by blocking multiple cancer-driving pathways. *Scientific Reports*, *7*(1), 43639. https://doi.org/10.1038/srep43639

Lin, C.-H., Li, H.-Y., Lee, Y.-C., Calkins, M. J., Lee, K.-H., Yang, C.-N., & Lu, P.-J. (2015). Landscape of Pin1 in the cell cycle. *Experimental Biology and Medicine (Maywood, N.J.)*, *240*(3), 403–408. https://doi.org/10.1177/1535370215570829

Lisio, M. A., Fu, L., Goyeneche, A., Gao, Z. H., & Telleria, C. (2019). High-grade serous ovarian cancer: Basic sciences, clinical and therapeutic standpoints. In *International Journal of Molecular Sciences* (Vol. 20, Issue 4). MDPI AG. https://doi.org/10.3390/ijms20040952

Lu, K. P. (2003). Prolyl isomerase Pin1 as a molecular target for cancer diagnostics and therapeutics. *Cancer Cell*, *4*(3), 175–180. https://doi.org/https://doi.org/10.1016/S1535-6108(03)00218-6

Lu, K. P., Suizu, F., Zhou, X. Z., Finn, G., Lam, P., & Wulf, G. (2006). Targeting carcinogenesis: A role for the prolyl isomerase Pin1? *Molecular Carcinogenesis*, *45*(6), 397–402. https://doi.org/https://doi.org/10.1002/mc.20216

Lu, K. P., & Zhou, X. Z. (2007). The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nature Reviews Molecular Cell Biology*, *8*(11), 904–916. https://doi.org/10.1038/nrm2261

Lucchetti, C., Caligiuri, I., Toffoli, G., Giordano, A., & Rizzolio, F. (2013). The Prolyl Isomerase Pin1 Acts Synergistically with CDK2 to Regulate the Basal Activity of Estrogen Receptor α in Breast Cancer. *PLOS ONE*, *8*(2), e55355-. https://doi.org/10.1371/journal.pone.0055355

Lusk, H., Burdette, J. E., & Sanchez, L. M. (2021). Models for measuring metabolic chemical changes in the metastasis of high grade serous ovarian cancer: fallopian tube, ovary, and omentum. *Molecular Omics*, *17*(6), 819–832. https://doi.org/10.1039/D1MO00074H

Manocha, A., & Jain, S. (2019). WT1 in astrocytomas: Comprehensive evaluation of immunohistochemical expression and its potential utility in different histological grades. *Indian Journal of Cancer*, *56*(3), 197–201. https://doi.org/10.4103/ijc.IJC_51_18

Matsumoto, K., Nishimura, M., Onoe, T., Sakai, H., Urakawa, Y., Onda, T., & Yaegashi, N. (2019). PARP inhibitors for BRCA wild type ovarian cancer; gene alterations, homologous recombination deficiency and combination therapy. *Japanese Journal of Clinical Oncology*, *49*(8), 703–707. https://doi.org/10.1093/jjco/hyz090

Matulonis, U. A., Sood, A. K., Fallowfield, L., Howitt, B. E., Sehouli, J., & Karlan, B. Y. (2016). Ovarian cancer. *Nature Reviews Disease Primers*, *2*(1), 16061. https://doi.org/10.1038/nrdp.2016.61

Mirsadraei, L., Hodkoff, A., Jones, K., Shabaik, A., Kader, A. K., Saenz, C. C., Montironi, R., Tacha, D. E., Fadare, O., & Hansel, D. E. (2017). Serous Carcinoma Mimicking Primary Urothelial Carcinoma on Clinical Evaluation and Pathology: A Potential Diagnostic Pitfall. *Archives of Pathology & Laboratory Medicine*, *142*(2), 168–177. https://doi.org/10.5858/arpa.2017-0004-OA

Moore, J. D., & Potter, A. (2013). Pin1 inhibitors: Pitfalls, progress and cellular pharmacology. *Bioorganic & Medicinal Chemistry Letters*, *23*(15), 4283–4291. https://doi.org/https://doi.org/10.1016/j.bmcl.2013.05.088

Mutch, D. G., & Prat, J. (2014). 2014 FIGO staging for ovarian, fallopian tube and peritoneal cancer. *Gynecologic Oncology*, *133*(3), 401–404. https://doi.org/https://doi.org/10.1016/j.ygyno.2014.04.013

Nonaka, D., Chiriboga, L., & Soslow, R. A. (2008). Expression of Pax8 as a Useful Marker in Distinguishing Ovarian Carcinomas From Mammary Carcinomas. *The American Journal of Surgical Pathology*, *32*(10). https://journals.lww.com/ajsp/Fulltext/2008/10000/Expression_of_Pax8_as_a_Useful_Marker_in.17.aspx

Notario, B., Zamora, M., Viñas, O., & Mampel, T. (2003). All-trans-Retinoic Acid Binds to and Inhibits Adenine Nucleotide Translocase and induces

Mitochondrial Permeability Transition. *Molecular Pharmacology*, *63*(1), 224. https://doi.org/10.1124/mol.63.1.224

Ochoa, W. F., Torrecillas, A., Fita, I., Verdaguer, N., Corbalán-García, S., & Gomez-Fernandez, J. C. (2003). Retinoic Acid Binds to the C2-Domain of Protein Kinase Cα. *Biochemistry*, *42*(29), 8774–8779. https://doi.org/10.1021/bi034713g

O'Neill, C. J., McBride, H. A., Connolly, L. E., Deavers, M. T., Malpica, A., & McCluggage, W. G. (2007). High-grade ovarian serous carcinoma exhibits significantly higher p16 expression than low-grade serous carcinoma and serous borderline tumour. *Histopathology*, *50*(6), 773–779. https://doi.org/https://doi.org/10.1111/j.1365-2559.2007.02682.x

Pang, R., Lee, T. K. W., Poon, R. T. P., Fan, S. T., Wong, K. B., Kwong, Y., & Tse, E. (2007). Pin1 Interacts With a Specific Serine-Proline Motif of Hepatitis B Virus X-Protein to Enhance Hepatocarcinogenesis. *Gastroenterology*, *132*(3), 1088–1103. https://doi.org/https://doi.org/10.1053/j.gastro.2006.12.030

Pang, R. W., Lee, T. K., Man, K., Poon, R. T., Fan, S.-T., Kwong, Y.-L., & Tse, E. (2006). PIN1 expression contributes to hepatic carcinogenesis. *The Journal of Pathology*, *210*(1), 19–25. https://doi.org/https://doi.org/10.1002/path.2024

Pang, R., Yuen, J., Yuen, M. F., Lai, C. L., Lee, T. K. W., Man, K., Poon, R. T. P., Fan, S. T., Wong, C. M., Ng, I. O. L., Kwong, Y. L., & Tse, E. (2004). PIN1 overexpression and β-catenin gene mutations are distinct oncogenic events in human hepatocellular carcinoma. *Oncogene*, *23*(23), 4182–4186. https://doi.org/10.1038/sj.onc.1207493

Pawson, T., & Scott, J. D. (2005). Protein phosphorylation in signaling – 50 years and counting. *Trends in Biochemical Sciences*, *30*(6), 286–290. https://doi.org/https://doi.org/10.1016/j.tibs.2005.04.013

Pei-Jung, L., Zhen, Z. X., Minhui, S., & Ping, L. K. (1999). Function of WW Domains as Phosphoserine- or Phosphothreonine-Binding Modules. *Science*, *283*(5406), 1325–1328. https://doi.org/10.1126/science.283.5406.1325

Peng, T., Zintsmaster, J. S., Namanja, A. T., & Peng, J. W. (2007). Sequence-specific dynamics modulate recognition specificity in WW domains. *Nature Structural & Molecular Biology*, *14*(4), 325–331. https://doi.org/10.1038/nsmb1207

Perren, T. J., Swart, A. M., Pfisterer, J., Ledermann, J. A., Pujade-Lauraine, E., Kristensen, G., Carey, M. S., Beale, P., Cervantes, A., Kurzeder, C., Bois, A. du, Sehouli, J., Kimmig, R., Stähle, A., Collinson, F., Essapen, S., Gourley, C., Lortholary, A., Selle, F., … Oza, A. M. (2011). A Phase 3 Trial of Bevacizumab in Ovarian Cancer. *New England Journal of Medicine*, *365*(26), 2484–2496. https://doi.org/10.1056/NEJMoa1103799

Piek, J. M. J., van Diest, P. J., Zweemer, R. P., Jansen, J. W., Poort-Keesom, R. J. J., Menko, F. H., Gille, J. J. P., Jongsma, A. P. M., Pals, G., Kenemans, P., & Verheijen, R. H. M. (2001). Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. *The Journal of Pathology*, *195*(4), 451–456. https://doi.org/https://doi.org/10.1002/path.1000

Piek, J. M. J., Verheijen, R. H. M., Kenemans, P., Massuger, L. F., Bulten, H., & van Diest, P. J. (2003). BRCA1/2-related ovarian cancers are of tubal origin: a hypothesis. *Gynecologic Oncology*, *90*(2), 491. https://doi.org/https://doi.org/10.1016/S0090-8258(03)00365-2

Pinch, B. J., Doctor, Z. M., Nabet, B., Browne, C. M., Seo, H.-S., Mohardt, M. L., Kozono, S., Lian, X., Manz, T. D., Chun, Y., Kibe, S., Zaidman, D., Daitchman, D., Yeoh, Z. C., Vangos, N. E., Geffken, E. A., Tan, L., Ficarro, S. B., London, N., … Gray, N. S. (2020). Identification of a potent and selective covalent Pin1 inhibitor. *Nature Chemical Biology*, *16*(9), 979–987. https://doi.org/10.1038/s41589-020-0550-9

Ping Lu, K., Hanes, S. D., & Hunter, T. (1996). A human peptidyl–prolyl isomerase essential for regulation of mitosis. *Nature*, *380*(6574), 544–547. https://doi.org/10.1038/380544a0

Prat, J. (2012). New insights into ovarian cancer pathology. *Annals of Oncology*, *23*, x111–x117. https://doi.org/https://doi.org/10.1093/annonc/mds300

Pruthi, S., Gostout, B. S., & Lindor, N. M. (2010). Identification and Management of Women With BRCA Mutations or Hereditary Predisposition for Breast and Ovarian Cancer. *Mayo Clinic Proceedings*, *85*(12), 1111–1120. https://doi.org/10.4065/mcp.2010.0414

Querleu, D., Planchamp, F., Chiva, L., Fotopoulou, C., Barton, D., Cibula, D., Aletti, G., Carinelli, S., Creutzberg, C., Davidson, B., Harter, P., Lundvall, L., Marth, C., Morice, P., Rafii, A., Ray-Coquard, I., Rockall, A., Sessa, C., van der Zee, A., … duBois, A. (2017). European Society of Gynaecological Oncology (ESGO) Guidelines for Ovarian Cancer Surgery. *International Journal of Gynecologic Cancer*, *27*(7), 1534. https://doi.org/10.1097/IGC.0000000000001041

Ranganathan, R., Lu, K. P., Hunter, T., & Noel, J. P. (1997). Structural and Functional Analysis of the Mitotic Rotamase Pin1 Suggests Substrate Recognition Is Phosphorylation Dependent. *Cell*, *89*(6), 875–886. https://doi.org/https://doi.org/10.1016/S0092-8674(00)80273-1

Reid, B. M., Permuth, J. B., & Sellers, T. A. (2017). Epidemiology of ovarian cancer: a review. *Cancer Biology & Medicine*, *14*(1), 9–32. https://doi.org/10.20892/j.issn.2095-3941.2016.0084

Rizzolio, F., Caligiuri, I., Lucchetti, C., Fratamico, R., Tomei, V., Gallo, G., Agelan, A., Ferrari, G., Toffoli, G., Klein-Szanto, A. J., & Giordano, A. (2013). Dissecting Pin1 and phospho-pRb regulation. *Journal of Cellular Physiology*, *228*(1), 73–77. https://doi.org/https://doi.org/10.1002/jcp.24107

Rizzolio, F., Lucchetti, C., Caligiuri, I., Marchesi, I., Caputo, M., Klein-Szanto, A. J., Bagella, L., Castronovo, M., & Giordano, A. (2012). Retinoblastoma tumor-suppressor protein phosphorylation and inactivation depend on direct interaction with Pin1. *Cell Death & Differentiation*, *19*(7), 1152–1161. https://doi.org/10.1038/cdd.2011.202

Russo Spena, C., de Stefano, L., Palazzolo, S., Salis, B., Granchi, C., Minutolo, F., Tuccinardi, T., Fratamico, R., Crotti, S., D'Aronco, S., Agostini, M., Corona, G., Caligiuri, I., Canzonieri, V., & Rizzolio, F. (2018). Liposomal delivery of a Pin1 inhibitor complexed with cyclodextrins as new therapy for high-grade serous ovarian cancer. *Journal of Controlled Release*, *281*, 1–10. https://doi.org/https://doi.org/10.1016/j.jconrel.2018.04.055

Russo Spena, C., de Stefano, L., Poli, G., Granchi, C., el Boustani, M., Ecca, F., Grassi, G., Grassi, M., Canzonieri, V., Giordano, A., Tuccinardi, T., Caligiuri, I., & Rizzolio, F. (2019). Virtual screening identifies a PIN1 inhibitor with possible antiovarian cancer effects. *Journal of Cellular Physiology*, *234*(9), 15708–15716. https://doi.org/https://doi.org/10.1002/jcp.28224

Rustighi, A., Zannini, A., Tiberi, L., Sommaggio, R., Piazza, S., Sorrentino, G., Nuzzo, S., Tuscano, A., Eterno, V., Benvenuti, F., Santarpia, L., Aifantis, I., Rosato, A., Bicciato, S., Zambelli, A., & del Sal, G. (2014). Prolyl-isomerase Pin1 controls normal and cancer stem cells of the breast. *EMBO Molecular Medicine*, *6*(1), 99–119. https://doi.org/10.1002/emmm.201302909

Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y.-C., Wulf, G., Rottapel, R., Yamaoka, S., & Lu, K. P. (2003). Regulation of NF-kappaB Signaling by Pin1-Dependent Prolyl Isomerization and Ubiquitin-Mediated Proteolysis of p65/RelA. *Molecular Cell*, *12*(6), 1413–1426. https://doi.org/10.1016/S1097-2765(03)00490-8

Sallum, L. F., Andrade, L., Ramalho, S., Ferracini, A. C., de Andrade Natal, R., Borsarelli, A., Brito, C., Sarian, L. O., & Derchain, S. (2018). WT1, p53 and p16 expression in the diagnosis of low-and high-grade serous ovarian carcinomas and their relation to prognosis. In *Oncotarget* (Vol. 9, Issue 22). www.oncotarget.comwww.oncotarget.com

Schenk, T., Stengel, S., & Zelent, A. (2014). Unlocking the potential of retinoic acid in anticancer therapy. *British Journal of Cancer*, *111*(11), 2039–2045. https://doi.org/10.1038/bjc.2014.412

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Scott, C., Becker, M., Haluska, P., & Samimi, G. (2013). Patient-Derived Xenograft Models to Improve Targeted Therapy in Epithelial Ovarian Cancer Treatment. *Frontiers in Oncology*, *3*. https://www.frontiersin.org/article/10.3389/fonc.2013.00295

Shaw, P. E. (2007). Peptidyl-prolyl cis/trans isomerases and transcription: is there a twist in the tail? *EMBO Reports*, *8*(1), 40–45. https://doi.org/10.1038/sj.embor.7400873

Shimazui, T., Schalken, J. A., Kawamoto, R., van Bockhoven, A., Oosterwijk, E., & Akaza, H. (2004). Role of complex cadherins in cell-cell adhesion evaluated by spheroid formation in renal cell carcinoma cell lines. In *ONCOLOGY REPORTS* (Vol. 11).

Singh, A., & Settleman, J. (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene*, *29*(34), 4741–4751. https://doi.org/10.1038/onc.2010.215

Smyrek, I., Mathew, B., Fischer, S. C., Lissek, S. M., Becker, S., & Stelzer, E. H. K. (2019). E-cadherin, actin, microtubules and FAK dominate different spheroid formation phases and important elements of tissue integrity. *Biology Open*, *8*(1), bio037051. https://doi.org/10.1242/bio.037051

Stuart, G. C. E., Kitchener, H., Bacon, M., duBois, A., Friedlander, M., Ledermann, J., Marth, C., Thigpen, T., & Trimble, E. (2011). 2010 Gynecologic Cancer InterGroup (GCIG) Consensus Statement on Clinical Trials in Ovarian Cancer: Report From the Fourth Ovarian Cancer Consensus Conference. *International Journal of Gynecologic Cancer*, *21*(4), 750. https://doi.org/10.1097/IGC.0b013e31821b2568

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/https://doi.org/10.3322/caac.21660

Torre, L. A., Trabert, B., DeSantis, C. E., Miller, K. D., Samimi, G., Runowicz, C. D., Gaudet, M. M., Jemal, A., & Siegel, R. L. (2018). Ovarian cancer statistics, 2018. *CA: A Cancer Journal for Clinicians*, *68*(4), 284–296. https://doi.org/10.3322/caac.21456

van Zyl, B., Tang, D., & Bowden, N. A. (2018). Biomarkers of platinum resistance in ovarian cancer: what can we use to improve treatment. *Endocrine-Related Cancer*, *25*(5), R303–R318. https://doi.org/10.1530/ERC-17-0336

Vaughan, S., Coward, J. I., Bast, R. C., Berchuck, A., Berek, J. S., Brenton, J. D., Coukos, G., Crum, C. C., Drapkin, R., Etemadmoghadam, D., Friedlander, M., Gabra, H., Kaye, S. B., Lord, C. J., Lengyel, E., Levine, D. A., McNeish, I. A., Menon, U., Mills, G. B., … Balkwill, F. R. (2011). Rethinking ovarian cancer: recommendations for improving outcomes. *Nature Reviews Cancer*, *11*(10), 719–725. https://doi.org/10.1038/nrc3144

Verdecia, M. A., Bowman, M. E., Lu, K. P., Hunter, T., & Noel, J. P. (2000). Structural basis for phosphoserine-proline recognition by group IV WW domains. *Nature Structural Biology*, *7*(8), 639–643. https://doi.org/10.1038/77929

Vergote, I., Tropé, C. G., Amant, F., Kristensen, G. B., Ehlen, T., Johnson, N., Verheijen, R. H. M., van der Burg, M. E. L., Lacave, A. J., Panici, P. B., Kenter, G. G., Casado, A., Mendiola, C., Coens, C., Verleye, L., Stuart, G. C. E., Pecorelli, S., & Reed, N. S. (2010). Neoadjuvant Chemotherapy or Primary Surgery in Stage IIIC or IV Ovarian Cancer. *New England Journal of Medicine*, *363*(10), 943–953. https://doi.org/10.1056/NEJMoa0908806

Voutsadakis, I. A. (2020). Further Understanding of High-Grade Serous Ovarian Carcinogenesis: Potential Therapeutic Targets. *Cancer Management and Research*, *12*, 10423–10437. https://doi.org/10.2147/CMAR.S249540

Wang, J., Liu, K., Wang, X. F., & Sun, D. J. (2017). Juglone reduces growth and migration of U251 glioblastoma cells and disrupts angiogenesis. *Oncology Reports*, *38*(4), 1959–1966. https://doi.org/10.3892/or.2017.5878

Wei, S., Kozono, S., Kats, L., Nechama, M., Li, W., Guarnerio, J., Luo, M., You, M.-H., Yao, Y., Kondo, A., Hu, H., Bozkurt, G., Moerke, N. J., Cao, S., Reschke, M., Chen, C.-H., Rego, E. M., Lo-Coco, F., Cantley, L. C., … Lu, K. P. (2015). Active Pin1 is a key target of all-trans retinoic acid in acute promyelocytic leukemia and breast cancer. *Nature Medicine*, *21*(5), 457–466. https://doi.org/10.1038/nm.3839

Wintjens, R., Wieruszeski, J.-M., Drobecq, H., Rousselot-Pailley, P., Buée, L., Lippens, G., & Landrieu, I. (2001). 1H NMR Study on the Binding of Pin1 Trp-Trp Domain with Phosphothreonine Peptides*. *Journal of Biological Chemistry*, *276*(27), 25150–25156. https://doi.org/https://doi.org/10.1074/jbc.M010327200

Wulf, G. M., Ryo, A., Wulf, G. G., Lee, S. W., Niu, T., Petkova, V., & Lu, K. P. (2001). Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the

transcriptional activity of c-Jun towards cyclin D1. *The EMBO Journal*, *20*(13), 3459–3472. https://doi.org/10.1093/emboj/20.13.3459

Yoon, N., Yoon, G., Park, C. K., & Kim, H.-S. (2016). Stromal p16 expression is significantly increased in malignant ovarian neoplasms. *Oncotarget*, *7*(40), 64665–64673. https://doi.org/10.18632/oncotarget.11660

Yu, J. H., Im, C. Y., & Min, S.-H. (2020). Function of PIN1 in Cancer Development and Its Inhibitors as Cancer Therapeutics. *Frontiers in Cell and Developmental Biology*, *8*. https://www.frontiersin.org/article/10.3389/fcell.2020.00120

Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., & Sal, G. del. (2002). The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature*, *419*(6909), 853–857. https://doi.org/10.1038/nature01120

Zannini, A., Rustighi, A., Campaner, E., & del Sal, G. (2019). Oncogenic Hijacking of the PIN1 Signaling Network. *Frontiers in Oncology*, *9*. https://www.frontiersin.org/article/10.3389/fonc.2019.00094

Zhang, Y., Cao, L., Nguyen, D., & Lu, H. (2016). TP53 mutations in epithelial ovarian cancer. *Translational Cancer Research*, *5*(6), 650–663. https://doi.org/10.21037/tcr.2016.08.40

Zhou, X. Z., Kops, O., Werner, A., Lu, P.-J., Shen, M., Stoller, G., Küllertz, G., Stark, M., Fischer, G., & Lu, K. P. (2000). Pin1-Dependent Prolyl Isomerization Regulates Dephosphorylation of Cdc25C and Tau Proteins. *Molecular Cell*, *6*(4), 873–883. https://doi.org/https://doi.org/10.1016/S1097-2765(05)00083-3

Zhou, X. Z., & Lu, K. P. (2016). The isomerase PIN1 controls numerous cancer-driving pathways and is a unique drug target. *Nature Reviews Cancer*, *16*(7), 463–478. https://doi.org/10.1038/nrc.2016.49

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